Rita Kundu · Rajiv Narula *Editors*

Advances in Plant & Microbial Biotechnology

Springer

Advances in Plant & Microbial Biotechnology

Rita Kundu • Rajiv Narula
Editors

Advances in Plant & Microbial Biotechnology

Springer

Editors
Rita Kundu
Department of Botany
University of Calcutta
Kolkata, West Bengal, India

Rajiv Narula
State University of New York
Albany, NY, USA

ISBN 978-981-13-6320-7 ISBN 978-981-13-6321-4 (eBook)
https://doi.org/10.1007/978-981-13-6321-4

Library of Congress Control Number: 2019934372

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Foreword

The field of Plant and Microbial Biotechnology is a broad domain which encompasses both the macroscopic and the microscopic members of the world. The utility of plant systems for the development of human welfare is realized from time immemorial. The application of plant biotechnology encompasses the fields for the effective production of value-added materials like food and biochemical and pharmaceutical products; it also emphasizes the control of plant growth and development and plant protection against several biotic and abiotic factors.

In the last century, there had been a drastic change in the concept of microbes as pathogens only to beneficial role as it leads to the generation of thousands aspects of research with microbes used in bioremediation of pollutants; utilization of wastes; amelioration of the nutritional conditions of soil; production of variety of fermented, probiotic food; and so on. There was a drastic paradigm shift in the traditional use of inorganic substances to the unique application of plant and microbial technology for the eco-friendly way of industrial production of materials, which gave rise to a volley of research areas.

The topic of the current issue of ***Advances in Plant & Microbial Biotechnology*** is a compilation of the contributions from leading workers and researchers from different arenas of plant and microbial biotechnology and is a successful outcome of the 1st International Conference on Biotechnology and Biological Sciences, BIOSPECTRUM 2017, organized by the Department of Biotechnology, University of Engineering & Management, Kolkata. This is an admirable issue comprising of 17 different research topics which would definitely inculcate the scientific knowledge of researchers and scientists working in the field of Plant and Microbial Biotechnology and aims to give an idea on the ongoing cutting edge research areas on these domains.

The book focuses on the background research for the development of technology to be handed over from "lab to land" for the improvement in the production of crops like rice, wheat, and sugarcane. Biogenic production of silver nanoparticles and its use in waste removal are also emphasized. The book contains the improved techniques for the production of various enzymes of commercial interests, plant hormones, sugar alcohols, hydroxy fatty acids, phytochemicals, and biofertilizers. The cytotoxic potentials and genetic toxicity potentials of various plant parts are meticulously analyzed. The book also focuses on the recent context of interspecies

interaction of the biofilm formed by the extracellular products of *Pseudomonas* sp. isolated from ocular infection. The role of siderophores for sustained maintenance of soil health is also discussed in the book.

This book is a nice blend of plant and microbial biotechnology focusing on various techniques and methodologies being used in the recent field of research. The authors used various analytical instrumentations accompanied by biochemical assay techniques providing results which can have a far-sighted impact on the societal benefit.

I am delighted to go through the chapters and hope the readers at large will be benefited from the technologies described to face various challenges in the field of industrial production of commodities of plant and microbial origin.

Rina Rani Ray
Associate Professor and Head
Department of Biotechnology
Maulana Abul Kalam Azad University of Technology
Kolkata, West Bengal, India

Preface

BIOSPECTRUM 2017 was organized by the Department of Biotechnology, University of Engineering & Management, Kolkata. This conference brought forth a unique bridging forum for the interaction among eminent academicians, scientists, researchers, and professionals from various branches of biological sciences, a platform to exchange knowledge and expertise enriching researchers with opportunities of networking and collaboration across the globe. This conference promoted an intense dialogue between academia and industry to bridge the gap between academic research, industry initiatives, and governmental policies. This was fostered through panel discussions, keynote lectures, invited talks, and industry exhibits where academia was exposed to state of practice and results from trials and current research trends. There was participation from different domains of biotechnology with plant biotechnology assuming a pivotal role.

This book is an assimilation of some of the newest and upcoming research topics in the field of plant biotechnology. Readers of this book will be immensely benefited in obtaining novel ideas which will open up newer directions in the field of research in plant biotechnology. This book offers original, peer-reviewed articles dealing with all aspects of fundamental and applied research in the field of plant biotechnology. The coverage extends to other current applied areas of molecular biology, genetics, biochemistry, and cell and tissue culture. This book provides a single platform for articles that discusses and describes the scope of modern technologies to address the increasing demands for high yielding crop production, encompassing plant tissue culture, role of genetic engineering, and extending the exploitability of plants to incorporate other sustainable uses.

Finally, the editors are indebted to the International Advisory Committee and the Technical Program Committee for their valuable guidance and support. We express our sincere thanks to the students for their consistent support and spirited participation; we express our heartfelt gratitude to the management, staff, and faculty members of the University of Engineering & Management, Kolkata, for making the conference a success.

Kolkata, West Bengal, India — Rita Kundu
Albany, NY, USA — Rajiv Narula

Acknowledgment

The book is the outcome of the International Conference on Biotechnology and Biological Sciences, BIOSPECTRUM 2017. The editors of the book would like to acknowledge the help of all the people involved in making this book, *Advances in Plant and Microbial Biotechnology*, more specifically to the authors and reviewers who helped in the whole process. Without their support, this book would not have become a reality.

First, the editors would like to thank each one of the authors for their research contribution. Our sincere gratitude goes to the chapters' authors who contributed their time and expertise to make this book.

Second, the editors are extremely thankful to the organizers of the international conference BIOSPECTRUM and the management, faculty, and staff members of the University of Engineering & Management (UEM), Kolkata, India. The editors are grateful to the chancellor, Prof. Dr. Satyajit Chakrabarti, for his motivation and encouragement.

Third, the editors wish to acknowledge the valuable contribution of the reviewers regarding the improvement of the quality, coherence, and content presentation of the chapters.

Introduction

Biotechnology is the use or manipulation of an organism or parts of an organism. By this definition, the origin of biotechnology dates back to the early era of civilization, when people first begin to cultivate food crops. While the early applications are certainly still employed today, modern biotechnology is primarily associated with molecular biology, cloning, and genetic engineering to increase the yield as well as to improve the quality of the crop. Within the last 50 years, several key discoveries revolutionized the biological sciences that enabled the rapid evolution of the biosciences. These discoveries enabled scientists to isolate and manipulate genes, which has facilitated the growth of the biotechnology industry.

Life on earth would not have flourished without plants. Plants are the source of oxygen which is required by all living beings for respiration. Plants absorb carbon dioxide and produce oxygen by photosynthesis. This not only maintains oxygen on earth but also decreases carbon dioxide content of the atmosphere which in turn reduces global warming which is an important environmental problem.

Plants are valued greatly for their therapeutic properties and considered as excellent sources of medicines. Plants have been used for medicinal purposes from prehistoric times. Among ancient civilizations, India has been known to be a rich repository of medicinal plants. The forest in India is the principal source of a large number of medicinal and aromatic plants, which are largely used as raw materials for the manufacture of drugs and perfumery products. About 8,000 herbal remedies have been codified in AYUSH systems in India. Ayurveda, Unani, Siddha, and folk (tribal) medicines are the major systems of indigenous medicines. Treatment with medicinal plants is considered safe as there are no or minimal side effects. These remedies are *in sync* with nature, which is the biggest advantage. The golden fact is that the use of herbal treatments is independent of any age groups and sexes.

Over the past few years, a number of techniques have been developed which have advanced basic research in plant sciences and their application in agriculture. The very first of these studies deals with modifications to the growth of plant cells, tissues, organs, and protoplasts in tissue culture. The second area which is fast gaining worldwide attention is genetic engineering. This emerging technology has led to the manipulation of plant genetic material which will facilitate breeding better quality crop varieties.

Plant biotechnology delivers significant and tangible benefits to farmers, consumers, and the environment around the globe. It has improved farm incomes through increased crop yields and reduced use of agrochemicals, protected natural habitats by increasing the production on existing cropland, and allowed for improving waterways and reducing soil erosion. Biotech crop varieties have significantly increased plant productivity along with protecting the environment from an increasing chemical burden.

The 11 research papers in the book comprise of different facets of advancements in research in plant biotechnology.

Ayurveda, the oldest Indian indigenous medicine system of plant drugs, is known from very early times for preventing or suppressing various tumors using natural drugs. A chapter illustrates the cytotoxic potential of *Moringa oleifera* Lam. Entomopathogenic fungi *Cordyceps* sp. are one of the unique and valuable sources of bioactive compounds which help to treat various diseases. An experimental work explains the strain improvement strategies of *Cordyceps* sp.

Another very important work emphasized on rice crop improvement by development of low-cost blue-green algal biofertilizer comprising of consortium of four blue-green algal strains, namely, *Anabaena variabilis*, *Nostoc muscorum*, *Tolypothrix tenuis*, and *Aulosira fertilissima*, using different carrier materials, i.e., fly ash (100%), soil (100%), montmorillonite (100%), fly ash + soil (1:1), and fly ash + montmorillonite (1:1).

Apart from medicines, plants have industrial application. In this respect, a chapter elaborates studying the modification introduced into the fatty acid molecule of *Camelina sativa* to enable it to become a potential resource to synthesis biolubricants. A chapter on production of bioethanol describes pretreatment procedures of rice straw for improving enzymatic hydrolysis which facilitates the conversion into ethanol. Lignocellulosic materials are the potential renewable energy resources for the production of transportation fuels. In one study, sorghum biomass was used as a model lignocellulosic substrate for the production of xylulosic ethanol.

This book can be used by students, academicians, and researchers working on the domain of plant biotechnology as a reference book.

Contents

About the Editors

Dr. Rita Kundu is a professor at the University of Calcutta's Department of Botany. With a specialization in Cell Biology and Genetics, she has been engaged in cancer biology research. She has studied cell death through apoptosis, autophagy, or any other way in cervical cancer cell lines using traditional medicinal plants and other organic sources (marine/freshwater algal compounds, microbial compounds, synthetic organic compounds) to regulate cell proliferation. Currently, her main interest is in identifying metal-resistant crop (rice) varieties in southern West Bengal and studying their stress responses. She is the author of numerous papers.

Dr. Rajiv Narula received his Bachelor's Degree in Botany from the esteemed Presidency College (now Presidency University), Kolkata. He went on to complete a Master's in Biotechnology at GGD University, Chhattisgarh, and a PhD in Environmental Engineering at Clarkson University, Potsdam, NY. After completing his doctoral thesis on Pathogen Reduction and Recycling of Bedding Materials on Dairy Farms (May 2011), he joined the State University of New York, Canton, in 2011, and is currently a member of its Environmental Science and Chemistry Department.

1 Dilute Acid Pretreatment Efficiency on Various Solid Loadings and Effect of Different Neutralizing Agents on Xylulosic Ethanol Production

Narendra Naik Deshavath, Bijayeeni Singh Deo, Jyothika Boddu, Komali Vykuntam, Vaibhav V. Goud, and Venkata Dasu Veeranki

Abstract

Lignocellulosic materials are the potential renewable energy resources for the production of transportation fuels. In the present study, sorghum biomass is used as a model lignocellulosic substrate for the production of xylulosic ethanol. Dilute sulfuric acid pretreatment was performed at different solid loadings (2.5% to 15% w/v) to hydrolyze the maximum hemicellulosic content of sorghum biomass. As a result, significant xylan conversion efficiency was achieved and xylose found to be a predominant sugar present in the acid hydrolyzate. The pretreatment-derived acid hydrolyzate was divided into five fractions, and each fraction was neutralized with different alkaline agents such as calcium hydroxide ($Ca(OH)_2$), potassium hydroxide (KOH), magnesium hydroxide ($Mg(OH)_2$), sodium hydroxide (NaOH), and ammonia (NH_3) to determine the effect of alkaline agents on *Pichia stipitis* growth and xylulosic ethanol production. Among all the alkaline agents, $Ca(OH)_2$ was found to be a significant neutralizing agent which showed comparatively higher ethanol yield and productivity during the fermentation.

Keywords

Pretreatment · Sorghum biomass · Solid loadings · Ethanol

N. N. Deshavath · V. V. Goud · V. D. Veeranki (✉)
Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India
e-mail: d.narendra@iitg.ac.in; vvgoud@iitg.ac.in; veeranki@iitg.ac.in

B. S. Deo
Utkal University, Bhubaneswar, Odisha, India

J. Boddu · K. Vykuntam
National Institute of Technology Durgapur, Durgapur, West Bengal, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_1

1.1 Introduction

Bioethanol is a substitute to petroleum-based fuels which can also conquer the fossil fuel depletion. Lignocellulosic biomass is the most abundant, economically viable renewable energy resource that can be utilized for the production of bioethanol [9]. Generally, agricultural residues such as corn stover, sorghum stalks, sugarcane bagasse, and wheat straw are the major sources of lignocellulosic biomass for bioethanol production. Cellulose, hemicellulose, and lignin are the major constituents of lignocellulosic biomass. Cellulose is a homopolymer which is made up of glucose units, whereas heteropolymeric structure of hemicellulose is made up of xylose, arabinose, and other organic acids [11].

In a traditional way, conversion of lignocellulosic biomass into bioethanol can be performed in three steps such as pretreatment, enzymatic hydrolysis, and fermentation. Pretreatment is an essential process which mostly hydrolyzes the hemicellulosic fraction, and the resulting residual solid biomass constitutes mostly cellulose which can be further hydrolyzed by cellulase (biocatalyst) for the production of glucose monomers. Xylose and glucose are found to be principal sugars formed during the pretreatment and enzymatic hydrolysis, respectively. As compared to enzymatic hydrolyzate, pretreatment-derived hydrolyzate has less significance in the production of bioethanol. Efficiency of prehydrolyzate fermentation was deterred due to the presence of sulfate and fermentative inhibitors such as furans, organic acids, and other phenolic compounds [6].

However, several processes were well established to decrease the fermentative inhibitor effect such as ion exchange, overliming, and treatment with activated carbon [1, 5, 10]. Among them, overliming is a well-known process which effectively decreases the fermentative inhibitor concentration. Overliming is a process where the prehydrolyzate pH increases up to 9, 10, and 11 by the addition of alkali ($Ca(OH)_2$) and then adjusted to cultivation pH of microorganisms. While increasing the pH of prehydrolyzate at elevated levels sugar loss can occur, it is a profound drawback of overliming process [8].

Therefore, in the present study, dilute sulfuric acid pretreatment of sorghum biomass was performed at different solid loadings. The pretreatment-derived acid hydrolyzate was neutralized with different alkaline agents and then subjected to fermentation by *Pichia stipitis* 3498.

1.2 Materials and Methods

1.2.1 Dilute Sulfuric Acid Pretreatment

Sorghum biomass pretreatment was carried out with 0.2 M sulfuric acid at 121 °C for 2 h reaction time with 2.5–15% (w/v) solid loading. After pretreatment, solid and liquid fractions were separated through 0.2 μm nylon membrane filter. The solid

portion was washed with distilled water to attain neutral pH and then dried at 45 ± 3 °C. The liquid portion was divided into five fractions and heated to 50 °C for 30 min. Among the five fractions, two fractions were neutralized by slow addition of $Ca(OH)_2$ and $Mg(OH)_2$, respectively. The remaining three fractions were neutralized with liquid form of 10N KOH, 10N NaOH, and 25% NH_3, respectively. These hydrolyzates were stored at −20 °C until fermentation. Prior to fermentation, centrifuge the hydrolyzates to remove the precipitate, and then supernatant was adjusted to cultivation pH (i.e., 6).

1.2.2 Microorganism and Seed Culture Preparation

Pichia stipitis 3948 strain was procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. *P. stipitis* was subcultured on modified YPDX agar plates which contains (g/L) yeast extract, 10; peptone, 20; glucose, 5; xylose, 15; and agar, 20, and incubated at 30 °C for 48 h. A colony from the plate was inoculated into 100 ml of autoclave sterilized YPDX liquid growth medium and incubated at 30 °C with 120 rpm for 18 h. Initial pH of the YPDX medium was adjusted to 6. The cells were harvested by centrifugation at 8000 rpm for 10 min and resuspended in sterile distilled water to adjust the final concentration of 40 g/L which served as inocula for bioethanol production. Cell growth was observed by measuring the absorbance at 600 nm (OD600) using Agilent Cary 100 UV-visible spectrophotometer.

1.2.3 Fermentation

Fermentation was carried out in sterile 25 mL Erlenmeyer flasks containing 10 mL of fermentation medium which include 0.2 mL of 50X concentrated nutrient solution (1.7 g of yeast nitrogen base, 1 g of urea, and 6.56 g of peptone in 20 mL of water), 0.5 mL of inocula gives an initial cell concentration of 2 g/L, and an appropriate quantity of hydrolyzate was added to reach the desired volume. Initial pH of the media was adjusted to 6 and incubated at 30 °C with 120 rpm.

1.2.4 Analytical Method

Quantification of pretreatment-derived carbohydrates, fermentative inhibitors, and fermentation-produced ethanol was carried out by high-performance liquid chromatography (HPLC) [3].

1.3 Result and Discussion

1.3.1 Pretreatment of Sorghum Biomass

According to our previous studies, dilute acid pretreatment was conducted with 5% solid loading, and the maximum hemicellulose conversion was attained at 121 °C with 0.2 M sulfuric acid for 120 min [2–4]. Therefore, in the present study, we made an attempt to increase solid loading of pretreatment process to meet the desired levels of sugar concentration for successful xylulosic ethanol production. According to the HPLC analysis, different types of carbohydrates were formed from their respective polymeric carbohydrates such as xylobiose, xylose, and arabinose derived from hemicellulose, cellobiose, and glucose derived from cellulose. Among all the carbohydrates, xylose is found to be the principal carbohydrate formed during the pretreatment (Fig. 1.1a). It is a fact that pretreatment is an essential step which significantly hydrolyzes the hemicellulose content and the percentage of xylan present in hemicellulose is comparatively higher than that of arabinose [3]. Hence, higher concentration of xylose is found (Fig. 1.1a).

From Fig. 1.1a, linear increase of carbohydrates concentration was observed by increasing the sorghum biomass solid loading (2.5–15%), with a R^2 value of 0.98 and above. Besides carbohydrates, fermentative inhibitors like furfural (a degradation product of pentose sugars), 5-hydroxymethyl furfural (a degradation product of glucose), and acetic acid (derived from acetylated xylan) were also formed (data not shown).

1.3.2 Neutralization of Acid Hydrolyzates for Xylulosic Ethanol Production

During the neutralization process, sugar loss was not found, and the possible stoichiometric chemical reactions are shown in Fig. 1.2. The resulting $MgSO_4$, Na_2SO_4, K_2SO_4, and $(NH_4)_2SO_4$ are solubilized in their respective hydrolyzates; however, $CaSO_4$ is a solid which could be removed through centrifugation. Apart from these, at industrial-scale production, moisture heat-sterilized medium is used for fermentation. Therefore, in the present study, instead of using filter sterilization method, autoclave sterilization of fermentation medium was performed. Generally, xylose degradation can be initiated when the temperature reaches above 120 °C [7]. Therefore, minute amounts of xylose (4–5 mg) loss could be possible during the autoclave sterilization of fermentation medium.

Since glucose is the preferred substrate, *P. stipitis* starts to utilize xylose after glucose consumption. Due to the presence of K_2SO_4, Na_2SO_4, and $(NH_4)_2SO_4$ in the fermentation medium, sugar consumption and ethanol production were delayed (Fig. 1.1b–d). It took 144 h to reach the maximum ethanol concentration (Table 1.1). In addition to this, 15%, 18%, and 27% of unconverted xylose were still present in the fermentation media of KOH-, NaOH-, and NH_3-treated hydrolyzate at 144 h. The presence of sulfate ions decreases the microbial metabolic growth which

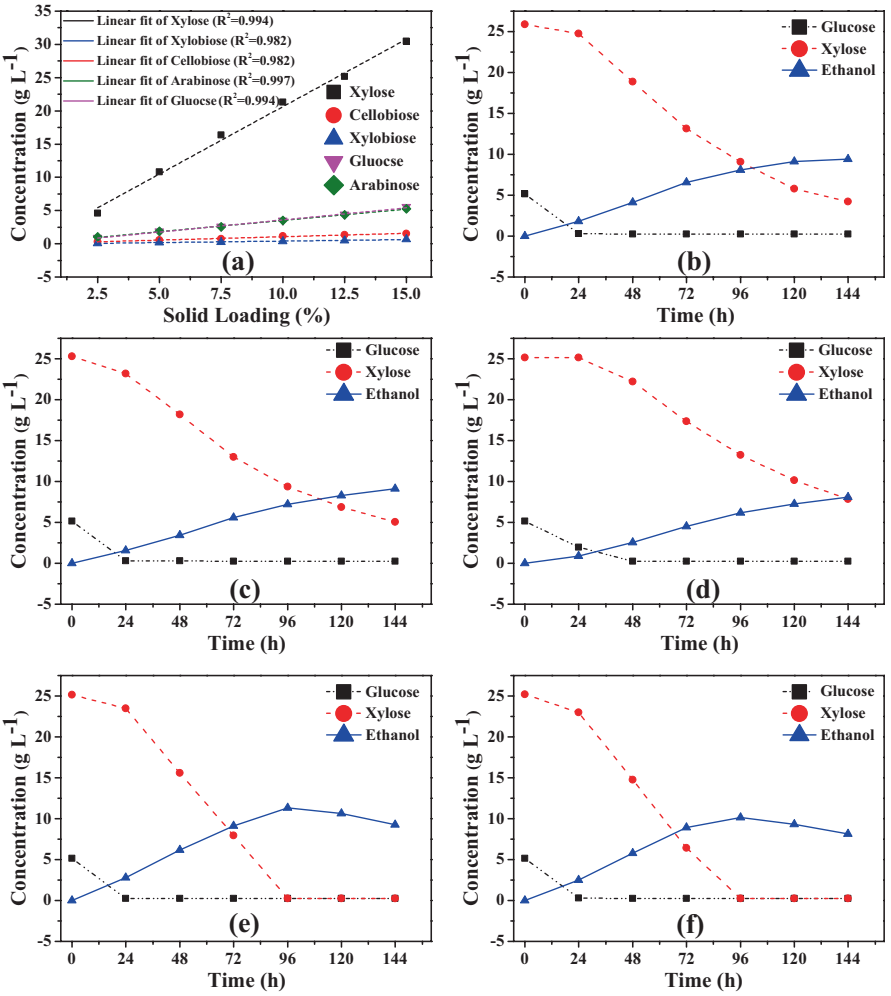


Fig. 1.1 (**a**) Pretreatment efficiency on different solid loadings for carbohydrates yield, (**b**) fermentation profiles of (**b**) KOH-, (**c**) NaOH-, (**d**) NH_3-, (**e**) $Ca(OH)_2$-, and (**f**) $Mg(OH)_2$-neutralized hydrolyzates: sugar utilization and ethanol production

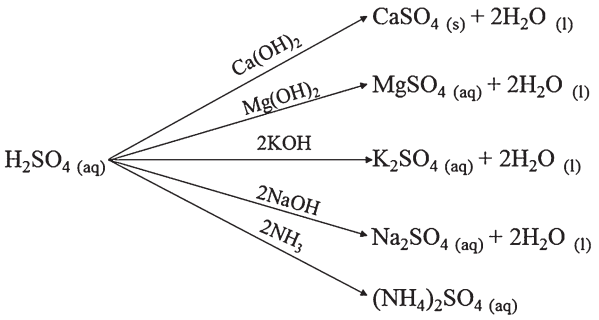


Fig. 1.2 Possible stoichiometric chemical reactions of different alkaline agents when reacted with H_2SO_4

Table 1.1 Summary of fermentation results from sorghum biomass pretreatment-derived hydrolyzate

	Different alkaline agents used for neutralization of acid hydrolyzates				
Parameters	$Ca(OH)_2$	$Mg(OH)_2$	KOH	NaOH	NH_3
Maximum ethanol concentration (g/L)	11.3	10.13	9.4	9.12	8.1
Fermentation time (h)	96	96	144	144	144
Ethanol yield on substrate (g_p/g_s)	0.37	0.33	0.30	0.30	0.26
Theoretical ethanol yield (%)	73.2	65.7	59.7	59	52.5
Ethanol productivity (g/L/h)	0.11	0.1	0.065	0.063	0.056
Total sugars utilized (%)	97	98	85	82	73

eventually affects the ethanol yield. Neutralization with $Ca(OH)_2$ and $Mg(OH)_2$ showed better results as compared to other neutralizing agents. $Ca(OH)_2$ forms calcium sulfate ($CaSO_4$) when reacted with H_2SO_4. Therefore, removal of sulfate in the form of $CaSO_4$ (prior to the fermentation) enhanced ethanol production (Fig. 1.1e). Highest ethanol concentration (11.3 g/L) and yield (0.37 g_p/g_s) are evidences of the $Ca(OH)_2$-treated hydrolyzate fermentation. To the best of our knowledge, this is the first report in which $Mg(OH)_2$ has been used for neutralization of acid hydrolyzate, and the results are found to be significant with 10.3 g/L of ethanol concentration (Figure 1.1f) along with 0.33 g_p/g_s ethanol yield. According to the literature, $MgSO_4$ has superior advantage in the yeast-based fermentation processes. Availability of Mg^{2+} ions in the fermentation medium significantly influenced the metabolic growth of microbes [12]. Previous studies also suggest that magnesium ions are important in stimulating the central pathway of carbohydrates catabolism, especially ethanol production process [12]. However, detailed summary of fermentation results are shown in Table 1.1 for better understanding the effect of different alkaline agents on xylulosic ethanol production.

1.4 Conclusions

During the pretreatment process, with increase in solid loading of sorghum biomass, the amount of carbohydrates concentration increased with a R^2 value of 0.98 and above. During fermentation, 11.3 g/L and 10.13 g/L ethanol production were observed in both $Ca(OH)_2$- and $Mg(OH)_2$-neutralized hydrolyzates, respectively, at 96 h fermentation period, whereas 9.4 g/L, 9.12 g/L, and 8.1 g/L ethanol concentrations were observed in KOH-, NaOH-, and NH_3-treated hydrolyzate fermentation, respectively, at 144 h. Prolonged fermentation is not feasible for the commercialization of ethanol production process; therefore, fermentation was stopped at 144 h.

Acknowledgment Authors would like to thank Miss Sushmita Mahanta, a summer trainee at Centre for the Environment, IIT Guwahati, for her help in proofreading.

References

1. Chandel AAK et al (2011) Detoxification of lignocellulosic hydrolysates for improved bioethanol production. Biofuel Production-Recent …, 2012, p. 989572. https://doi.org/10.1155/2012/989572
2. Deshavath NN, Dasu VV et al (2017a) Development of dilute sulfuric acid pretreatment method for the enhancement of xylose fermentability. Biocatal Agric Biotechnol 11(March):224–230. https://doi.org/10.1016/j.bcab.2017.07.012
3. Deshavath NN, Mohan M et al (2017b) Dilute acid pretreatment of sorghum biomass to maximize the hemicellulose hydrolysis with minimized levels of fermentative inhibitors for bioethanol production. 3 Biotech. Springer Berlin Heidelberg 7(2):1–12. https://doi.org/10.1007/s13205-017-0752-3
4. Deshavath NN et al (2018) Chemical composition analysis of various genetically modified sorghum traits: pretreatment process optimization and bioethanol production from hemicellulosic hydrolyzates without detoxification. Biochem Pharmacol Elsevier B.V. https://doi.org/10.1016/j.jece.2018.08.002
5. Gírio FM et al (2010) Hemicelluloses for fuel ethanol: a review. Bioresour Technol 101(13):4775–4800. https://doi.org/10.1016/j.biortech.2010.01.088
6. Huang CF et al (2009) Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of Pichia stipitis. Bioresour Technol 100(17):3914–3920. https://doi.org/10.1016/j.biortech.2009.02.064
7. Liu X et al (2012) Quantification of glucose, xylose, arabinose, furfural, and HMF in corncob hydrolysate by HPLC-PDA-ELSD. Carbohydr Res Elsevier Ltd 353:111–114. https://doi.org/10.1016/j.carres.2012.03.029
8. Mohagheghi A, Ruth M, Schell DJ (2006) Conditioning hemicellulose hydrolysates for fermentation: effects of overliming pH on sugar and ethanol yields. Process Biochem 41(8):1806–1811. https://doi.org/10.1016/j.procbio.2006.03.028
9. Naik SN et al (2010) Production of first and second generation biofuels: a comprehensive review. Renew Sust Energ Rev 14(2):578–597. https://doi.org/10.1016/j.rser.2009.10.003
10. Palmqvist E, Hahn-Hägerdal B (2000) Fermentation of lignocellulosic hydrolysates I: inhibitors and mechanism of inhibition. Bioresour Technol 74(1):25–33
11. Rowell R (2012) Handbook of wood chemistry and wood composites, 2nd edn. CRC Press, Boca Raton. https://doi.org/10.1201/b12487
12. Walker GM (1994) The roles of magnesium in biotechnology. Crit Rev Biotechnol 14(4):311–354

Effect of BGA Biofertilizers Using Different Carrier Materials on Rice Crop

2

Rajinder Kaur and Dinesh Goyal

Abstract

The present study laid emphasis on rice crop improvement by development of low-cost blue-green algal biofertilizer comprising of consortium of four ARM blue-green algal strains, viz. *Anabaena variabilis* (ARM 441), *Nostoc muscorum* (ARM 442), *Tolypothrix tenuis* (ARM 443) and *Aulosira fertilissima* (ARM 444) using different carrier materials, i.e. fly ash (100%), soil (100%), montmorillonite (100%), fly ash + soil (1:1) and fly ash + montmorillonite (1:1). Pot trial was conducted to study their effect on rice cultivar PUSA 1121 using nonsterile soil with a control without inoculation of blue-green algal consortium. At the time of harvest (after 90 days of inoculation), consortium of ARM culture showed highest nitrogen content (0.149%) and carbon (0.39%), respectively, at treatment T3 involving fly ash + soil (1:1) followed by highest grain yield (g per pot) of 14.3 and 12.75 which was recorded in treatment T3, fly ash + soil (1:1) as compared to control. Therefore in the present study, fly ash with combination of soil (1:1) was observed as a good carrier material in place of soil or MMT alone for showing highest nitrogen, carbon and phosphorus content promoting cheap and adaptable method by farmers for organic farming.

Keywords

Blue-green algae · Biofertilizers · Soil · Fly ash · Soil properties · Rice

R. Kaur (✉)
Department of Biotechnology, Beant College of Engineering and Technology, Gurdaspur, Punjab, India

D. Goyal
Department of Biotechnology, Thapar University, Patiala, Punjab, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_2

2.1 Introduction

Indian agriculture was mostly organic before the advent of the Green Revolution; however, widespread adoption of nutrient-responsive and high-yielding varieties and use of inorganic fertilizers, weedicides and insecticides only resulted in high crop yield [1]. It has been reported that excessive utilization of chemical fertilizers has made nitrogen as the second limiting factor after water, for which the use of biofertilizers becomes necessary to prevent soil deterioration and maintain soil fertility. Biofertilizers are best developed substitute for chemical fertilizers such as cyanobacteria which are capable of fixing nitrogen [2]. Amongst the array of biofertilizers developed for different crops, cyanobacteria, popularly known as blue-green algae, constitute the most important inputs in rice cultivation [4, 10]. In addition, the use of cyanobacteria as biofertilizers can improve plant growth and crop yield as they add organic matter to soil, thus improving soil texture [6]. Application of such algal biofertilizers has proved to be sustainable, eco-friendly, cheap and easily manageable and improves the nutrient status as well as soil health [3]. Carrier-based algal biofertilizers can make the rice production system more viable and reduce the ecological hazards caused due to synthetic fertilizers and can serve as one of the components of integrated plant nutrient supply system [7]. In the present study, consortium of four ARM blue-green algal strains *Anabaena variabilis* (ARM 441), *Nostoc muscorum* (ARM 442), *Tolypothrix tenuis* (ARM 443) and *Aulosira fertilissima* (ARM 444) procured from the Indian Agricultural Research Institute, New Delhi, were prepared using different carrier materials (fly ash (100%), soil (100%), montmorillonite (100%), fly ash + soil (1:1) and fly ash + montmorillonite (1:1)). Their effect on rice cultivar PUSA 1121 was examined in pot experiment under glasshouse conditions, using nonsterile soil with a control without inoculation of blue-green algal consortium.

2.2 Methodology

For development of low-cost BGA biofertilizers using different carrier materials, electrostatic precipitator (ESP) coal fly ash was collected from GGSS Thermal Power Plant, Ropar, Punjab. Wood charcoal and montmorillonite were purchased from local market located in Patiala, Punjab, as per required quantity. Garden soil was collected from experimental plot Science & Technology Entrepreneurs' Park (STEP), Thapar University, Patiala. Soil, charcoal and montmorillonite were crushed into fine powder and air-dried for their physiochemical properties. Comparative study of physicochemical and mineralogical content was done before the pot trial of each carrier material [9]. Different treatments of fly ash (100%), soil (100%), montmorillonite (100%), fly ash + soil (50:50%) and fly ash + montmorillonite (50:50) on rice cultivar PUSA 1121 were examined by pot experiment using nonsterile soil with a control without inoculation of blue-green algal consortium. At the time of harvest (after 90 days of inoculation), soil total nitrogen (%), organic carbon (%) and phosphorus content (mg kg^{-1}) and grain yield were examined with respective to control.

Table 2.1 Influence of consortium of blue-green algal inoculants on soil physicochemical properties in pot experiment with rice crop after 90 DAT (days after transplanting)

Treatments		BGA consortium of ARM cultures			
		pH	Organic Carbon (%)	Total Nitrogen (%)	Ava. P ($mgkg^{-1}$)
T1	Soil 100%	8.44 ± 0.5b	0.29 ± 0.04 bc	0.075 ± 0.005 d	12.95 ± 1.3 c
T2	MMT 100%	8.33 ± 0.24 c	0.31 ± 0.01 b	0.095 ± 0.012 c	11.69 ± 1.10 d
T3	Fly ash + soil (50:50)	8.21 ± 0.58e	0.39 ± 0.11 a	0.149 ± 0.034 a	14.98 ± 0.96 b
T4	Fly ash + MMT (50:50)	8.29 ± 0.17d	0.33 ± 0.02 b	0.137 ± 0.001 b	15.74 ± 0.52 a
T5	Fly ash + MMT (10:50)	8.13 ± 0.22f	0.30 ± 0.001 bc	0.120 ± 0.011 b	11. 25 ± 0.95 d
T6	Fly ash 100%	7.68 ± 0.51g	0.24 ± 0.07 ab	0.092 ± 0.047 b	13.01 ± 0.62 c
Control	Soil without inoculum	8.44 ± 0.6a	0.21 ± 0.001 c	0.007 ± 0.001 e	4.54 ± 0.32 e
	L.S.D ($P<0.05$)	**0.036**	**0.054**	**0.003**	**0.379**

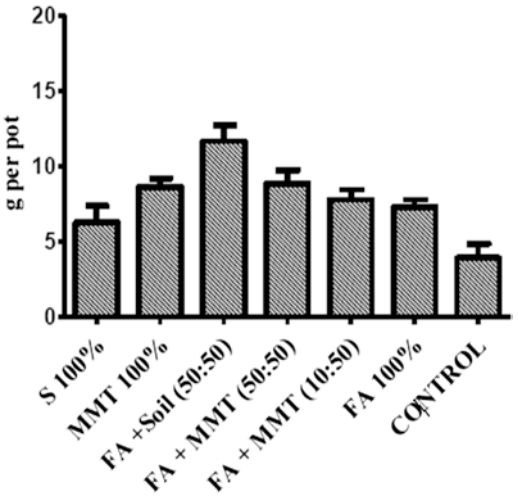


Fig. 2.1 Effect of inoculation of consortium of BGA (ARM cultures) on grain yield of rice crop (g per pot)

2.3 Result and Discussion

Pot trials on PUSA 1121 examined that consortium of ARM-procured cultures with the combination of fly ash and soil (1:1) T3 showed nitrogen content of 0.149% and carbon of 0.39%, respectively (Table 2.1), followed by highest grain yield (g per pot) of 14.3 and 12.75 that was recorded in treatment T3, fly ash +soil (1:1) as compared to control (Fig. 2.1). Physicochemical properties of soil before the inoculation of BGA consortium have been shown in Table 2.2. Significant enhancement of organic C and total N and P content over control could be contributed by growth-promoting

Table 2.2 Physicochemical analysis of soil used in pot culture experiment before the transplantation of rice and inoculation of BGA consortium

Parameters	Mean ± SE
pH	8.45 ± 0.07
Organic Carbon (%)	0.20 ± 0.01
Total Nitrogen (%)	0.011 ± 0.004
Ava. Phosphorus ($mgkg^{-1}$)	5.41 ± 0.6
Water Holding capacity (%)	33 ± 0.1

role of cyanobacterial inoculants in the rhizospheric zone of rice fields [8] and fly ash which itself contained some available P [5].

2.4 Conclusion

The present investigation concluded that fly ash with combination of soil (1:1) was observed as a good carrier material in place of soil or MMT alone for showing highest nitrogen, carbon and phosphorus content promoting cheap and adaptable method by farmers for organic farming. Incorporation of fly ash-based algal biofertilizers to the soil can enhance more crop productivity with reduced dosage of urea for long-term sustainability of soil nutrients.

References

1. Pabbi S (2008) Cyanobacterial biofertilizers (review). J Eco-friendly Agric 3(2):95–111
2. Elanwar M, Osman H, Mostafa M, El-Sheekh M, El-Naggar AH, Gheda SF (2010) Effect of two species of cyanobacteria as biofertilizers on some metabolic activities, growth, and yield of pea plant. Biol Fertil Soils 46:861–875
3. Pereira I, Ortega R, Barrientos L, Moya M, Reyes G, Kramm V (2009) Development of a biofertilizer based on filamentous nitrogen –fixing cynobacteria for rice crops in Chile. J Appl Phycol 21:135–144
4. Dhar DW, Prasanna R, Singh BV (2007) Comparative performance of three carrier based blue green algal biofertilizers for sustainable rice cultivation. J Sustain Agric 30:41–50
5. Gaind and Gaur (2002) Impact of fly ash and phosphate solubilising bacteria on soybean productivity. Bioresour Technol 85:313–315
6. Maqubela P, Pearson NSM, Muchaonyerwa P, Acqui LPD, Pardo MT (2010) Effects of cyanobacteria strains selected for their bioconditioning and biofertilization potential on maize dry matter and soil nitrogen status in a South African soil. Jpn Soc Soil Sci Plant Nutr 56:552–559
7. Mittra BN, Karmakar S, Swain KD, Ghosh CB (2005) Fly ash – a potential source of soil amendment and a component of integrated plant nutrient supply system. International Ash Utilization Centre for Applied Energy Research, University of Kentucky, Paper#28. http://www.flyash.info
8. Prasanna R, Jaiswal P, Nayak S, Sood A, Kaushik BD (2009) Cyanobacterial diversity in the rhizosphere of rice and its ecological significance. Indian J Microbiol 49:89–97
9. Kaur R, Goyal D (2014) Mineralogical comparison of coal fly ash with soil for use in agriculture. J Mater Cycles Waste Mater 18(1):186–200
10. Valiente EF, Ucha A, Quesada A, Leganes F, Carreres R (2000) Contribution of N2 fixing cyanobacteria to rice production: availability of nitrogen from 15N-labelled cyanobacteria and ammonium sulphate to rice. Plant Soil 221:107–112

3 Identification of Differentially Expressed Terminal Heat Stress-Associated Proteins in Developing Grains in Wheat (*Triticum aestivum* L.)

Davinder Sharma, Ratan Tiwari, Vijay Kumar Gupta, Jagadish Rane, and Rajender Singh

Abstract

Terminal heat stress (THS) causes abrupt modulation in the expression of stress-related proteins in developing seeds. These differential expressions are thought to enhance thermotolerance. Hence, a field experiment was conducted with three spring wheat genotypes, Raj 4014 (heat sensitive), K 7903 (heat escaper) and WH 730 (heat tolerant), for the identification of THS-associated proteins. To expose the plants to different levels of temperatures at the time of grain filling, the crop was sown during the second week of November and during the first week of January. The average ambient temperatures during the grain growth phase between anthesis and physiological maturity were 25.5 °C and 24.9 °C when sown in November and 31.3 °C and 32.0 °C when sown in January in 2012–2013 and 2013–2014, respectively. SDS-PAGE revealed considerable differences in grain proteome at different stages of grain filling in response to THS. Results showed that RAJ 4014 and K 7903 had very high homology in terms of qualitative pattern of protein bands as compared to WH 730. RAJ 4014 and K 7903 showed the expression of two new THS-responsive proteins (~ 40 and 105 kDa) at 7 days post anthesis (DPA) under THS. These protein bands appeared in WH 730 subsequently at 14 DPA but with low intensity. While protein bands of ~ 90 and 42 kDa appeared in K 7903 at 7 DPA, other two genotypes

D. Sharma
Presenting author, ICAR-Indian Institute of Wheat & Barley Research, Karnal, Haryana, India

R. Tiwari · R. Singh (✉)
ICAR-Indian Institute of Wheat & Barley Research, Karnal, Haryana, India

V. K. Gupta
Department of Biochemistry, Kurukshetra University, Kurukshetra, Haryana, India

J. Rane
School of Drought Stress, ICAR-National Institute of Abiotic Stress Management, Baramati, Maharashtra, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_3

showed these bands at 14 DPA under both normal and THS conditions. These information could help in designing a strategy for developing heat-tolerant cultivars in molecular breeding programmes.

Keywords
Terminal heat stress · Differentially expressed proteins · SDS-PAGE · Thermotolerance

3.1 Introduction

As a cool season crop, wheat is sensitive to high temperature, but with the availability of extensively adapted semidwarf cultivars, wheat cultivation has spread into temperate regions nearness to the equator especially in Southeast Asia. Some cultivars of wheat have versatile characters to survive above threshold level temperatures. Wheat evolved appropriate mechanisms such as escape, avoidance and/or staying green to cope with terminal heat stress (THS) by abrupt modulation in the expression of stress-related proteins in developing seeds. These differential expressions are thought to enhance thermotolerance [1]. Several studies have provided evidence that THS provokes cessation of conventional protein synthesis, accompanied by an increased synthesis of stress-related proteins, mainly, such as heat-shock proteins (HSPs) [2].

HSPs act as network of chaperone machinery, in which many chaperones act in concert or interact with other stress response mechanisms for facilitating protein refolding and stabilizing the polypeptides which improve and establish the normal physiological processes under high temperature stress to ensure normal growth and development [3]. HSPs can be broadly grouped as high molecular weight (HMW) HSPs and low molecular weight (LMW) HSPs. HMW-HSPs have essential functions in providing protection against aggregation and misfolding of non-native polypeptides under heat stress conditions. However, LMW-HSPs constitute complexes with unfolded proteins and other HSPs for stabilization of unfolded proteins [4]. Expression of specific stress-related proteins is genotypic dependent, and SDS-PAGE protein banding patterns could be important to investigate different thermotolerance mechanisms mentioned above. The study was undertaken to compare the SDS-PAGE protein profile in three wheat genotypes having different thermotolerance mechanism with a view to generate information which could provide a novel strategy for improving heat tolerance in molecular breeding programmes.

3.2 Materials and Methods

The experiment was conducted at Indian Institute of Wheat and Barley Research (IIWBR), Karnal (29° 42′ N, 77° 2′ E), in the Indo-Gangetic Plain in Northwestern India, with mildly alkaline soil (Typic ustochrept) over a period of 2 years

(2012–2013 and 2013–2014). Experiments were laid out in a randomized complete block design with ten replications. To expose the plants to different levels of temperatures at the time of grain filling, the crop was sown under optimal irrigated conditions during the second week of November as control and under very late sown (LS) irrigated conditions during the second week of January. The average ambient temperatures during the grain growth phase between anthesis and physiological maturity ranged from 25.1 to 25.4 °C when sown in November and 32.0 to 32.6 °C when sown in January in 2012–2013 and 2013–2014, respectively. Three spring wheat genotypes, viz. Raj 4014 (heat sensitive) [5, 6], K 7903 (heat escaper) [5, 6] and WH 730 (heat tolerant) [5–7], with a wide range of genetic background were chosen. As days to flowering in selected genotypes ranged from 7 to 12 days in each experiment, previous observations were used for staggered sowing to ensure synchrony in flowering and thus nearly the same level and magnitude of exposures to ambient temperatures during grain filling across the genotypes. Sowing was done with the IIWBR dibbler to ensure precision in planting [5, 6]. Dibbling tool was used to place 3 seeds at 1 locus without any overlap at 6.5 cm depth within a small experimental unit of 4 rows of 50 cm length with 20 cm space between the rows and 10 cm between the plants within the rows. Seeds were hand-planted in uniformly created cavities at the rate of single seed/cavity at 72 seeds/plot. Two weeks after sowing, 2 of 3 plants were uprooted to finally keep 1 plant/locus and 24 plants/plot. Developing spikes were sampled in the morning hours, at 5 days gap from anthesis to 35 DPA. All grains from each spikelet were removed, frozen and stored at −80 °C. To extract crude protein, 12 grains were homogenized with a pestle in a precooled mortar that contained 2.5 mL frozen extraction medium consisting of 100 mM HEPES-NaOH (pH 7.5), 2 mM EDTA, 50 mM 2-mercaptoenthanol and 12.5% (v/v) glycerol. The homogenate was filtered through multilayered cheese cloth and followed by centrifugation at 12,000 g for 15 min at 4 °C. The supernatant obtained was used for the SDS-PAGE protein profiling. Soluble protein from developing seed samples of each treatment determined by Lowry method [8] and the samples of each corresponding to 12 μg protein were mixed with sample buffer and resolved on 10% SDS-PAGE. The protein bands were resolved using the Hoefer SE600 electrophoresis unit.

3.3 Result and Discussion

One-dimensional SDS-PAGE revealed considerable differences in grain proteome at different stages of grain filling in response to terminal heat stress. Heat stress induced both qualitative (presence/absence) and quantitative (differential expression) alterations in the protein profile (Fig. 3.1). More than 30 protein bands were detected which included both common and specific bands among the investigated genotypes. An examination of the protein profile of genotypes grown under both the sowing conditions revealed that RAJ 4014 and K 7903 had very high homology in terms of qualitative pattern of protein bands as compared to WH 730.

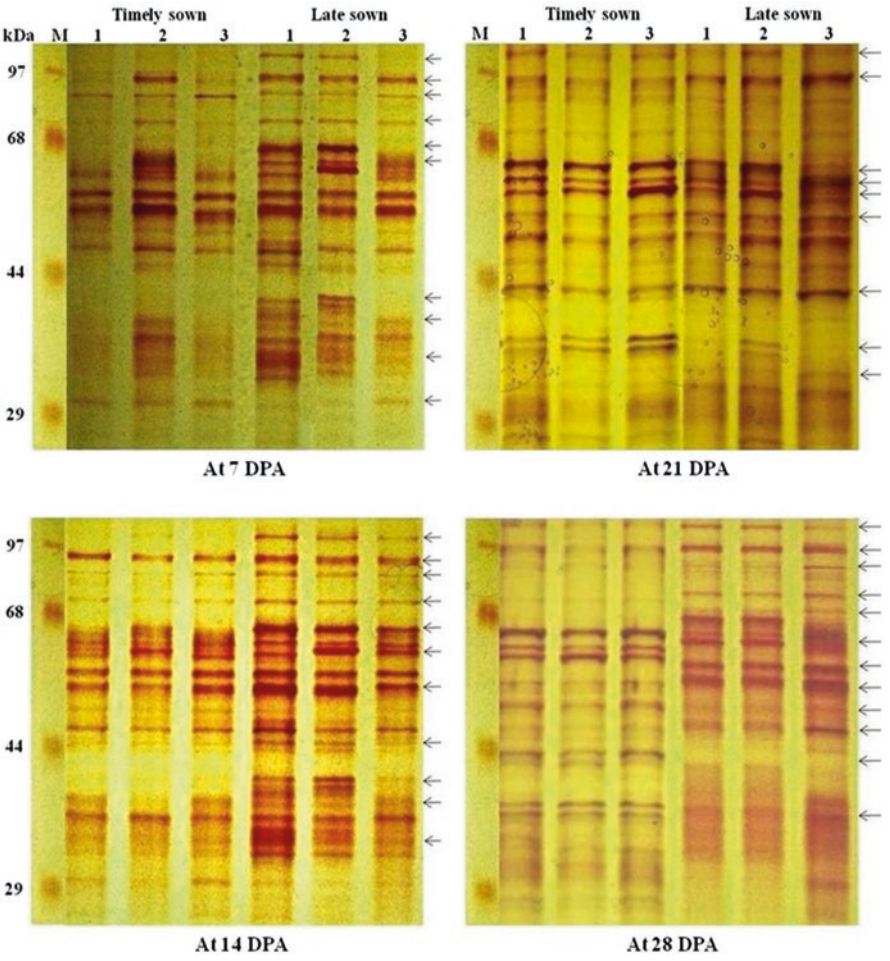


Fig. 3.1 SDS-PAGE protein profiling of three different wheat genotypes (1- K 7903, 2- RAJ 4014 and 3- WH730) at 7, 14, 21 and 28 days post anthesis of grain filling under timely and late sown conditions. A mixture of markers (M) was used for SDS-PAGE. Arrows show the change in expression of existing/new proteins. 12 μg of proteins was loaded in each well of the polyacrylamide gel (10%) for SDS-PAGE

There was much quantitative variation in protein bands among the genotypes. At 7 and 14 DPA, almost all protein bands showed upregulation in both tolerant (K 7903 and WH 730) and sensitive (RAJ 4014) genotypes under late sown as compared to the timely sown condition (Fig. 3.1). RAJ 4014 and K 7903 showed the expression of two new heat stress-responsive proteins (~ 40 and 105 kDa) at 7 DPA under late sown condition. The expression of these proteins was high in RAJ 4014 as compared to K 7903. These protein bands appeared in WH 730 subsequently at 14 DPA but with less expression. On the contrary, protein bands (~ 90 and 42 kDa)

were present in K 7903 only under the timely and the late sown condition, respectively, at 7 DPA, while these were highly expressed in all the three genotypes at 14 DPA under both the sowing conditions. A protein band (~ 30 kDa) showed downregulation under late sown condition in all the three genotypes. Few proteins (~ 68, 70 and 60 kDa), which were upregulated during early grain filling under late sown condition, showed their downregulation at 21 DPA (Fig. 3.1). On the other hand, protein bands (~ 40 and 90 kDa) were downregulated under timely sown condition. In addition, a protein of ~ 40 kDa disappeared from all genotypes under both the sowing conditions. As the grain filling progresses, some new protein bands (~ 68 and 72 kDa) appeared, while some previously existed protein bands (~ 40 and 44 kDa) disappeared under late sown condition at 28 DPA (Fig. 3.1).

Besides these, few protein bands which were upregulated previously showed their downregulation under both sowing conditions. Protein profiling showed both qualitative and quantitative changes under heat stress (Fig. 3.1). These heat stress-responsive changes were predicted to be related to starch biosynthesis enzymes, heat-shock proteins or anti-oxidant enzymes. The differences in SDS-PAGE protein profile under heat stress are in agreement with the earlier results reported in wheat [9–12]. Wheat and barley proteome analysis under heat stress also showed the similar pattern of expression of heat stress-related proteins [12]. A positive association of HSP with different intensity of temperature stress (from 25 to 46 °C) has been reported in wheat [13, 14]. Similarly, [9] reported that several HMW-HSPs (83–94 KDa) and LMW-HSPs (16–42 KDa) appeared in wheat developing seeds after heat shock in Mustang and Sturdy varieties of wheat with distinct levels of acquired thermotolerance. A growth stage-specific expression profile revealed the role of Hsp 70 *gene* in THS tolerance [15]. SDS-PAGE profile study revealed that differential SDS-PAGE protein profile exhibited new set of proteins during late sown in thermotolerant cultivars while few proteins were observed constantly in all wheat cultivars in both timely and late sown conditions. These unique proteins could adjust the tolerance mechanism of wheat under high temperature at different stages of grain filling and could be used as a stage-specific screening marker to identify high-temperature-tolerant wheat genotypes.

Acknowledgements We gratefully acknowledge the financial support from ICAR for Network Project on Transgenic in Crop: Functional Genomics in Wheat.

References

1. Farooq M, Bramley H, Palta JA, Siddique KHM (2011) Heat stress in wheat during reproductive and grain-filling phases. Crit Rev Plant Sci 30(6):491–507
2. Lin JS, Kuo CC, Yang IC, Tsai WA, Shen YH, Lin CC, Liang YC, Li YC, Kuo YW, King YC, Lai HM (2018) MicroRNA160 modulates plant development and heat shock protein gene expression to mediate heat tolerance in Arabidopsis. Front Plant Sci 9:68
3. Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M (2013) Physiological, biochemical and molecular mechanisms of heat stress tolerance in plants. Int J Mol Sci 14(5):9643–9684

4. Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot 61(3):199–223
5. Sharma D, Singh R, Rane J, Gupta VK, Mamrutha HM, Tiwari R (2016) Mapping quantitative trait loci associated with grain filling duration and grain number under terminal heat stress in bread wheat (Triticum aestivum L.). Plant Breed 135(5):538–545
6. Sharma D, Tiwari R, Gupta VK, Rane J, Singh R (2018) Genotype and ambient temperature during growth can determine the quality of starch from wheat. J Cereal Sci 79:240–246
7. Pandey GC, Mamrutha HM, Tiwari R, Sareen S, Bhatia S, Siwach P, Tiwari V, Sharma I (2015) Physiological traits associated with heat tolerance in bread wheat (Triticum aestivum L.). Physiol Mol Biol Plants 21(1):93–99
8. Lowry OH, Rosebrough NJ, Farrand AL, Randall RJ (1951) Protein measurement with the Folin Phenol reagent. J Biol Chem 193:265–270
9. Krishnan M, Nguyen HT, Burke JJ (1989) Heat shock protein synthesis and thermal tolerance in wheat. Plant Physiol 90(1):140–145
10. Sharma NPM, Babu SS, Udayakumar M (2007) Heat shock response of wheat cultivars differing in thermotolerance. Indian J Plant Physiol 12(4):327–336
11. Chauhan S, Srivalli S, Nautiyal AR, Khanna-Chopra R (2009) Wheat cultivars differing in heat tolerance show a differential response to monocarpic senescence under high temperature stress and involvement of serine proteases. Photosynthetica 47(4):536–547
12. Kumar RR, Goswami S, Sharma SK, Singh K (2012) Protection against heat stress in wheat involves change in cell membrane stability, antioxidant enzymes, osmolytes, H_2O_2 and transcript of heat shock protein. Int J Plant Physiol Biochem 4(4):83–91
13. Zivey M (1987) Genetic variability for heat shock protein in common wheat. Theor Appl Genet 74(2):209–213
14. Satbhai R, Kale A, Naik R (2016) Study on effect of high temperature stress in wheat genotypes using SDS protein profile. J Wheat Res 8(1):51–58
15. Hasan M, Barthakur S (2014) Hsp70 based gene expression biomarker shows growth stage specific genotypic diversity in Indian wheat (Triticum aestivum L.) cultivars. Annu Agric Res New Series 35(3):233–243

The Genetic Toxicity Potential of Heavy Metals (Zn, Cu) on *Vigna radiata*, *Triticum aestivum*, and *Cicer arietinum*

4

Aparajita Shilpie and Kamal Nayan Mishra

Abstract

Optimum amount of heavy metal in soil is essential for proper growth of plants. However, presence of these metals in higher concentration is detrimental and harmful for plant kingdom. Accumulation of heavy metals in soil can inhibit the growth as well as ability of absorbance of nutrients from soil in several plants. Toxic tolerance and response toward these heavy metals vary among plant species, and this variation is directly related to genetic constitution of plant genome. The present study is a comparative account on the effect of $ZnSO_4$ and $CuSO_4$ on *Vigna radiata*, *Triticum aestivum*, and *Cicer arietinum* plants exposed to heavy metals. The plants were treated with different concentrations of $ZnSO_4$, $CuSO_4$, and combined $ZnSO_4$ and $CuSO_4$. Different concentrations of Zn and Cu independently showed significant effect on different parts of plants. A significant synergetic effect was also observed in treated groups when compared with controlled plants. This study contributes valuable information on effect of heavy metals present in excess amount which lead to the changes in genotypic and the phenotypic characteristics of plants.

Keywords

Genetic toxicity · Toxic tolerance · Heavy metals · Plant growth

A. Shilpie (✉)
IIT Roorkee, Roorkee, Uttarakhand, India

K. N. Mishra
Pandit Ravishankar Shukla University, Raipur, Chhattisgarh, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_4

4.1 Introduction

Heavy metals are highly toxic environmental pollutants having harsh effects on plants, animals, and human being. The factors which are generally responsible for heavy metal contamination in environment are anthropogenic (viz., mining, smelting operation, and agriculture) as well as natural activities (viz., earthquake, volcanic activity), and these are taken by human being and other animals through the food chain, thus affecting them [20]. Optimum ranges of trace metals are necessary for growth, but below and above the optimum level, they cause deficiencies and toxicity, respectively. They also affect enzymatic activities in plants and animals. Al, B, Cu, Ni, Zn, As, Ba, Cd, Cr, Hg, Mo, Ni, Pb, and Se are the metals which generally cause harsh effects in plants as well as in animals including human [13, 14]. Plants are good specimens to judge and estimate the environmental pollution and toxicity caused by heavy metals. Phytotoxicity of metals is well proved [10, 19, 24]. Toxic heavy metal toxicity affects the plant growth; reduces the yielding capability, growth of root and shoot, homeostasis, and rate of nutrient absorption; and also gets accumulated in plants [18]. Plants give some indication of heavy metal toxicity by producing some biomarkers or endpoints which can be detected and examined at different levels of molecular and cellular organizations to check and control the heavy metal toxicity in an environment [5, 6].

Heavy metal gives rise to some genotoxic effects; they can be classified into four types: mutagenesis, clastogenesis, aneugenesis, and recombinogenesis [17]. Response against heavy metals in a plant can be checked by various ways like observing morphological and molecular changes [18]. Among the heavy metals, excess amount of zinc in soil affects the metabolic activities of plants; it reduces the growth of both root and shoot and causes senescence and gives rise to chlorosis in the younger leaves; it reaches to older leaves after prolonged exposure [7–9]. Copper (Cu) is a metal which is considered as a micronutrient for plants [22] and helps in CO_2 assimilation and ATP synthesis. But high concentration of Cu in soil has some cytotoxic effect; it produces stress and damage to plants, inhibits the growth of plant, and causes leaf chlorosis [12].

4.2 Materials and Methods

The experiment was performed on three plants, mung bean (*Vigna radiata*), wheat (*Triticum aestivum*), and gram (*Cicer arietinum*), which were grown in pot, in controlled conditions. The concentrations of copper and zinc which were tested in soil

Table 4.1 The soil concentration of the Cu/Zn pre- and posttreatment

Soil concentration	Pretreatment (mg/kg)	Posttreatment (mg/kg)
Copper	58.75	58.38
Zinc	354.62	352.44

Table 4.2 The concentration of $ZnSO_4$, $CuSO_4$, and combined $ZnSO_4$ and $CuSO_4$

Heavy metals	Concentration 1	Concentration 2	Concentration 3
Control	Distilled water	Distilled water	Distilled water
$CuSO_4$	50 µM	175 µM	200 µM
$ZnSO_4$	0.25 mM	0.625 mM	1 mM
$ZnSO_4$:$CuSO_4$	0.75 mM:150 µM	0.75 mM:300 µM	1.5 mM:150 µM
	(1:1)	(1:2)	(2:1)

before sowing the seed were shown in Table 4.1. And they are treated regularly with different concentrations of $ZnSO_4$, $CuSO_4$, and combined $ZnSO_4$ and $CuSO_4$ [1, 11, 23] given in Table 4.2. Not much changes in concentration of Cu/Zn in soil profile (before and after treatment) had been observed in concentration due to the reason that treatments were given to the plants by spraying method on their leaves; there were no contact of soil with heavy metal solutions. The percentage of seed germination was calculated after 24 h in room temperature (Table 4.3). After 10 days, plants were taken out from pot, washed with distilled water and nonionic detergent Tween 20. Plants were cut into three parts, viz., leaves, stems, and roots, and DNA was isolated from each part separately by modified CTAB method [2, 16]. DNA of controlled and treated samples was compared by the process of agarose gel electrophoresis. Due to treatment of heavy metals, growth and development of plants deteriorated; changes in protein function must be a reason behind this. That is why protein estimation was performed by the Bradford method [4] (Fig. 4.1).

Table 4.3 Seed germination percentage was calculated on the basis of the number of seed germinated

Heavy metals	Concentration 1	Seed germination percentage (%)	Concentration 2	Seed germination percentage (%)	Concentration 3	Seed germination percentage (%)
Control	Distilled water	*Vigna radiata*: 92	Distilled water	*Vigna radiata*: 96	Distilled water	*Vigna radiata*: 92
		Triticum aestivum:100		*Triticum aestivum*:96		*Triticum aestivum*: 96
		Cicer arietinum: 96		*Cicer arietinum*:92		*Cicer arietinum*: 96
$CuSO_4$	50 µM	*Vigna radiata*:72	175 µM	*Vigna radiata*: 68	200 µM	*Vigna radiata*: 60
		Triticum aestivum:88		*Triticum aestivum*:72		*Triticum aestivum*:68
		Cicer arietinum:80		*Cicer arietinum*:72		*Cicer arietinum*:64
$ZnSO_4$	0.25 mM	*Vigna radiata*: 76	0.625 mM	*Vigna radiata*: 72	1 mM	*Vigna radiata*: 68
		Triticum aestivum:84		*Triticum aestivum*:88		*Triticum aestivum*:76
		Cicer arietinum:80		*Cicer arietinum*:84		*Cicer arietinum*:68
$ZnSO_4$:$CuSO_4$	0.75 mM:150 µM	*Vigna radiata*: 80	0.75 mM:300 µM	*Vigna radiata*: 76	1.5 mM:150 µM	*Vigna radiata*: 60
		Triticum aestivum:88		*Triticum aestivum*:80		*Triticum aestivum*:64
		Cicer arietinum:84		*Cicer arietinum*:72		*Cicer arietinum*:68

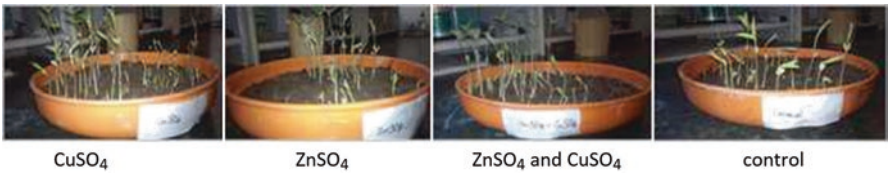


Fig. 4.1 Variation in seed germination rate during treatment of heavy metals (3 days) along with control samples

4.3 Results (Figs. 4.2, 4.3, and 4.4; Graphs 4.1, 4.2, and 4.3)

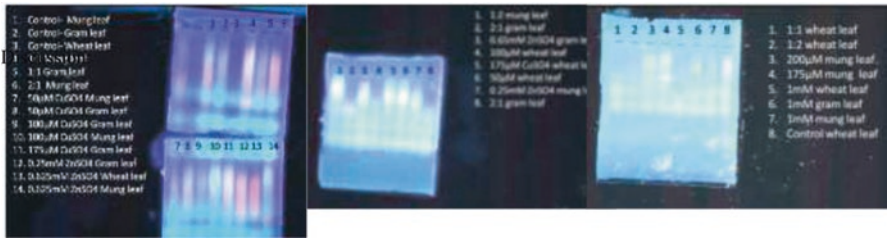


Fig. 4.2 Leaf samples: comparison between absorbance of leaf samples (gram, wheat, moong) treated with different concentrations of Zn and Cu

Fig. 4.3 Root samples: comparison between absorbance of root samples (gram, wheat, moong) treated with different concentrations of Zn and Cu

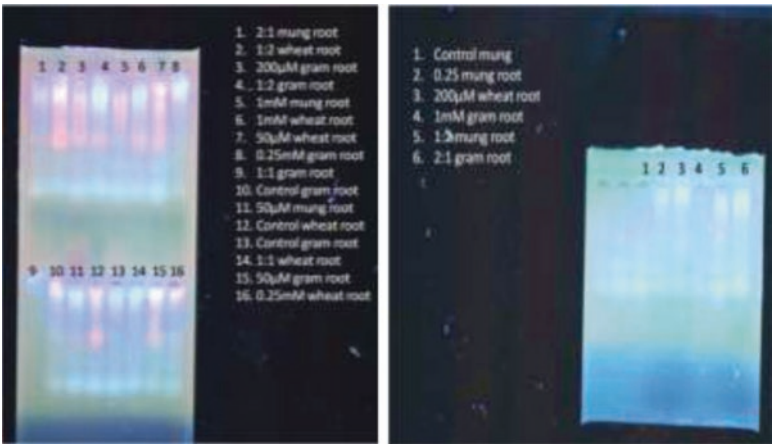
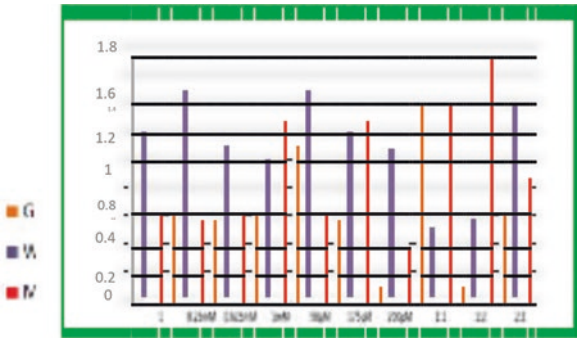
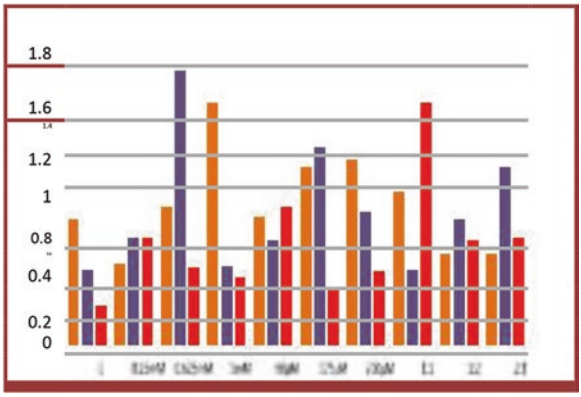


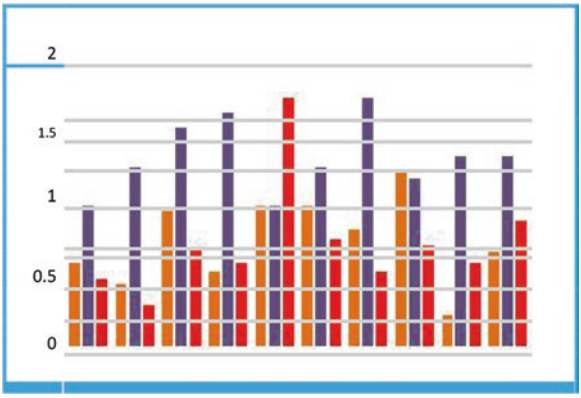
Fig. 4.4 Stem samples: comparison between absorbance of stem samples (gram, wheat, moong) treated with different concentrations of Zn and Cu

Graph 4.1 DNA electrophoretic gel picture of leaf samples from treated plants



Graph 4.2 DNA electrophoretic gel pictures of stem samples from treated plants





Graph 4.3 DNA electrophoretic gel picture of root samples from treated plants

4.4 Discussion

The same result was observed by Verma et al. [23]. They used various concentrations of $CuSO_4$ solution, viz., 50, 200, 500, and 1000 μM, and found that less concentration (<50 μM) of it is necessary for seedling growth in mung bean. Higher concentration affects adversely. And he also found that there was high protein content in root than shoot as compared to control plants.

Ashagre et al. [3] performed the same experiment on tomato's growing seed to check the effect of copper and zinc. And he found high concentration ($p < 0.05$) decreases the rate and amount of germination, length of root and shoot, as well as capacity to endure the stress conditions. Minimum growths were observed at 600 ppm and maximum in controlled samples.

Most of the experimental reports [1, 15, 21] explained the same result in different plants.

4.5 Conclusion

Heavy metals affect the plant cells adversely. The total protein content was found to be varying as compared to control samples, along with varying concentrations of heavy metals. The DNA was also affected by heavy metals. This may be due to the adverse effects of heavy metal on the metabolic activities. Senescence was found in younger leaves.

References

1. Ahmad N, Alatar AA, Faisal M, Khan MI, Fatima N, Anis M, Hegazy AK (2015) Effect of metals (Cu and Zn) on the development of sarpagandha (*Rauvolfia serpentina*) cultured in vitro. Biol Plant 59(1):11–17

2. Allen GC, Flores-Vergara MA, Krasnyanksi S, Kumar S, Thompson WF (2006) A modified protocol for rapid DNA isolation form plant tissues using cetyltrimethylammonium bromide. Nat Protoc 1(5):2320–2325
3. Ashagre H, Almaw D, Feyisa T (2013) Effect of copper and zinc on seed germination, phytotoxicity, tolerance and seedling vigor of tomato (*Lycopersicon esculentum* L. cultivar Roma VF). Int J Agric Sci Res 2(11):312–317
4. Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
5. Briat JF, Lebrun M (1999) Plant responses to metal toxicity. Comptes Rendus de l'Academie des Sciences/Life Sci 322:43–54
6. Burton MAS (1986) Biological monitoring of environmental contaminants, a technical report. MARC, London
7. Choi JM, Pak CH, Lee CW (1996) Micronutrient toxicity in French marigold. J Plant Nutr 19:901–916
8. Ebbs SD, Kochian LV (1997) Toxicity of zinc and copper to Brassica species: implications for phytoremediation. J Environ Qual 26:776–781
9. Fontes RLS, Cox FR (1998) Zinc toxicity in soybean grown at high iron concentration in nutrient solution. J Plant Nutr 21:1723–1730
10. Foy CD, Chaney RL, White MC (1978) The physiology of metal toxicity in plants. Annu Rev Plant Physiol 29:511–566
11. Johnson SE, Lauren JG, Welch RM, Duxbury JM (2005) A comparison of the effects of micronutrient of seed priming and soil fertilization on the mineral nutrition of chickpea (Cicer arietinum), Lentil (Lens culinaris), Rice (Oryza sativa) and Wheat (Triticum aestivum) in Nepal. Exp Eng 41:427–448
12. Lewis S, Donkin ME, Depledge MH (2001) Hsp70 expression in Enteromorpha intestinalis (Chlorophyta) exposed to environmental stressors. Aquat Toxicol 51:277–291
13. Logan TJ, Traina SJ (1993) Trace metals in agricultural soils. In: Allen HE, Perdue EM, Brown DS (eds) Metals in groundwater. Lewis Publishers, Boca Raton, pp 309–347
14. Mehera A, Farago ME (1994) Metal ions and plant nutrition. In: Farago ME (ed) Plants and the chemical elements: biochemistry, uptake tolerance and toxicity. VCH, Weinheim, pp 32–66
15. Mittal N, Vaid P, Kaur A (2015) Effect on amylase activity and growth parameters due to metal toxicity of iron, copper and zinc. Indian J Appl Res 5(4):662–664
16. Murray HG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. Nucleic Acids Res 8:4321–4325
17. Panda BB, Panda KK (2000) Genotoxicity and mutagenicity of metals in plants. In: Prasad MNV, Strzalka K (eds) Physiology and biochemistry of metal toxicity and tolerance in plants. Springer, The Netherlands, pp 3–172
18. Pirselova B (2011) Monitoring the sensitivity of selected crop to lead, cadmium and arsenic. J Stress Physiol Biochem 7(4):31–38
19. Prasad MNV (1997) Trace metals. In: Plant ecophysiology. Wiley, New York, pp 263–273
20. Rajeshwari TR, Sailaja N (2014) Impacts of heavy metals on environmental pollution. J Chem Pharm Sci 3(0974-2115):175–181
21. Singh D, Shakya N, Katiyar DK, Verma A, Narayan R, Niyazi R (2010) Impact of copper toxicity on black gram and its remedial approach for minimization of metal toxicity. Res Environ Life Sci 3(3):133–138
22. Thomas F, Malick C, Endreszl EC, Davies KS (1998) Distinct responses to copper stress in the halophyte, Mesembryanthemum crystallium. Physiol Plant 102:360–368
23. Verma JP, Singh V, Yadav J (2011) Effect of copper sulphate on seed germination, plant growth and peroxidase activity of Mung bean (*Vigna radiata*). Int J Bot 7(2):200–204
24. Woolhouse HW (1983) Toxicity and tolerance in the response of plants to metals. In: Lang O, Nobel PS, Osmond CB, Zeigler H (eds) Encyclopedia of plant physiology, Physiological plant ecology III, vol 12. Springer-Verlag, Berlin, pp 245–300

5 Production, Optimization, and Characterization of Siderophore by *Pseudomonas aeruginosa* (C_3) Isolated from Rhizospheric Soil

Chhaya Verma, Apoorva Dixit, and Rajesh Kumar

Abstract

In this study production of siderophore was analyzed in fluorescent pseudomonad named as C_3 (*Pseudomonas aeruginosa*). Out of ten isolates, this strain was selected for optimization of siderophore production; it produced 51.45 SU (siderophore unit). Findings of this experiment showed that siderophore production was increased when growth medium was added with potassium nitrate, 0.073 mg/ml $FeCl_3$, 200 ppm cadmium, sucrose (C source), and 0.5% NaCl in shaking condition at pH 6 after 5 days of incubation. Siderophore production was increased with incubation time and cadmium concentration, but after 6 days, siderophore production was reduced. Further, siderophore extraction was done by crystallization process, formed by adding methanol after maintaining pH 3 by mixing of sulfuric acid, ferrous sulfate, and 50% ammonium sulfate. Produced crystal was purified and characterized TLC (thin layer chromatography) and FTIR (Fourier-transform infrared spectroscopy) analysis. In TLC analysis the specific spot from the extracted siderophore was found to correspond with a spot of standard siderophore with the same Rf value. In FTIR analysis similar peak was observed as reported in earlier studies. This study was based on siderophore production, optimization, and characterization of extracted siderophore.

Keywords

Siderophore · *Pseudomonas* · Optimization · FTIR

C. Verma · A. Dixit · R. Kumar (✉)
Rhizosphere Biology Laboratory, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, Uttar Pradesh, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_5

5.1 Introduction

Iron is a transition metal and essential micronutrient for almost all organisms which consist of bacteria and fungi and plants in the various metabolic and informational cellular pathways. In plant the iron is essential for many processes such as respiration, photosynthesis, and nitrogen fixation. However, microorganisms are unable to obtain enough iron present in soil as immobilized form and cannot be transported in the cells. For utilization of unavailable iron, the plant and microbes produced low molecular weight (<10 KD) iron-chelating secondary metabolites termed as "siderophore" [1]. Siderophore has various applications in the field of agriculture, medicine, etc. In present time the siderophore production in microbes is analyzed by using the CAS agar assay [2]. In this assay competition was developed between the ferric complex of an indicator dye chrome azurol S (CAS) and a chelator or siderophore for iron. Iron is removed from CAS by siderophore because of higher affinity of siderophore for iron.

Ionic forms of iron are insoluble under physiological conditions and hence are difficult to assimilate for microorganisms, although iron is abundantly present in the environment. Three to four gram of iron is generally present in human body, out of which hemoglobin and myoglobin acquire the major share of 70%. To make available siderophore for biotechnological and medicinal application is important to increase siderophore production by evaluating process parameter. Many different environmental factors affect the synthesis of siderophore, notably the chemical nature of the organic carbon and energy source, metals, Fe^{3+}, amino acids, and organic nitrogen sources [3, 4]. Any factor influencing siderophore production influences the performance of PGPR in plant growth promotion and phytopathogen suppression [5]. Therefore, present study was aimed to regulate the cultural parameter for improvement of siderophore production and characterization.

5.2 Materials and Methods

All the ingredients and media used in these experiments were purchased from Himedia Laboratories Pvt Ltd (India) and Rankem Pvt Ltd (India). The strain C_3 used in this experiment was collected from Rhizosphere Biology Laboratory and characterized by morphological and biochemical methods.

5.2.1 Siderophore Production

Siderophore production by different strains of *Pseudomonas aeruginosa* was tested by chrome azurol S (CAS) agar assay. Formation of yellow-orange zone around the colonies indicates the siderophore production. CAS-shuttle assay was used for the quantitative estimation of siderophore [2]. In this method, 3–4-day grown culture broths were centrifuged at 10,000 rpm in cooling centrifuge at 4 °C for 10 min. After this obtained cell-free supernatant was mixed with 0.5 ml CAS solution in

1 ml of culture filtrate. The developing blue color was determined after 20 min of incubation by using spectrophotometer at absorbance 630 nm. The percentage of siderophore units was calculated by standard formula.

5.2.2 Optimization of Siderophore

There are several parameters affecting production of siderophore used for the optimization studies; such factors are different nitrogen and carbon source, different NaCl and $FeCl_3$ concentration, pH, and days, etc. For nitrogen source potassium nitrate, ammonium sulfate, ferrous ammonium sulfate, and glycine were added in CAS media. In analysis of effects of carbon source, dextrose, glucose, sucrose, lactose were added in CAS media. Three parameters of $FeCl_3$ 0.135 mg/ml, 0.270 mg/ml, and 0.067 mM and for NaCl effects 0.5, 1, 3, 5% NaCl were used in the media. Different concentrations of cadmium 0, 50, 100, and 200 ppm were used. Siderophore production was also checked in shaking and static condition. The effect of days on siderophore production was determined by incubating the flask at different days such as 3, 6, and 9 days.

5.2.3 Crystallization and Characterization of Siderophore

For study of chemical structure, siderophore was extracted from culture supernatant of C_3 strain as crystal form by modified method of Tank et al. [6]. Produced crystal was purified and characterized by TLC and FTIR analysis. In TLC analysis the specific spot from the extracted siderophore was found to correspond with a spot of standard siderophore with the same Rf value. In FTIR analysis similar peak was observed as reported in earlier studies.

5.3 Result and Discussion

Siderophore production of isolate C_3 was done on solid CAS blue agar plates. Production of siderophore was observed with the formation of clear orange color zone around the growth. In quantitative analysis the result showed that C_3 strain produces high amount of siderophore approximately 51.45 SU. In presence of $FeCl_3$ the growth of *Pseudomonas* increases, and it is vital for its growth. However, De Villegas [7] stated that above 10 μM concentration of $FeCl_3$ has a negative effect on siderophore production, and other study favors that highest siderophore production occurs at iron concentration above 50 μg/mL [8]. Our results show maximum siderophore production at 0.027 M concentration, and at 0.27 no production was reported (Fig. 5.1d). Sodium chloride is also an important growth factor like other supplement to the medium. The concentration of siderophore was maximum at 3% NaCl than other concentrations. The 1% and 8% also showed positive result, but no production was found at 5% (Fig. 5.1c).

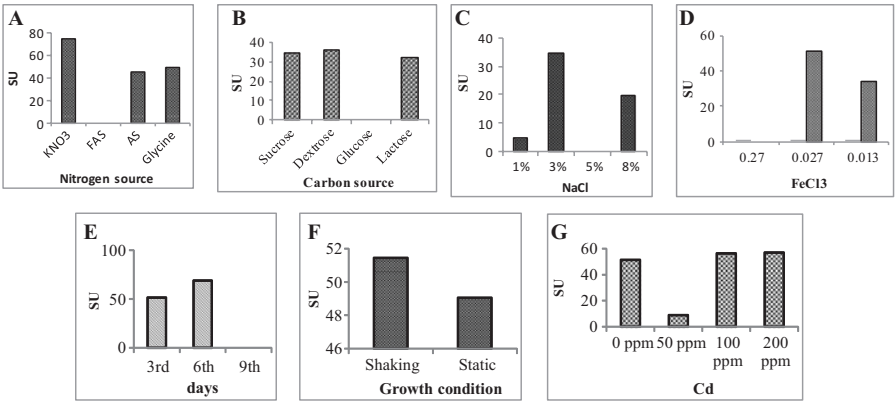


Fig. 5.1 Siderophore production at different parameters including (**a**) nitrogen source, (**b**) carbon source, (**c**) NaCl, (**d**) $FeCl_3$, (**e**) days, (**f**) growth condition (shaking and static), (**g**) cadmium concentration

Efficiency of biosynthesis of siderophore in strains was also affected by the carbon sources. Four different sugars (sucrose, dextrose, glucose, and lactose) were used for optimization of siderophore production. In Fig. 5.1b, it was found that dextrose, sucrose, and lactose were good for C_3, but lactose was useless for siderophore production. However, Sharma et al. [9] reported that glucose stimulated siderophore production in *Pseudomonas*. Impact of nitrogen sources on siderophore production was studied by addition of various nitrogenous compounds like urea, potassium nitrate, ammonium sulfate, glycine, and FAS (ferrous ammonium sulfate) supplemented to the medium in which FAS show negative siderophore production, while remaining source had good response (Fig. 5.1a).

Different cadmium concentrations are used to check the production of siderophore. Cadmium concentrations 0, 50, 100, and 200 ppm were used for the optimization of siderophore. In this test at low concentration (50 ppm) of cadmium, very little amount was produced, but at high concentration (200 ppm), siderophore was produced positively as shown in Fig. 5.1g. At different incubation conditions, we found that siderophore production was higher at shanking condition than static because of oxygen availability (Fig. 5.1f).The inoculated culture media was incubated for various days such as 3, 6, and 9 days at 30 °C (Figure 5.1e). Siderophore production was enhanced day by day and reduced after 9th day of incubation. As time spends, iron is depleted from the media. Therefore, increase in pH may be coincidental to the increase in siderophore concentration. Along with siderophore production, the pH of media was increased from 6.8 to 10. Sayyed et al. [3] reported that siderophore production was declined if more iron content was found in CAS medium.

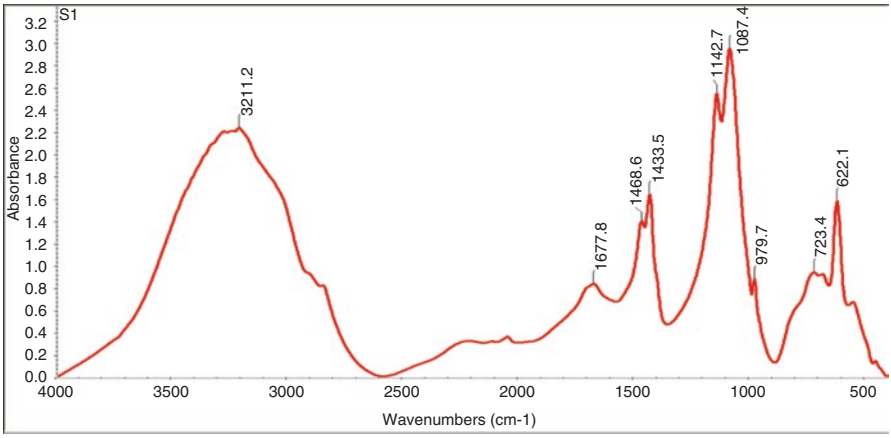


Fig. 5.2 FTIR characterization of siderophore crystal

5.3.1 Crystallization and Characterization of Siderophore

Obtained crystal was transparent, bright fine needle shaped, and white in appearance. The crystal of extracted siderophore was analyzed by using FTIR analysis on KBr pellets with the 4000–400 cm^{-1} range of wave numbers. Results of FTIR show that crystals obtained had hydroxamate functional group which correlated with the peaks obtained from FTIR analysis of PBHA crystals and pyoverdine (Fig. 5.2). Finding showed that the standard crystals are hydroxamate crystal.

5.4 Conclusion

The present study depends on siderophore production and optimization in florescent pseudomonad isolated from rhizosphere. The isolate C_3 was selected for study because it produced maximum amount of siderophore. Optimization of growth condition and media composition was done for maximum siderophore production. Maximum production was observed at different conditions, but in respect of incubation time, maximum production was reported at 6th day of incubation. Extracted siderophore was characterized by FTIR and TLC analysis. The use of siderophore as biocontrol agent against selected plant pathogens is an emerging idea, and such field of study is an environment-friendly alternative for chemical pesticides.

References

1. Saharan BS, Nehra V (2011) Plant growth promoting rhizobacteria: a critical review. Life Sci Med Res 21:1–30
2. Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

3. Sayyed RZ, Chincholkar SB (2010) Growth & siderophore production *Alcaligenes faecalis* is influenced by heavy metals. Indian J Microbiol 50(1):179–182
4. Sayyed RZ, Naphade BS, Chincholkar SB (2005) Ecologically competent rhizobacteria for plant growth promotion & disease management. In: Rai MK, Chikhale NJ, Thakare PV, Wadegaonkar PA, Ramteke AP (eds) Recent trends in biotechnology. Scientific Publisher, Jodhpur, pp 1–16
5. Sharma S, Kaur M (2010) Antimicrobial activities of rhizobacterial strains of *Pseudomonas* and *Bacillus* strains isolated from rhizosphere soil of carnation. Indian J Microbiol 50:229–232
6. Tank N, Rajendran N, Patel B, Saraf M (2012) Evaluation and biochemical characterization of a distinctive pyoverdin from a *Pseudomonas* isolated from chickpea rhizosphere. Braz J Microbiol 43:639–664
7. Díaz de Villegas ME, Villa P, Frías A (2002) Evaluation of the siderophores production by *Pseudomonas aeruginosa* PSS. Rev Latinoam Microbiol 44(3–4):112–117
8. Manninen M, Sandholm TM (1993) Methods for the detection of *Pseudomonas* siderophores. J Microbiol Methods 19:223–234
9. Sharma T, Kumar N, Rai N (2016) Production and optimization of siderophore producing *Pseudomonas* species isolated from Tarai region of Uttarakhand. Int J Pharm Biol Sci 7(1):306–314

Development of Marker in the Soft Gold Mushroom *Cordyceps* spp. for Strain Improvement

6

Loknath Deshmukh, Diva Gupta, and Sardul Singh Sandhu

Abstract

Natural drugs play extensive role and are the basis of traditional systems for cure and treatment of diseases. Entomopathogenic fungi *Cordyceps* Spp. are one of the unique and valuable sources of bioactive compounds which help in treatment of various diseases like nervous disorders, cardiovascular diseases, tumors, ageing, hypo-sexuality, etc. A significant decrease in natural production of *Cordyceps* Spp. has been observed in the last few decades from protected biosphere reserves, due to thorough and illegal harvesting. This compels the necessity of artificial cultivation and strain improvement strategies. For identifying improved strains of the fungi, different methods using antifungal agents, UV irradiation and antibiotic compound have been carried out for effective selection systems. The two fungi did not show any marker when treated with UV-irradiated conidia for ammonia assimilation. Further when these isolates were treated with some antifungal agents, again no remarkable marker was found against any antifungal agents. Consecutively, an antibiotic hygromycin was also tested against the isolates. The gradient decrease in radial growth was observed on increasing the concentration of antibiotic from 200 to 900 μg/ml showing a radial zone of 15mm to 4mm in *Cordyceps sinensis*. While the isolate *Cordyceps militaris* is found to be highly susceptible showing no growth in any of the concentrations of hygromycin. This may be the evidence for the presence of selectable marker gene *hph* in the isolates and can be used as a selectable marker tool for selection in the strain improvement of *Cordyceps* Spp.

Keywords

Cordyceps Spp. · Strain improvement · Marker · Hygromycin

L. Deshmukh (✉) · D. Gupta · S. S. Sandhu
Fungal Biotechnology and Invertebrate Pathology Laboratory, Department of Biological Science, R.D. University, Jabalpur, Madhya Pradesh, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_6

6.1 Introduction

Today's research is paying attention towards strain improvement strategies for increasing productivity to broaden the application potential. Fungi are second largest and diverse group of organisms existing in nature after insects [1]. Medicinal mushrooms are thought to produce bio-metabolites seizing various medicinal functions including antitumor, antioxidant, radical scavenging, immunomodulating, cardiovascular, anti-hypercholesterolemic, detoxification, antiviral, antibacterial, antiparasitic, antifungal, hepatoprotective and antidiabetic effects [2]. The awareness on development of genomic assets of entomopathogenic fungi are emerging day by day [3, 4]. Genus *Cordyceps* is an entomopathogenic higher fungus parasitizes on larva of Lepidoptera and has been used as traditional medicine since ancient times [5, 6]. Hike in price of wild *Cordyceps* mushroom and capsule, i.e., $32,000/kg and $5.8/g, respectively, justified the name "soft gold mushroom". This hike is due to imbalance in supply and demand in the marketplace [7, 8]. The collection difficulties and insufficient data availability regarding its production compel the necessity of artificial cultivation and strain improvement strategies for this mushroom so that large amount of biomass for functional foods and medicines becomes easy to harvest [9].

The genetic makeup and molecular biology of these fungi are indispensable to understand the in vitro production of fruiting body and bio-metabolites like cordycepin and polysaccharides. In favour of enhancing the metabolic capacities of *Cordyceps* Spp., genetic improvement and manipulation are mandatory. The selection and identification of these genetic changes can be established inside the organisms through some selectable and non-selectable markers [10]. In the present work, the methods used for detecting the presence of markers in the genome of *C. militaris* and *C. sinensis* are UV irradiation, antifungal drugs and antibiotics.

6.2 Materials and Methods

6.2.1 Sample Collection and Maintenance

Cordyceps sinensis was collected from Pithoragarh District of Uttarakhand, and lyophilized culture of *Cordyceps militaris* (3936) was procured from MTCC Chandigarh, India. Both fungal isolates were grown on different media to optimize their higher growth rate at incubation of 20°C for 10 days. Pure cultures were maintained on optimized media PPDA (Potato dextrose agar supplemented with peptone) for *C. sinensis* and SMYEA (Sabouraud maltose agar supplemented with yeast extract) for *C. militaris* at 4°C for further experimental purpose.

6.2.2 Marker Development

Different methods were used to develop an efficient marker to achieve targeted function and their responsible altered gene performance.

6.2.2.1 UV Irradiation

Fungal suspensions of both the cultures were made in 0.05% Tween-80 solution (1×10^5 conidia ml^{-1}) and were spread on sterilized media plates. All plates were exposed under UV radiation (15 cm apart) for different time intervals ranging from 15 to 120 min in protected UV chamber. All UV-irradiated plates were incubated under dark condition at 20 °C. Further, UV-irradiated grown colonies were tested for nitrogen assimilation under different nitrogen sources (sodium nitrate, sodium nitrite, ammonium chloride, glutamate).

6.2.2.2 Antifungal Sensitivity Test

Antifungal agents like griseofulvin, ketoconazole and clotrimazole were selected. Fungal cultures were grown on media supplemented with antifungal agents in different concentration range from 0.5 to 2.5 mg/ml. Growth patterns of inoculated cultures were measured by means of colonial diameter with zone measuring scale (Himedia Laboratories, India).

6.2.2.3 Antibiotic Sensitivity Test

To test the sensitivity of *C. militaris* and *C. sinensis* against selected antibiotic, cultures were inoculated on different concentrations of hygromycin B antibiotic (200–900 μg/ml) supplemented in media plates. Growth pattern of fungus determines the sensitivity of antibiotic.

6.3 Results

6.3.1 *Development of Marker*

The effect of UV irradiation on both the cultures was determined. 27 and 31 colonies of *C. militaris* and *C. sinensis*, respectively, were procured from UV-irradiated culture. A total of 58 colonies were tested and observed for the screening of nitrogen assimilation, but none of colonies showed any marker when treated with UV-irradiated conidia. All colonies were grown normally when growth media was supplemented with different nitrogen sources. This result indicated that no nitrogen assimilation gene is defected by UV irradiation (Fig. 6.1).

In this sequence, when these isolates (*C. militaris* and *C. sinensis*) were treated with three antifungal agents like griseofulvin, ketoconazole and clotrimazole, no remarkable marker was found against any of the tested antifungal agents. Both cultures were showing moderate resistance against the used three antifungal agents; no sensitivity was shown by *C. militaris* and *C. sinensis* (Fig. 6.2).

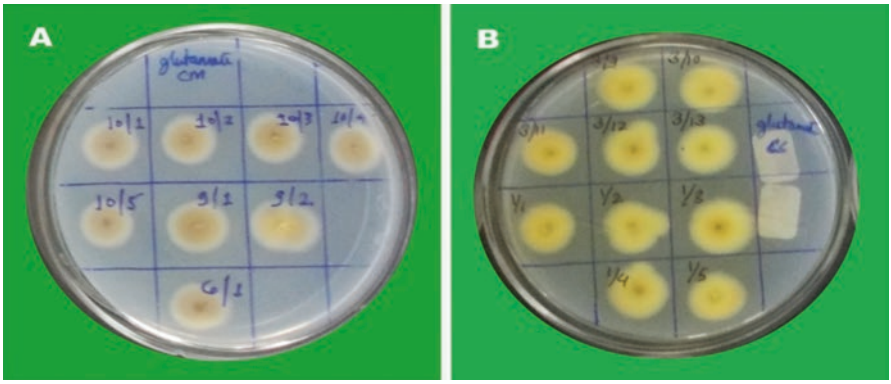


Fig. 6.1 UV-irradiated colonies tested under nitrogen sources. (**a**) *C. militaris,* (**b**) *C. sinensis*

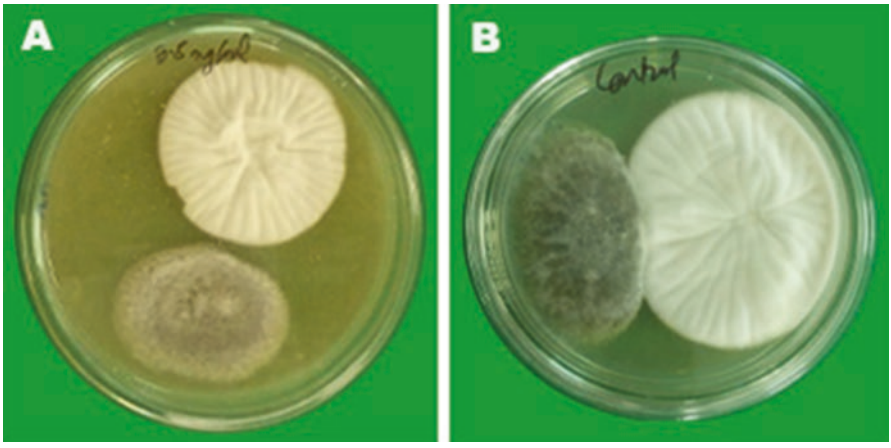


Fig. 6.2 Antifungal sensitivity test of *C. militaris* and *C. sinensis* (**a**) Test plate, (**b**) Control

The antibiotic hygromycin B was used to test sensitivity against the isolates (*C. militaris* and *C. sinensis*). The minimum inhibitory concentration of hygromycin B on the growth of *C. militaris* and *C. sinensis* was successfully determined. 200 µg/ml concentration of hygromycin B was completely able to inhibit the growth of *C. militaris* which suggested that *C. militaris* was completely sensitive on this concentration. It is apparent that *Cordyceps militaris* is highly susceptible and shows no growth in any of the concentrations of hygromycin (Fig. 6.3). However, a ramping decrease in radial growth was observed on increasing the concentration of antibiotic from 200 µg/ml to 900 µg/ml showing a radial zone of 15 mm to 4 mm in *Cordyceps sinensis* as depicted in Table 6.1 and Graph 6.1 This might be the evidence of the presence of selectable marker gene *hph* in the isolates and can be used as a selectable marker tool for selection in the strain improvement of aforesaid *Cordyceps* Spp.

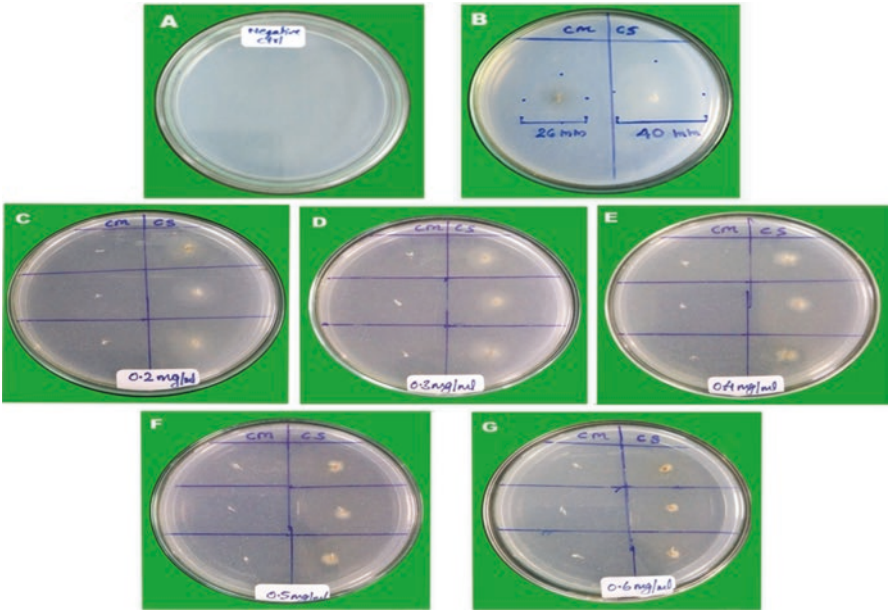


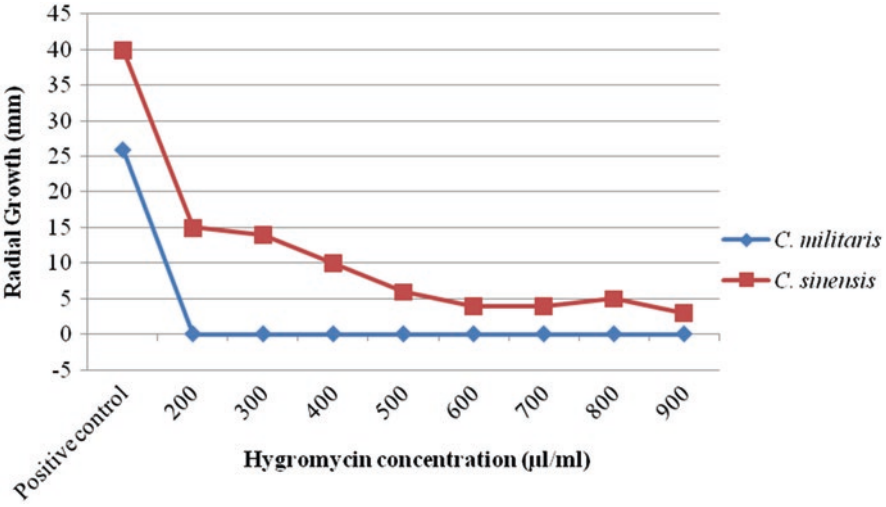
Fig. 6.3 Sensitivity test of antibiotic hygromycin B on *C. militaris* and *C. sinensis* (**a**) Negative control, (**b**) positive control, (**c**, **d**, **e**, **f** and **g**) growth of *C. militaris* and *C. sinensis* on growth media containing increasing order of hygromycin concentration

Table 6.1 Antibiotic sensitivity test of *C. militaris* and *C. sinensis* by hygromycin B

S.No	Hygromycin concentration (µg/ml)	*C. militaris* (mm)	*C. sinensis* (mm)	S.No	Hygromycin concentration (µg/ml)	*C. militaris* (mm)	*C. sinensis* (mm)
1.	Positive control	26	40	6.	600	00	4
2.	200	00	15	7.	700	00	4
3.	300	00	14	8.	800	00	5
4.	400	00	10	9.	900	00	3
5.	500	00	6	–			

6.4 Discussion

The market demand of *Cordyceps* Spp. regarding its medicinal value is increasing sharply [11]. Strain improvement techniques for increased production of this species are very crucial. For generating fungal mutants, inactivation of gene is found to be a very feasible one [12]. Strain improvement techniques like transformation and protoplast fusion require markers for selection of transformants and fusants. The provision for genetic engineering of strain requires homologous integration of gene, a recyclable marker which is a bidirectional positive selection system of transformants [13]. Most of the cultures in laboratories tolerate ampicillin, chloramphenicol, erythromycin and tetracycline like antibiotics at their much high dose



Graph 6.1 Graphical representation of effect of hygromycin B on *C. militaris* and *C. sinensis* growth on solid media

for resistance cassettes; hence aminoglycoside resistance marker like hygromycin B from *Streptomyces hygroscopicus* is used for positive selection [14]. In a study by Zheng et al. [15], growth of *Cordyceps militaris* JM4 was found to be inhibited by hygromycin B at the concentration of 400 µg/ml, and transformants of *C. militaris* were detected in selective PPDA plates by the presence of hygromycin B resistance gene in its integration DNA. The development of marker inside an organism can be versatile in function to understand the genetic diversity and structure. The mating system and ecology in *Cordyceps* Spp. can also be used in the study by these markers [10].

6.5 Conclusion

In the present work, an attempt to develop markers for *C. militaris* and *C. sinensis* was done. While looking for nitrogen assimilation defect, the UV-irradiated colonies of cultures did not show any of the defects. The cultures were also found resistant to antifungal agents like griseofulvin, ketoconazole and clotrimazole. The antibiotic sensitivity test of *C. militaris* and *C. sinensis* with hygromycin B showed positive selection system for improved strains. *C. militaris* was found highly susceptible to hygromycin, whereas *C. sinensis* shows a gradient decrease in radial growth from 15 mm to 4 mm on increasing concentration of 200 µg/ml to 900 µg/ml. Therefore, these can serve as valuable tool for genetic analysis of strains and also for selection of transformants. This will help for desirable strain development with higher production rate as well as resource conservation of this important medicinal mushroom *Cordyceps*.

Acknowledgements The authors owe huge gratitude to the Vice Chancellor, R.D. University Jabalpur, (M.P.). An eternal gratefulness to the Head, Department of Biological Science, R.D. University, Jabalpur, (M.P.), for allowing the authors to complete this research. We thank Bio-Design Innovation Centre, Department of Biological Science, R.D. University, Jabalpur, (M.P.), India, for providing facilities and funding to complete this research and bringing it to the world.

References

1. Mueller GM, Bills GF, Foster MS (eds) (2004) Biodiversity of fungi: inventory and monitoring methods. Elsevier Academic Press, Boston
2. Wasser SP (2014) Medicinal mushroom science: current perspectives, advances, evidences, and challenges. Biomed J 37(6):345–356
3. Wichadakul D, Kobmoo N, Ingsriswang S et al (2015) Insights from the genome of *Ophiocordyceps polyrhachis-furcata* to pathogenicity and host specificity in insect fungi. BMC Genomics 16(2015):881
4. Chiu CP, Hwangb TL, Chane Y et al (2016) Research and development of *Cordyceps* in Taiwan. Food Sci Human Wellness 5(2016):177–185
5. Seth R, Haider SZ, Mohan M (2014) Pharmacology, phytochemistry and traditional uses of *Cordyceps sinensis* (Berk.) Sacc: a recent update for future prospects. Indian J Tradit Knowl 13(3):551–556
6. Nakamura K, Shinozuka K, Yoshikawa N (2015) Anticancer and antimetastatic effects of cordycepin, an active component of *Cordyceps sinensis*. J Pharmacol Sci 127(1):53–56
7. Winkler D (2008) Yartsa gunbu (*Cordyceps sinensis*) and the fungal commodification of Tibet's rural economy. Econ Bot 62(3):291–305
8. Au D, Wang L, Yang D, Mok DK, Chan AS, Xu H (2012) Application of microscopy in authentication of valuable Chinese medicine I—*Cordyceps sinensis*, its counterfeits, and related products. Microsc Res Tech 75(1):54–64
9. Shih IL, Tsai KL, Hsieh C (2007) Effects of culture conditions on the mycelia growth and bioactive metabolite production in submerged culture of *Cordyceps militaris*. Biochem Eng J 33(3):193–201
10. Kim KY, Hong IP, Kang JH, Kim KH, Kim I, Lee HJ (2015) Development of twelve novel microsatellite markers in the medicinal mushroom *Cordyceps militaris* (Ascomycota: Clavicipitaceae) for its strain identification and genetic analysis. Entomol Res 45(6):328–331
11. Paterson RR (2008) *Cordyceps*: a traditional Chinese medicine and another fungal therapeutic biofactory? Phytochem 69(7):1469–1495
12. Fang W, Zhang Y, Yang X et al (2004) *Agrobacterium tumefaciens*-mediated transformation of *Beauveria bassiana* using an herbicide resistance gene as a selection marker. J Invertebr Pathol 85(1):18–24
13. Steiger MG, Vitikainen M, Uskonen P et al (2011) Transformation system for *Hypocrea jecorina* (*Trichoderma reesei*) that favors homologous integration and employs reusable bidirectionally selectable markers. Appl Environ Microbiol 77(1):114–121
14. Kalivoda EJ, Horzempa J, Stella NA et al (2011) New vector tools with a hygromycin resistance marker for use with opportunistic pathogens. Mol Biotechnol 48(1):7–14
15. Zheng Z, Huang C, Cao L, Xie C, Han R (2011) *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in medicinal fungus *Cordyceps militaris*. Fungal Biol 115(3):265–274

7 Optimization of Microwave-Assisted Pretreatment of Rice Straw with $FeCl_3$ in Combination with H_3PO_4 for Improving Enzymatic Hydrolysis

Bikash Kumar and Pradeep Verma

Abstract

Pretreatment is a key step to alter the recalcitrance structure of lignocellulosic biomass for enhancing enzymatic hydrolysis. Rice straw is an agricultural residue which is one of the potential substrate for ethanol production. In the present work, optimization of microwave-assisted pretreatment of rice straw in $FeCl_3$ solution with H_3PO_4 was performed. The effect of concentration of $FeCl_3$ and H_3PO_4 along with pretreatment time was evaluated. The optimal pretreatment condition was found as follows: 250mM $FeCl_3$, 3%H_3PO_4, 155°C, and 20 minutes. The pretreated pulp was subjected to enzymatic hydrolysis using commercial cellulase for assessing effectiveness of pretreatment system. The maximum saccharification per pulp and per biomass was observed as 98.9% and 66.4%, respectively, under enzyme load of 3 FPU/g of substrate after incubation for 48 h.

Keywords

Microwave · Enzymatic hydrolysis · Lignocellulosic biomass · Delignification

7.1 Introduction

The rapid increase in energy demand, fast depletion of fossil fuel reservoirs, and environment pollution caused by use of fossil fuels have forced the government and scientific community to search for alternative sources of energy generation that are inexpensive, eco-friendly, and renewable and can efficiently replace conventional fossil fuels [1]. Lignocellulosic biomass is regarded as one of the most promising alternative to fossil fuel. It can be transformed into biofuel and various

B. Kumar · P. Verma (✉)
Bioprocess and Bioenergy Laboratory, Department of Microbiology, Central University of Rajasthan, Ajmer, Rajasthan, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_7

value-added products which can offer sustainable system to help meet up this global necessity. Rice is one of staple crop of the world with annual production of 481.54 million metric tons of which India contributes 106.50 million metric tons (2016–2017) [2]. Each kilogram of rice obtained after harvesting generates 1–1.5 kg of the straw [3]. Based on this it can be estimated that about 106.5–159.8 million metric tons of rice straw was produced in India (2016–2017) of which a part goes as cattle feed. However, the remaining part is waste and disposal of which is a major concern. Most often these residues are burnt resulting in pollution. Thus, utilization of rice straw for its conversion to biofuel is one approach that can help in lignocellulosic waste management. The bioconversion of lignocellulosic biomass to ethanol involved three major steps: first pretreatment for the opening of the crystalline structure of cellulose by breaking down of lignin, second conversion of cellulose to glucose by hydrolysis with combination of enzymes, and third fermentation of sugars to ethanol [4]. Pretreatment is one of the most essential steps in the cost-effective conversion of lignocellulosic biomass to bioethanol or other bio-based products. The major goal of the pretreatment is to improve the accessibility of cellulose to the hydrolytic enzymes. Pretreatment breaks the physical barrier by disrupting the cell wall, removing hemicellulose or lignin fractions, reducing the cellulose crystallinity, improving porosity in the biomass structure, and increasing the accessible surface area. As a consequence, hydrolytic enzymes can easily access the cellulose fibers and act with higher efficiency. Previously various physical methods such as ball milling [5], irradiation [6], and chemical pretreatment methods such as ammonia [7], alkali [8], dilute acids [9], and organosolv process [10] have been utilized frequently which can help to modify the structural framework of lignocellulosic biomass and improve the saccharification of the cell wall carbohydrates [11]. However, the existing pretreatment technologies have certain limitations such as formation of inhibitory compounds which hinder fermentation, inadequate separation of cellulose and lignin, and considerable production of wastes [12].

In order to disrupt the recalcitrance structures of lignocelluloses, microwave heating has been used [13]. Unlike conventional heating microwave irradiation has offered advantage over conventional heating methods due to nonthermal or thermal effects [14]. Microwave irradiation has increased reaction rate and reduced reaction time. Verma and Chaturvedi [15] have carried out studies using microwave pretreatment of lignocellulosic biomass especially for the efficient enzymatic hydrolysis of woody biomass. Similar approach has also been used by Sindhu et al. [16] where they used microwave-assisted surfactant pretreatment of chilli postharvest residue for the production of bioethanol and biopolymer. On the other hand, some microwave sensitizer chemicals can enhance the effect of irradiation, which will result in improved delignification with negligible or no carbohydrate degradation [17]. However only few studies have been carried out especially for the enzymatic hydrolysis of rice straw using microwave system and microwave pretreatment. Liu et al. [18] demonstrated effect of $FeCl_3$ pretreatment on corn stover. In the present study,

optimization of microwave pretreatment of rice straw with $FeCl_3$ in combination with H_3PO_4 at different pretreatment time was evaluated. Enzymatic hydrolysis of the pretreated pulp was performed to assess the impact of the microwave-assisted $FeCl_3$ pretreatment on rice straw.

7.2 Materials and Methods

7.2.1 Materials

The chemicals used were purchased from HiMedia, India, and Merck, India. The chemicals are of analytical grades. Rice straw was collected from local farms of Rajasthan, India. They were washed with water in order to remove dirt and mud, followed by oven drying at 50 °C. They were grounded in an electric grinder, sieved to 2–5 mM mesh size, and stored in airtight polythene bags until use.

7.2.2 Microwave Pretreatment of Rice Straw Using $FeCl_3$

Rice straw (2g) was soaked in aqueous solution of $FeCl_3$ of different concentrations 50–400 mM for 24 h. The solid to liquid ratio was maintained at 20:1 (w/w) in all the experiment .The treatment was performed in a microwave reactor with a 700W magnetron "Microwave Reaction system SOLV, Multiwave Pro" (Make: Anton Paar, Austria). The program was set up as heating as fast as possible with high stirring during attaining of the desired temperature and holding temp for desired time with fast stirring, then followed by cooling the system to 70 °C with slow stirring before opening the system. After the reaction, the pulp fraction was separated by vacuum filtration and washed three times with 150 ml of distilled water. The pulp fraction or pulp yield was calculated by using equation

$$\text{Pulp Yield}(\%) = \frac{Wi - Wf}{Wi} * 100$$

where Wi and W_f are weight of substrate before and after pretreatment, respectively.

7.2.3 Effect of H_3PO_4 Concentrations

Rice straw (2g) was treated with different concentrations of H_3PO_4 (0.5–5%) at 155 °C for 30 min to study the effect of H_3PO_4 concentration on saccharification obtained from pulp fractions.

7.2.4 Effect of Pretreatment Time

Rice straw (2g) samples soaked in 250 mM $FeCl_3$ for 24 h and 3%H_3PO_4 were added just before start of reaction which were subjected to microwave treatment at 155 °C for 10, 20, and 30 min in order to study effect of pretreatment time. The pretreated pulp was subjected to enzymatic hydrolysis using commercial cellulase.

7.2.5 Enzymatic Saccharification of Pulp

The enzymatic saccharification of pulp was performed as method suggested by Verma et al. [17]. The wet pulp fraction was hydrolyzed with a commercial cellulase preparation, "ONOZUKA R-10" from *Trichoderma viride* (HiMedia). The cellulase enzyme loading was 1FPU/g substrate. Enzymatic hydrolysis was performed at a substrate concentration of 2% in 0.05 M sodium acetate buffer (pH 4.5) containing 0.02% sodium azide at 50 °C in rotary shaker water bath (Tempo, India) at 140 rpm for 48 h. The saccharification ratio per pulp was calculated according to the NREL LAP-009 procedure[19]. After enzymatic hydrolysis, 1ml sample was collected from each tube; the samples were placed in 1.5 ml eppendorf tubes, and then the solutions were centrifuged at 5000 rpm for 5 min. The appropriate dilutions were made to estimate sugar yield using dinitrosalicylic assay [20].

The saccharification ratio per pulp was evaluated based on how much pulp fraction was susceptible to the enzymatic hydrolysis. The saccharification per biomass was based on the weight percentage of reducing sugar to the original biomass. All enzymatic hydrolysis experiments were performed in duplicate.

7.2.6 Effect of Enzyme Load on Saccharification Yield on Pretreated Pulp

Rice straw (2g) was pretreated with 250 mM $FeCl_3$ in combination with 3% H_3PO_4 at 155 °C for 20 min, and then the obtained pretreated pulp fractions were hydrolyzed with different concentrations i.e. 1, 3, and 5 FPU/g (filter paper unit per gram of substrate) of commercial cellulase "ONOZUKA R-10" in order to examine maximum saccharification yield for evaluating the optimum enzyme dose. The control set was incubated without addition of enzyme.

7.3 Results and Discussion

7.3.1 Microwave Pretreatment of Rice Straw Using $FeCl_3$

In the present work, we compared the effect of different concentrations of $FeCl_3$ (50–400 mM) on microwave treatment of rice straw at 155 °C for 30 min. The maximum pulp yield of 90.2% was observed for control (soaked in distilled water) which

may be because of minimum breakdown of lignocellulosic constituents of rice straw. With increase in concentration of $FeCl_3$, pulp yield gradually decreased which may be due to loss of lignin (data not shown) and breakdown of carbohydrate polymer. The obtained pulp was subjected to enzymatic hydrolysis using commercial cellulase. The maximum saccharification per pulp and per biomass of 43.1% and 25.3%, respectively, (Fig. 7.1a) was obtained with 250 mM $FeCl_3$.

The results clearly show that with an increase in $FeCl_3$ concentration from 50 to 250 mM, the saccharification yield gradually increased; however above 250 mM decrease in the saccharification yield was observed which may be due to loss of cellulosic part. The saccharification yield per biomass was 10.1-fold higher than the control, which was better than the results obtained by Lü and Zhou [21]. However, they pretreated rice straw in microwave at 140 mM $FeCl_3$, 160 °C for 19 min followed by utilization of *Trichoderma viride* and *Bacillus pumilus* for the production of reducing sugars.

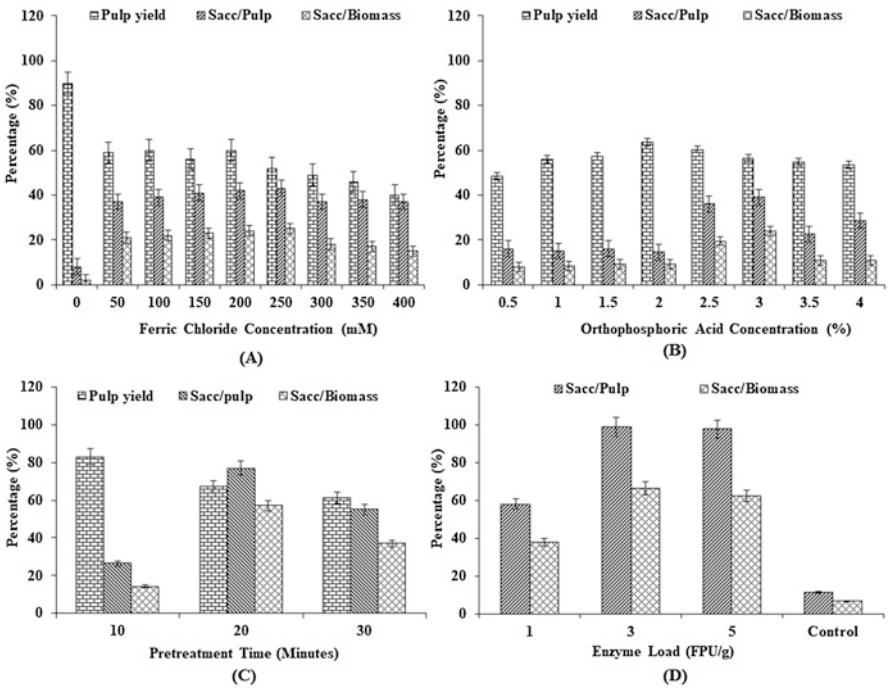


Fig. 7.1 (**a**) Pulp yield and saccharification yields of microwave pretreated rice straw in different concentrations (50–400 mM) of $FeCl_3$ at 155 °C for 30 min. (**b**) Pulp yield and saccharification yields of microwave pretreated rice straw in different H_3PO_4 concentrations (0.5–4%) at 155 °C for 30 min. (**c**) Pulp yield and saccharification yields of microwave pretreated rice straw in 250 mM $FeCl_3$ with 3% H_3PO_4 at 155°C for different time intervals. (**d**) Effect of enzyme dose on saccharification yield of the pretreated pulp obtained after microwave pretreatment of rice straw with 250 mM $FeCl_3$ and 3% H_3PO_4 at 155 °C for 20 min

7.3.2 Effect of H_3PO_4 Concentrations

The rice straw was subjected to addition of different concentrations (0.5–4%) of H_3PO_4 for microwave treatment at 155 °C for 30 min. The maximum pulp yield of 63.7% was obtained by addition of 2% H_3PO_4. The maximum saccharification per pulp and per biomass obtained was 39.01% and 24.03%, respectively, (Fig.7.1b) for the pretreatment under 3% acid concentration.

7.3.3 Effect of Pretreatment Time

The rice straw was soaked in 250 mM $FeCl_3$ for 24 h; 3% H_3PO_4 was added with just before start of pretreatment. The pretreatment was performed at 155 °C for different pretreatment time, i.e., 10, 20, and 30 min. The maximum pulp yield of 82.97% was obtained after pretreatment of 10 min, whereas pulp yield of 67.13% and 61.43% for 20 and 30 min, respectively. Maximum saccharification per pulp and per biomass of 77.03% and 57.09%, respectively (Fig. 7.1c), was obtained for pretreatment for 20 min which was much higher as compared to saccharification yields for pretreatment for 10 and 30 min. This can be explained as low pretreatment time (10 min) caused low delignification of lignocellulosic biomass due to inefficient breakdown of lignin carbohydrate complexes that resulted in less accessibility of cellulase enzyme to cellulosic part. The pulp yields for 20 and 30 min of pretreatment are in comparable range, but the saccharification yield for 30-min pretreatment is relatively low. It can be due to loss of cellulosic component with long pretreatment time (30 min) which finally affects the overall saccharification yield. The optimum pretreatment time obtained was 20 min, which is comparable to the results obtained by Lü and Zhou [21] where the optimum irradiation time was obtained as 19 min.

7.3.4 Effect of Enzyme Dose on Saccharification Yields

The enzymatic hydrolysis was performed for pulp obtained by microwave pretreatment of rice straw in 250 mM $FeCl_3$ and 3% H_3PO_4 at 155 °C for 20 min with different cellulase enzyme load, i.e., 1, 3, and 5 FPU/g of substrate. The maximum saccharification per pulp and per biomass of 98.9% and 66.4%, respectively, was obtained at enzyme load of 3 FPU/g after 48 h of incubation (Fig. 7.1d). The saccharification per pulp and per biomass by enzymatic hydrolysis of pretreated rice straw with enzyme load of 5 FPU/g was obtained as 97.8 and 62.3, respectively, which are comparable to the saccharification yields obtained for enzyme load of 3 FPU/g. The enzyme load higher than 3 FPU/g did not result in any increase in saccharification yield; it may be due to its adsorption onto the substrate that restricted the diffusion process through the structure as explained by Martín et al. [22] where enzyme loading higher than 15 FPU/g did not result in any increase in initial rate.

7.4 Conclusion

The conditions for microwave-assisted $FeCl_3$ in combination with H_3PO_4 pretreatment of rice straw were optimized. The optimal conditions were found as follows: 250 mM $FeCl_3$ concentration, 3% H_3PO_4 concentration, 155 °C pretreatment temperature, and 20-min pretreatment time. Enzymatic hydrolysis of pretreated pulp with enzyme load (3 FPU/g) for 48 h resulted in maximum saccharification per pulp and per biomass as 98.9% and 66.4%, respectively. The pretreatment step and cost of enzyme are rate-limiting step of the commercial biofuel production. The present work gives an efficient pretreatment strategy resulting in high amount of reducing sugars at low enzyme load. It will lead to develop cost-effective lignocellulosic biorefinery concept.

Acknowledgment The authors acknowledge the financial support provided by Department of Biotechnology, Government of India through project sanction wide no: BT/PR7333/PBD/26/373/2012.

References

1. Wan C, Li Y (2011) Effectiveness of microbial pretreatment by *Ceriporiopsis subvermispora* on different biomass feedstocks. Bioresour Technol 102(16):7507–7512
2. USDA (2017) World Rice Production 2016/2017. Date of Access: 26 June 2017
3. Sawangkeaw R, Ngamprasertsith S (2017) Rice straw as feedstock for biorefineries. In: Timayev A, Kadyrov G (eds) Rice and rice straw: production, cultivation and uses. Nova Science Publishers, New York, pp 141–191
4. Binder JB, Raines RT (2010) Fermentable sugars by chemical hydrolysis of biomass. Proc Natl Acad Sci 107(10):4516–4521
5. Hideno A, Inoue H, Tsukahara K et al (2009) Wet disk milling pretreatment without sulfuric acid for enzymatic hydrolysis of rice straw. Bioresour Technol 100(10):2706–2711
6. Bak JS, Ko JK, Han YH et al (2009) Improved enzymatic hydrolysis yield of rice straw using electron beam irradiation pretreatment. Bioresour Technol 100(3):1285–1290
7. Zhong C, Lau MW, Balan V et al (2009) Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. Appl Microbiol Biotechnol 84(4):667–676
8. Zhang Q, Cai W (2008) Enzymatic hydrolysis of alkali-pretreated rice straw by *Trichoderma reesei* ZM4-F3. Biomass Bioenergy 32(12):1130–1135
9. Sumphanwanich J, Leepipatpiboon N, Srinorakutara T (2008) Evaluation of dilute-acid pretreated bagasse, corn cob and rice straw for ethanol fermentation by*Saccharomyces cerevisiae*. Ann Microbiol 58(2):219–225
10. Zhao X, Cheng K, Liu D (2009) Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl Microbiol Biotechnol 82(5):815–827
11. Chaturvedi V, Verma P (2013) An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products. 3 Biotech 3(5):415–431
12. Harmsen P, Huijgen W, López L et al (2010) Literature review of physical and chemical pretreatment processes for lignocellulosic biomass, Food & Biobased Research. Wageningen UR, Food & Biobased Research, Wageningen , pp 1–49
13. Hu Z, Wen Z (2008) Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment. Biochem Eng J 38(3):369–378

14. Kim HM, Choi YS, Lee DS et al (2017) Production of bio-sugar and bioethanol from coffee residue (CR) by acid-chlorite pretreatment. Bioresour Technol 236:194–201
15. Verma P, Chaturvedi V (2017) A comparative assessment of autoclave and microwave-assisted peroxometal complex in delignification of wood biomass for enhanced sugar production. In: Pandey KK, Ramakantha V, Chauhan SS et al (eds) Wood is good: current trends and future prospects in wood utilization. Springer, Singapore, pp 383–390
16. Sindhu R, Binod P, Mathew AK et al (2016) A novel microwave assisted surfactant pretreatment of chili post -harvest residue for the production of bioethanol and biopolymer. Ann Agri Crop Sci 2(1):30–35
17. Verma P, Watanabe T, Honda Y et al (2011) Microwave-assisted pretreatment of woody biomass with ammonium molybdate activated by H_2O_2. Bioresour Technol 102(4):3941–3945
18. Liu L, Sun J, Li M et al (2009) Enhanced enzymatic hydrolysis and structural features of corn stover by $FeCl_3$ pretreatment. Bioresour Technol 100(23):5853–5858
19. Brown L, Torget R (1996) Enzymatic saccharification of lignocellulosic biomass. Chemical analysis and testing task laboratory analytical procedure(LAP)-009, NREL, USA, pp 1–18
20. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428
21. Lü J, Zhou P (2011) Optimization of microwave-assisted $FeCl_3$ pretreatment conditions of rice straw and utilization of *Trichoderma viride* and *Bacillus pumilus* for production of reducing sugars. Bioresour Technol 102(13):6966–6971
22. Martín C, De Moraes Rocha GJ, Dos Santos JRA et al (2012) Enzyme loading dependence of cellulose hydrolysis of sugarcane bagasse. Quim Nova 35(10):1927–1930

8

Profiling Indolic Auxins Produced by the Strains of *Aspergillus* Using Novel HPTLC Technique

Dhavalkumar Patel, Anoshi Patel, Disha Vora, Kinjal Desai, Sudeshna Menon, Sebastian Vadakan, and Dweipayan Goswami

Abstract

Fungi are recognized to have dealings with plants by several known mechanisms, one of which is through the production of phytohormones, auxins. Indole-3-acetate (IAA), indole-3-butyrate (IBA), and indole-3-pyruvate (IPyA), i.e., indolic auxins, are most widely yield by the strains of fungi through which they interact with plants. Fungal strains produce these indolic auxins by tryptophan (Trp)-dependent and tryptophan-independent pathways. Under present study, we found *Aspergillus flavus* strain PGFW, *Aspergillus niger* strain BFW, and *Aspergillus caespitosus* strain DGFW as the utmost effective IAA-producing strains from the rhizosphere of well-growing wheat plant which was determined by spectrophotometric method that uses Salkowski reagent. This method though has a flaw that it is not specific to IAA but develops color by reacting with all the other indolic derivatives. We found that for the spectrophotometric method, the absorption maxima (λ_{max}) of the mixture containing indolic compounds tend to shift when compared to pure standard. To overcome this limitation, high-performance thin-layer chromatography (HPTLC)-based etiquette is technologically advanced for the first time to precisely detect and calculate the amount of IAA and IBA in the assortment of 100 to 1000 ng per spot ignoring other Trp derivatives. HPTLC analysis showed that all the three strains under current study could produce indolic auxins by Trp-dependent and Trp-independent pathways, but the amount of indolic auxins produced was enhanced in presence of Trp. These strains may act as phytoaugmentor or phytopathogen, but for their mode of action, they produce various indolic auxins which can be profiled by the novel method described in this paper.

D. Patel · A. Patel · D. Vora · K. Desai · S. Menon · S. Vadakan · D. Goswami (✉)
Department of Biochemistry and Biotechnology, St. Xavier's College (Autonomous), Ahmedabad, Gujarat, India
e-mail: dweipayan.goswami@sxca.edu.in

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_8

Keywords
Aspergillus spp. · Indolic compounds · HPTLC

8.1 Introduction

Fungi residing in the rhizosphere are known to yield auxins like indole-3-acetic acid (IAA) and benefit plant growth [3]. Such fungi live symbiotically with plants and supports plant growth in return plant provide sugars and amino acids to the fungi for their survival. Such fungi are named plant growth-promoting fungi (PGPF) [10]. The microbial biosynthesis of auxins has undertaken penetrating examination which has reasoned that diverse strains of fungi produce IAA from tryptophan (Trp) while other fungi produced IAA even in the absence of Trp [7].

Spectrophotometric technique is the most generally used to distinguish indolic subordinates delivered by microorganisms and parasites. The spectrophotometric strategy utilizes the old-fashioned Salkowski reagent ($FeCl_3$ broken down in perchloric corrosive/sulfuric corrosive) which reacts to the indolic subordinates to create color complexes [11]. This procedure is basic yet profoundly uncertain as it gives a non-particular color complex response with all the indolic subordinates fashioned by the growth and gives detection of aggregate indolic content rather than exact amount of IAA. High-performance thin-layer chromatography (HPTLC) is recently reported to be very truthful in distinguishing indolic units created by microbes [7].

Here for the first time, we are reporting the method development to analyze indolic molecules from fungi using HPTLC. For the study we chose three efficient IAA-producing strains from the rhizospheric soil of wheat plant. We also compared the senility of HPTLC method with routinely spectrophotometric.

8.2 Experimental

8.2.1 Materials and Reagents

Indolic auxins, standard IAA (99.0%) and standard IBA (99.0%), were procured from Hi-Media. Solvents isopropanol, n-butanol, methanol, and ammonia solution (25% v/v) were procured from Merck Ltd. Aluminum backed silica gel 60F254 TLC foils with 0.25-mm thickness were purchased from Merck (Darmstadt, Germany).

8.2.2 Instrumentation

The HPTLC apparatus used in the experimentations was obtained from CAMAG (Muttenz, Germany) which essentially contained of two parts: Linomat 5 which is a

programmed applicator snug with a Hamilton syringe (100 µl) for precise stacking of samples onto TLC foils; second is Scanner 3 for the scanning of HPTLC foils after drying.

8.2.3 Standard Solutions

Standard solutions of both the auxins (IAA and IBA) were prepared in pure methanol in a range of 100 µg/ml.

8.2.4 Strains of PGPF Used for Study

Three strains, *Aspergillus flavus* strain PGFW (KY964054), *Aspergillus niger* strain BFW (KY964055), and *Aspergillus caespitosus* strain DGFW (KY964056), capable to produce IAA-producing strains from the rhizospheric soil of *Triticum aestivum* (wheat) plant (22°61′N, 72°93′E).

8.2.5 Assessment of the IAA Produced by PGPF Strains by Means of the Traditional Spectrophotometric Method

The preliminary IAA-producing capability of these strains was estimated using conventional spectrophotometric method which involves the use of Salkowski reagent [8]. Fungal isolates were cultivated in the potato dextrose broth enhanced with Trp (1 mg/ml of broth) at 25 ± 2 °C for 168 h. From each of these isolates, their culture supernatants were assorted with Salkowski reagent (50 ml, 35% of perchloric acid and 1 ml 0.5 M $FeCl_3$ solution) in the proportion of 1:1. Pink color develops indicating the production of IAA, its optical density was taken at 530 nm. Concentration of the IAA bent was assessed against the standard curve of IAA in the array of 10–100 µg/ml.

8.2.6 Extraction of Indolic Auxins

Indolic auxins were haul out from culture supernatants as described by [6]. Briefly, fungal strains were grown for 168 h in the potato dextrose broth enhanced with Trp (1 mg/ml of broth) at 25 ± 2 °C. Culture supernatants were brought to pH 2.5 by means of 1 N HCl and mined thrice using equivalent volume of ethyl acetate. A portion of ethyl acetate was air desiccated and redissolved in one tenth volume of methanol [8]. These methanolic extracts were further used for HPTLC study.

8.2.7 HPTLC Procedure

8.2.7.1 Sample Application

Sample loading on TLC foils (sized 10 × 15 cm) was completed using a Linomat 5 applicator (CAMAG, Muttenz, Germany) which confined Hamilton 100 µl syringe.

8.2.7.2 Calibration Curves

Calibration curves of IAA and IBA (100 µg/ml) were set as per the technique previously developed by Goswami et al. [7] within the range of limit of detection (LOD) and limit of quantitation (LOQ). Each standard, i.e., IAA and IBA were loaded from 4 to 20 µl, was distinctly on TLC in the form of bands (size 10 × 10 cm). After proper loading of the standard, TLC plate was air dehydrated for 600 s, and the developed band were envisioned under UV with a wavelength of 254 nm. Pre-saturated twin chambers with the solvent system were used for development of TLC (containing standard). Solvent system used as mobile phase was isopropanol/n--butanol/ammonia/water [10:6:3:1 (v/v)]. TLC plates were air desiccated, and the developed band was scanned with Scanner 3 (Camag) in absorbance-reflectance mode at 254 nm.

8.2.7.3 Densitometric Analysis of Chromatogram

For quantitative densitometric examination, apex region of developed spots for distinct sample was measured by direct scanning at 254 nm utilizing the Camag TLC Scanner 3 with deuterium source at an inspection rate of 20 mm/s. The slit measurement setting was 6 mm in length into 0.45 mm in width, and data resolve was 100 µm per step utilizing filter factor Savitzky-Golay 7, pattern refinement was set to the least slant, and show scaling was set to programmed.

8.2.7.4 Determination of IAA from Extracted Indolic Auxins from Fungal Supernatant

Fungal methanolic extract (containing indolic auxins formed by fungal strains) and indolic standards (IAA and IBA) were laden on single TLC plate. On TLC plate (sized 15 × 10 cm), 16 bands were laden on entire TLC, where 2 bands laden with individual standards, 2 bands laden with combination of standards auxins (IAA and IBA), and 2 bands laden with each fungal methanolic extract in different volumes. For *Aspergillus flavus* strain PGFW, *Aspergillus niger* strain BFW, and *Aspergillus caespitosus* strain DGFW, the methanolic extracts laden per band were 20 µl and 30 µl correspondingly. TLC plate was developed in pre-saturated twin rack chamber as mentioned above densitometric analysis was completed to perceive and calculate IAA and IBA in the methanolic fungal extracts; measured values were equated with the values attained by means of the spectrophotometric method.

8.3 Results

8.3.1 IAA Production by PGPF Strains Examined by Means of Spectrophotometric Method

Spectrophotometric analysis recommended that all the three strains under current study could produce indolic auxins under both the conditions (culture medium with and without supplementation of Trp). Maximum values of IAA produced by these strains are represented in Table 8.1.

8.3.2 HPTLC

8.3.2.1 Calibration Curves

TLC plate laden with standard indoles were allowed to advance in twin rack chamber pre-saturated with solvent system isopropanol/n-butanol/ammonia/water [10:6:3:1 (v/v/v)] for 75 min. On development, the TLC plate was air desiccated and studied for retention factor (R_f) values underneath short UV (254-nm wavelength) where R_f value of IAA was 0.63 and R_f value of IBA was 0.70. For concocting calibration curve, each standard was laden independently extending from 400 to 2000 ng per band. Densitometric study presumed that Trp possessed linear correlation with concentration of standard laden and peak zone of the developed band in the range of 100–1000 ng per spot. Standard auxins exhibited parallel linearity in the array of 100–1000 ng per band and 100–500 ng per band of IAA and IBA correspondingly. Qualified visualization of standard IAA and IBA standards on solitary TLC is revealed in Fig. 8.1. Further, LOD and LOQ were determined for each standard auxin (IAA and IBA) as of the calibration curve where which were 96.1 ng and 291.2 ng per spot correspondingly for IAA and 83.4 ng and 252.7 ng per spot correspondingly for IBA.

8.3.2.2 Determination of IAA and IBA from Fungal Supernatant Containing Indolic Auxins

On precise standardization, this method was rummage sale to perceive and quantify IAA and IBA from fungal extracts. TLC after development is shown in Fig. 8.2. Bands 1 and 2 are laden with standard IAA (20 µl and 30 µl individually), bands 3 and 4 are laden with standard IBA (20 µl and 30 µl individually), bands 5 and 6 are laden with extracted indolic auxin from *A. flavus* without Trp in media (20 µl and 30 µl individually), bands 7 and 8 are laden with extracted indolic auxin from *A. flavus* strain PGFW in media supplemented with Trp (20 µl and 30 µl respectively), bands 9 and 10 are laden with extracted indolic auxins from *A. niger* strain BFW without Trp in media (20 µl and 30 µl individually), bands 11 and 12 are laden with extracted indolic auxin from *A. niger* strain BFW in the media supplemented with Trp (20 µl and 30 µl individually), bands 13 and 14 are laden with extracted indolic auxins from *A. caespitosus* strain DGFW without Trp in media (20 µl and 30 µl individually), and bands 15 and 16 are laden with extracted indolic auxin from *A.*

Table 8.1 HPTLC analysis of IAA from three Aspergillus spp.

Strains	Culture media	Sample loaded	Area under curve	ng of IAA per band	ng of IAA per ml sample	µg of IAA per sample	µg of IAA per ml of broth	µg of IAA per ml of broth	Spectrophotometric results showing production (µg of IAA per ml of broth)
A. flavus strain PGFW	without Trp	20	10173.00	3681.30	184065.01	184.07	18.41	17.58 ± 1.17	85.67 ± 4.33
		30	13519.10	5024.04	167467.90	167.47	16.75		
	with Trp	20	16628.90	6271.95	313597.51	313.60	31.36	30.10 ± 1.78	112.29 ± 8.34
		30	22566.50	8654.61	288487.16	288.49	28.85		
A. niger strain BFW	without Trp	20	7551.90	2629.49	131474.72	131.47	13.15	12.26 ± 1.26	82.67 ± 3.22
		30	9498.60	3410.67	113689.14	113.69	11.37		
	with Trp	20	12703.60	4696.79	234839.49	234.84	23.48	23.25 ± 0.33	139.72 ± 7.44
		30	18205.00	6904.41	230147.14	230.15	23.01		
A. caespitosus strain DGFW	without Trp	20	8534.50	3023.80	151189.81	151.19	15.12	14.55 ± 0.81	106.62 ± 6.44
		30	11445.90	4192.09	139736.49	139.74	13.97		
	with Trp	20	13552.10	5037.28	251863.96	251.86	25.19	26.54 ± 1.92	156.08 ± 5.44
		30	21855.60	8369.34	278978.06	278.98	27.90		

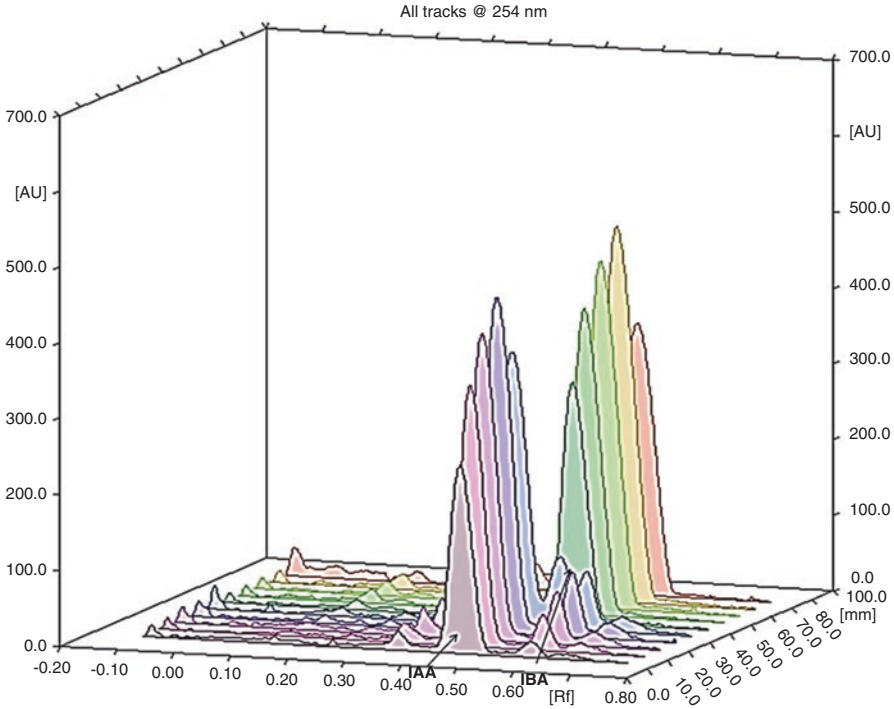


Fig. 8.1 Displays the 3D densitogram of the TLC foil (same) where concentration of peak defines the area underneath the curve. It can be understood that as the concentration of standard auxin (IAA and IBA) increases as the zone underneath the curve for an equivalent band also rises

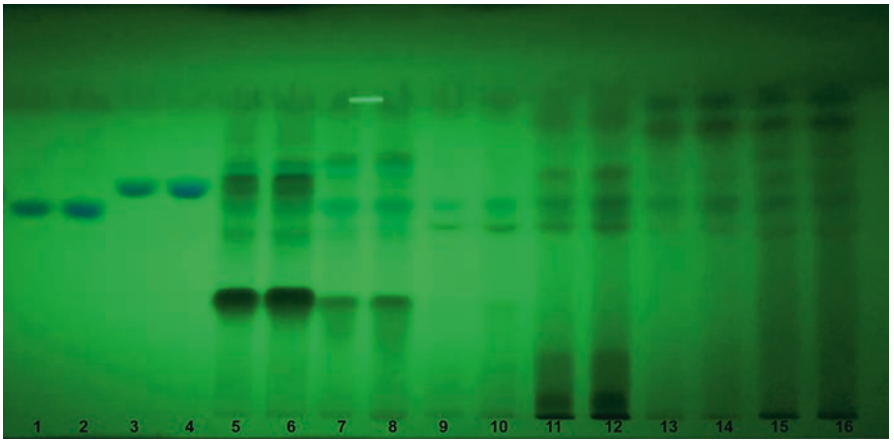


Fig. 8.2 Display the image of TLC foil under 254-nm UV light

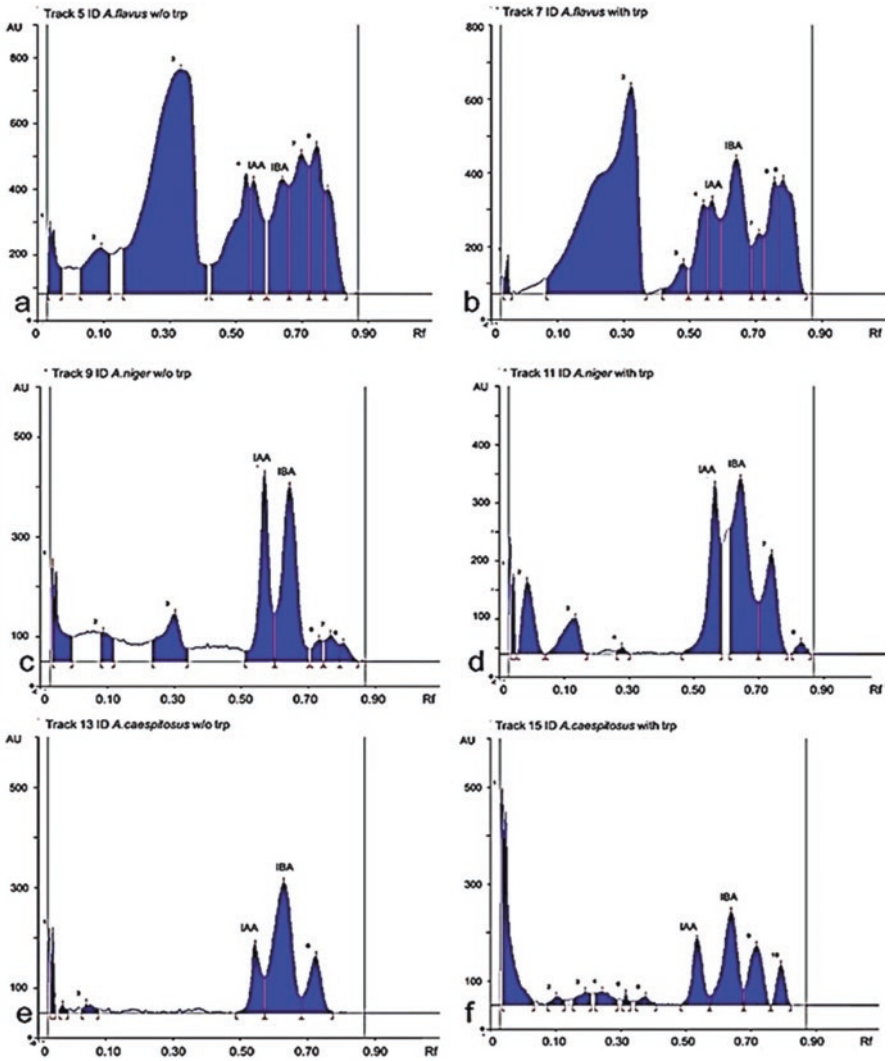


Fig. 8.3 Displays the 2D densitogram of fungal extracts containing indolic auxin compounds (**a**) band laden with extract of *A. flavus* strain PGFW without Trp (track 5 of this figure), (**b**) track laden with extract of *A. flavus* strain

caespitosus strain DGFW in media supplemented with Trp (20 µl and 30 µl individually). Area under the curves of IAA and IBA yield by fungal isolates are shown in Fig. 8.3. These values are used to calculate IAA produced by fungal isolates which is represented in Table 8.1.

It was found strains produced IAA or IBA or both. Their actual production detected by spectrophotometric method is much greater than the HPTLC-derived values. This is because fungal isolates produce several other molecules other than

IAA and IBA (Fig. 8.2) which may be falsely detected as IAA by the spectrophotometric method.

8.4 Discussion

Spectrophotometric technique is the most extensively used to perceive indolic derivatives from Trp among the existing approaches. The spectrophotometric technique uses the outmoded Salkowski reagent ($FeCl_3$ dissolved in perchloric acid/sulfuric acid) which responds to the indolic auxins to develop color [1, 6, 10]. This technique is easy and simple but extremely imprecise as it gives a non-precise color response with all the indolic auxin produced by fungi and make available detection of entire indole content relatively than precise detection of both IAA and IBA. Therefore, the spectrophotometric technique provides inaccurate amounts of IAA/IBA produced by PGPFs [13]. Excluding the spectrophotometric technique, thin-layer chromatographic (TLC) technique [8, 9, 12] and high-performance liquid chromatographic (HPLC) approaches are also castoff [12]. TLC affords merely qualitative detection of indolic auxins, while HPLC is very delicate [2], but needs extremely purified samples which make procedure of sample preparation monotonous; HPLC study also necessitates longer time intervals for detection and standardization [4, 5].

8.5 Conclusion

Hence, in this paper we account the use of high-performance thin-layer chromatography (HPTLC) for the immediate detection and quantification of IAA produced by quite a few PGPFs. We are also providing comparison of its sensitivity with outmoded spectrophotometric technique. To the best of our data, this is the foremost report which describes quantification of IAA and IBA from fungal isolates by means of HPTLC.

Acknowledgments Authors are thankful to the Gujarat State Biotechnology Mission (GSBTM) for providing the funding under FAP 2016 GSBTM/MD/PROJECTS/SSA/5041/2016-17 project and St. Xaveir's College (Autonomous), Ahmedabad-380009 for providing necessary facilities.

References

1. Akbari GA, Arab SM, Alikhani HA, Allakdadi I, Arzanesh MH (2007) Isolation and selection of indigenous *Azospirillum* spp. and the IAA of superior strains effects on wheat roots. World J Agric Sci (WJAS) 3(4):523–529
2. Beni A, Soki E, Lajtha K, Fekete I (2014) An optimized HPLC method for soil fungal biomass determination and its application to a detritus manipulation study. J Microbiol Methods 103:124–130

3. De-Palma M, D'Agostino N, Proietti S, Bertini L, Lorit M, Ruocco M, Tucci M (2016) Suppression Subtractive Hybridization analysis provides new insights into the tomato (*Solanum lycopersicum* L.) response to the plant probiotic microorganism *Trichoderma longibrachiatum* MK1. J Plant Physiol 190:79–94
4. Dhandhukia PC, Thakkar VR (2008) Separation and quantitation of jasmonic acid using HPTLC. J Chromatogr Sci 46(4):320–324
5. Dhandhukia PC, Thakker JN (2011) Quantitative analysis and validation of method using HPTLC. In: High-Performance Thin-Layer Chromatography (HPTLC). Springer-Verlag, Berlin/Heidelberg, pp 203–221
6. Glickmann E, Dessaux Y (1995) A critical examination of the specificity of the salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol 61(2):793–796
7. Goswami D, Thakker JN, Dhandhukia PC (2015) Simultaneous detection and quantification of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) produced by rhizofungi from l-tryptophan (Trp) using HPTLC. J Microbiol Methods 110:7–14
8. Goswami D, Vaghela H, Parmar S, Dhandhukia P, Thakker JN (2013) Plant growth promoting potentials of *Pseudomonas* spp. strain OG isolated from marine water. J Plant Interact 8(4):281–290
9. Hartmann A, Singh M, Klingmüller W (1983) Isolation and characterization of Azospirillum mutants excreting high amounts of indoleacetic acid. Can J Microbiol 29(8):916–923
10. Karnwal A (2009) Production of indole acetic acid by fluorescent *Pseudomonas* in the presence of L-tryptophan and rice root exudates. J Plant Pathol Microbiol 91:61–63
11. Meiners SJ, Phipps KK, Pendergast TH, Canam T, Carson WP (2017) Soil microbial communities alter leaf chemistry and influence allelopathic potential among coexisting plant species. Oecologia 203(4):1155–1165
12. Swain MR, Naskar SK, Ray RC (2007) Indole-3-acetic acid production and effect on sprouting of yam (*Dioscorea rotundata* L.) minisetts by *Bacillus subtilis* isolated from culturable cowdung microflora. Pol J Microbiol 56(2):103
13. Szkop M, Bielawski W (2013) A simple method for simultaneous RP-HPLC determination of indolic compounds related to fungal biosynthesis of indole-3-acetic acid. Antonie Van Leeuwenhoek 103(3):683–691

9 Comparative Analysis of Cytotoxic Potential of Crude Extracts and Fractionated Isolates from *Moringa oleifera* Lam.

Kinjal Desai and Vincent Braganza

Abstract

According to World Health Organization, most of the world's population depends upon plants as an important element in primary healthcare systems. Ayurveda is India's oldest indigenous medicine system of plant drugs. It is known from for preventing or suppressing various tumors using natural drugs, one such being *Moringa oleifera* Lam. This plant has reported antioxidant properties for both fruits and leaves (Luqman S, Srivastava S, Kumar R, Maurya AK, Chanda D, Evid Based Complement Alternat Med 2012(December):e519084. https://doi.org/10.1155/2012/519084, 2011); moreover, its extracts have exhibited anticancer properties in vitro in case of hepatocarcinoma as well as antitumor-promoting activities for skin cancer in rat models (Guevara AP, Vargas C, Sakurai H, Fujiwara Y, Hashimoto K, Maoka T, Kozuka M, Ito Y, Tokuda H, Nishino H, Mutat Res/Genet Toxicol Environ Mutagen 440(2):181–188. https://doi.org/10.1016/S1383-5718(99)00025-X, 1999).

The aim of this study is to investigate the antiproliferative activity of various extracts from *Moringa oleifera* Lam. and isolate the active compounds. Fifteen extracts were prepared form dried leaves of *Moringa oleifera* Lam. using five solvent systems and three methods of extraction. In vitro screening was done using *Schizosaccharomyces pombe* and MCF-7 cell line. The fractionation of active crude extracts was performed by silica gel column chromatography and fractions evaluated for cytotoxicity. The aqueous, methanolic and hydromethanolic extracts exhibited cytotoxicity against MCF-7 cell line at lower concentrations compared to lymphocytes. Our findings showed the following: (a) the crude extracts of *Moringa oleifera* Lam. exhibited cytotoxic potential; (b) the extracts

K. Desai
Department of Biochemistry, St Xavier's College, Ahmedabad, Gujarat, India

V. Braganza (✉)
Loyola Centre for Research and Development, Ahmedabad, Gujarat, India
e-mail: vincentbraganza@xrf.res.in

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_9

were selectively more toxic to tumor cell line compared to normal lymphocytes. (c) The crude extract showed better anti-proliferative activity compared to fraction separated. The reason could be that the compounds in crude extract have a synergistic effect resulting in better activity.

Keywords
Antiproliferative · *Schizosaccharomyces pombe* MCF-7 · Cytotoxic potential

9.1 Introduction

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years [3]. Several groundbreaking and important drugs have been isolated from plants. Some significant examples are quinine (antimalaria), morphine (pain reducer), Taxol and camptothecin (anticancer). Screening over 110,000 compounds from 35,000 plants by the National Cancer Institute for about 25 years led to the isolation from the bark of the Pacific yew tree, *Taxus brevifolia*, Taxol® (paclitaxel), a cytotoxic diterpene alkaloid [4]. Studies of Taxol against several cancer cells established its excellent anticancer potential, especially against leukemia cells [5].

Moringa oleifera Lam., belonging to family Moringaceae, is one of the most commonly cultivated species. The common name for this plant is drumstick tree, and it is widely cultivated in tropical and subtropical regions. It is a drought-resistant tree whose fruits and leaves are used in culinary preparations. It has also been used in herbal medicine [6].

All the plant parts have been used for various ailments, like the seeds are considered to be antipyretic and are reported to have antimicrobial activity. The leaves and its extract are believed to reduce blood glucose levels and are used to treat sores and burns. The roots of the plant have anti-inflammatory activities [7]. Reported phytochemical evaluation of *Moringa oleifera* Lam. has indicated the presence of various phytosterols, glucosinolates, and isothiocyanates [8]. The leaves of this plant exhibit antioxidant properties due to the presence of phenolics [9].

Biological activity and cytotoxicity of drugs, chemicals, and extracts are very commonly assessed using various model systems. Yeast has been one of the earliest species to be used as a model organism. Due to its sequence homology and conserved gene sequences, it serves as a good model for correlation with mammalian systems [10] so can be used for screening of new therapeutics. Another commonly used system is cell lines. This is generally a preliminary screening before clinical trials and also a methodology to narrow down to a specific fraction or chemical or drug that is effective. Thus, in our study, extracts that showed cytotoxicity in the prescreening study using mutant yeasts were selected for further screening using cell lines to confirm their therapeutic potential.

The focus of modern science is to identify and isolate active principles from plants. Their isolation can be achieved using bioassay-guided fractionation. The

process involved would be preparing crude extracts and fractionating the compounds and assessing each fraction for desired biological activity. Once such active fraction is obtained, further characterization would enable identification of the compounds. Hence, leading to discovery of new therapeutics form plants. Many such examples are reported of bioassay-guided fractionation where active principles have been successfully identified and isolated. For example, 2-methoxy-1, 4-naphthoquinone (MNQ), an antimicrobial compound, was identified and isolated from *Impatiens balsamina* L. using bioassay-guided fractionation [11]. Hence, fractionation, isolation, and identification are crucial steps for drug development from natural products coupled with efficacy and toxicity studies.

9.2 Materials and Method

9.2.1 Plant Material and Preparation of Crude Extracts

Plant materials originally bought from government nursery, Gandhinagar, and planted at the greenhouse of the Xavier Research Foundation were used for the study. The leaves were air dried under shade and crushed finely. This powder was then used for all extraction purposes. Three different experimental conditions [12–14], (i) lower temperatures (25 °C) (ii) elevated temperature and (iii) sonication, have been used for extraction. Moreover, five solvent systems were used which were (i) water, (ii) methanol, (iii) ethyl acetate, (iv) chloroform/methanol (1:1), and (v) water/methanol (1:1). The solvents were selected for the whole azeotropic range varying from polar to nonpolar solvents.

The entire range of solvents was used for each of the experimental conditions. For each of the conditions, 1gram of powder was weighed and suspended in 20 ml of selected solvent [15, 16].

The extraction procedure involved continuous agitation and changing of solvent until solvents were rendered colorless. The extracts obtained were pooled, and the solvent was allowed to evaporate in a pre-weighed crucible. The residue was resuspended in tissue culture grade DMSO to make a stock of 10mg/ml and stored at 4 °C [13].

9.2.2 Culturing Yeast and Cytotoxicity Assay

The study involved use of genetically modified yeast strain as a prescreening tool to check the effectiveness of the crude extracts. The yeast used for this study was *Schizosaccharomyces pombe* purchased from National Collection for Yeast Culture (NCYC) with accession number 1683. This yeast that has its *cdc 2* gene modified has an altered cell cycle. This model has been used to screen for antiproliferative activity of various crude extracts using methyl thiazole tetrazolium (MTT) assay [17, 18]. Moreover, MTT assay was performed for wild-type yeast as well as to ensure selective toxicity of extracts against mutant yeast. The media used for culturing yeast comprised of yeast extract, peptone, dextrose (YEPD) broth. For each

assay, a fresh inoculum was introduced into YEPD broth and allowed to grow until it gave an absorbance of 0.9 at 660 nm. After the incubation, 100µl of broth with cells (~10^6) were transferred to 96-well microtiter plate. The crude extracts (in triplicates) at concentrations of 50, 100, 150, and 200 µg per well were added. Blank wells contained YEPD without cells. Yeast cells without any extract were kept as control. DMSO was used as vehicle control. Vinblastine at concentration of 0.16 µM (IC_{50} of vinblastine) was used as positive control. After exposure of 24 h, 20 µl of MTT dye (2.5 mg/ml) was added to each well. The plate was then incubated in dark for 4 h. In order to dissolve the formazan crystals formed post the incubation, 100 µl of DMSO was added, and the plate agitated for 5 min. The absorbance was read at 570 nm using a microplate reader. Based on the absorbance, cell viability was calculated as shown below:

$$\text{Cell viability} = \frac{\text{ODof sample} - \text{OD of blank}}{\text{OD of yeast cells} - \text{OD of blank}}$$

Results of MTT assay for yeast were used to select extracts to be screened in the cell lines.

9.2.3 Culturing MCF-7 Cell Line and Screening Assay

The study was done on MCF-7 cell line purchased from National Cell Culture Collection (NCCS), Pune. The cell line was cultured using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in T-75 flasks. The flasks were maintained at 37 °C, 5% CO_2 in completely humidified atmosphere. The cells were used for cytotoxicity assay when they reached a confluence of 90%. These cells were resuspended at a concentration of 4×10^5 cells/ml and aliquoted into 96-well plate (100 µl/well). After 24 h, the cultivated cells were treated with crude extracts at concentrations of 0.1, 1.0, 10, and 100 µg/ml. Cells were exposed to vinblastine 0.16 µM (positive control) and DMSO 1% (vehicle control). Phosphate buffered saline (PBS) was added to three wells serving as control. MTT assay was performed 48 h after exposure [19]. The toxicity assessment of crude extracts was done using peripheral blood lymphocyte culture (PBLC) [20]. 1.0 ml of heparinized blood was withdrawn from a healthy donor and transferred to microfuge tube containing 0.1 ml of phytohemagglutinin (PHA). The tube was then incubated at 4 °C for 45 min and centrifuged at 350×g for 10 min. The erythrocytes settled at the bottom of the tube, and colorless fluid supernatant was transferred to T-75 flask containing RPMI-1640 supplemented with 20 mM glutamine and 1.5% sodium bicarbonate. The flask was incubated at 37 °C in a 5% CO_2 and humidified incubator for 48 h. After incubation, plated in a 96-well plate at density of 10^6 cells/ml (100 µl/well). The crude extracts were then introduced at concentrations of 0.1, 1.0, 10, and 100 µg/ml and incubated for 24 h. MTT assay was performed as mentioned earlier to assess cytotoxicity. The cell viability was calculated. Therefore, our study focuses on identifying cytotoxicity of crude extracts on breast cancer cell line and toxicity

assessment on lymphocytes as well. Based on the results, the crude extracts that exhibited toxicity against the tumor cell line and non-cytotoxic to lymphocytes were used for further experiments.

9.2.4 Column Chromatography and Fractionation of Crude Extracts

The crude extracts exhibiting cytotoxicity selectively against the cell lines were fractionated using silica gel column chromatography. Figure 9.1 depicts the scheme for fractionation of selected extract.

9.2.5 Statistical Analysis

The cytotoxicity assay was performed using MTT assay. The cytotoxic potential was correlated to decrease in viable number of cells. A dose-response curve was generated, and the different doses were compared using one-way ANOVA. Moreover, the various extracts were also compared to find significant difference in their efficacy. Analysis for the same was done using one-way ANOVA.

The inhibitory concentration (IC_{50}) is essential to compare efficacy of a compound to standards or other similar active principles. IC_{50} was calculated for all the extracts using regression analysis on a percent inhibition versus dose plot.

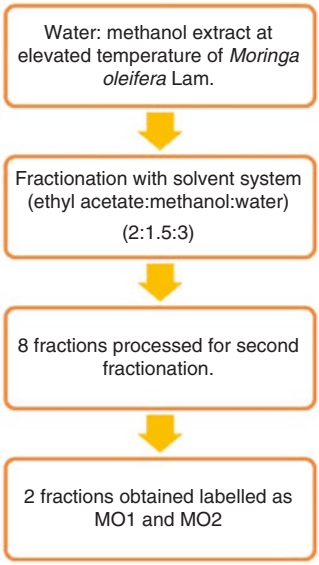


Fig. 9.1 Fractionation scheme for crude extract of *Moringa oleifera* Lam.

9.3 Results

9.3.1 Cytotoxicity Assay Using Yeast

The cell viability was calculated after performing MTT assay of yeast cells both wild and mutant exposed to various extracts at dose of 50–200 µg/ml. A dose-response curve was plotted, and linear regression analysis was performed to obtain IC_{50} values.

This study evaluates cytotoxicity potential of the extracts for both wild-type and mutant yeast cells to assess selective cytotoxicity. Table 9.1 compares IC_{50} of crude extracts of *Moringa oleifera* Lam. for mutant and wild-type yeast. The water, methanol, and water/methanol extracts had a significant difference (p value < 0.01) between IC_{50} values for wild-type and mutant yeast in all three methods employed for extract preparation.

9.3.2 MTT Assay of MCF-7 Cell Line

Based on the results obtained, the water, water/methanol, and methanol extracts for all three plants were chosen for study in cell line MCF-7.

The IC_{50} values for crude extracts of *Moringa oleifera* Lam. were calculated from dose-response curves and compared between MCF-7 cells and lymphocytes. The water/methanol extract at elevated temperature had an IC_{50} of 244.6 µg/ml and was least toxic to lymphocytes compared to other extracts. The methanol extract by sonication and at 25 °Cexhibited greater cytotoxicity at lower dose and were less

Table 9.1 IC_{50} values of crude extracts of *Moringa oleifera* Lam. for wild-type and mutant yeast

Method employed	Solvent system used	IC_{50} (µg/ml)		Significance
		Mutant	Wild type	
25 °C	Water	204.087	650.862	*
	Methanol	302.664	534.948	**
	Ethyl acetate	315.964	241.945	ns
	Chloroform/methanol	275.792	273.321	*
	Water/methanol	234.449	1157.89	**
Elevated temperature	Water	296.761	475.309	***
	Methanol	196.724	442.826	**
	Ethyl acetate	246.505	293.285	ns
	Chloroform/methanol	186.799	221.464	ns
	Water/methanol	144.225	479.292	**
Sonication	Water	287.270	407.896	**
	Methanol	192.607	391.567	***
	Ethyl acetate	234.059	240.956	ns
	Chloroform/methanol	243.971	237.273	ns
	Water/methanol	218.541	482.635	***

p values, *ns* = nonsignificant: *p* value > 0.05, **p* value < 0.05, ***p* value < 0.01, ****p* value < 0.001

Table 9.2 IC_{50} values of crude extracts of *Moringa oleifera* Lam. for MCF-7 and lymphocytes

Method employed	Solvent system used	IC_{50} (μg/ml)		Significance
		MCF-7	PBMC	
25 °C	Water	9744.8	1556.1	**
	Methanol	6502.4	1929.1	**
	Water/methanol	2213.3	14650.7	**
Elevated temperature	Water	2202.6	8082.1	**
	Methanol	7516.3	1418.5	**
	Water/methanol	244.6	1993.0	**
Sonication	Water	10881.1	3524.2	**
	Methanol	1130.0	170.71	**
	Water/methanol	1318.8	3640.9	**

p values, ns = nonsignificant: *p* value > 0.05, **p* value < 0.05, ***p* value < 0.01, ****p* value < 0.001

toxic to lymphocytes. The sonication extract was toxic to lymphocytes as seen in Table 9.2. Based on these results, the water/methanol extract (elevated temperature) was selected for fractionation and further study.

9.3.3 Cytotoxicity Assessment of Fractions and Comparison with Crude Extract

The cytotoxicity of fraction MO1 and MO2 was expressed as percent cell viability and compared to the cytotoxicity of water/methanol extract that was subjected to fractionation. As seen in Fig. 9.2, the fractions MO1 and MO2 were not able to reduce the viability even up to 70% at dose of 100 μg/ml, whereas an equivalent reduction in cell viability a dose of 100 μg/ml was seen in case of crude extract.

9.4 Discussion

Moringa oleifera Lam. has been used traditionally for treatment of various ailments including cancer. Anticancerous activity of leaves of this plant and their extracts against a variety of cell lines have been reported [21–23]. However, our report on breast cancer cell line with a huge range of extracts and simultaneous toxicity assessment with lymphocytes is first of its kind. The present study evaluated the cytotoxic effects of crude extracts prepared by various methods and solvent systems for three plants using fission yeast. The cdc2 gene in fission yeast is highly conserved to the human cdc2 and plays a central role in regulating the onset of S-phase of cell cycle as well as for initiation of mitosis. Hence, it works as a key checkpoint regulator in yeast cell cycle. The alteration in this gene would lead to loss of regulation of cell cycle, a situation similar to cancer cells, and therefore such mutant yeast was used for screening plant extracts in our study. Similar studies using yeast have been reported for various chemical agents [24]. Moreover, the ability of plumbagin

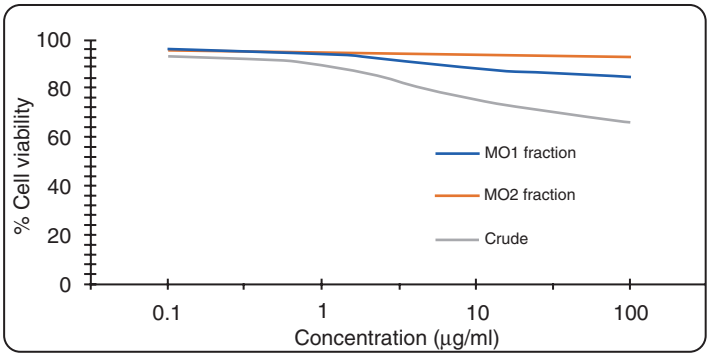


Fig. 9.2 Cell viability of crude extract and fraction MO1 and MO2 of *Moringa oleifera* Lam., after 48 h exposure at a dose of 0.1–100 µg/ml

(plant based compound) as an anticancer agent and the pathway it affects was studied using a yeast system [25].

Moreover, the study also focused on assessing the cytotoxicity of crude extracts of this plant not only against MCF-7 cell line but also toxicity assessment for normal peripheral blood monocytes (PBMCs). This will ensure the selectivity of extract to affect cancerous cell rather than normal cells. This finding is essential in process of drug development.

9.5 Conclusion

The cytotoxicity analysis has revealed the ability of water methanolic extract (elevated temperature) to be a better cytotoxic agent than the fractions. The probable reasons could be that in the crude extract compounds are exerting a synergistic effect and hence more effective. Even traditional systems of medicine like Ayurveda, traditional Chinese medicine, and European phytotherapy generally believe that a synergy of all ingredients of plants will bring about the maximum of the therapeutic efficacy. Moreover, the water methanolic extract (elevated temperature) was more toxic to MCF-7 compared to lymphocytes indicating selective action against cancerous cells, hence can be used a therapeutic agent. The mode of action of the extract can be further explored using specific metabolite assays and in vivo studies can be performed as well.

References

1. Luqman S, Srivastava S, Kumar R, Maurya AK, Chanda D (2011) Experimental assessment of Moringa oleifera leaf and fruit for its antistress, antioxidant, and scavenging potential using in vitro and in vivo assays. Evid Based Complement Alternat Med 2012(December):e519084. https://doi.org/10.1155/2012/519084

2. Guevara AP, Vargas C, Sakurai H, Fujiwara Y, Hashimoto K, Maoka T, Kozuka M, Ito Y, Tokuda H, Nishino H (1999) An antitumor promoter from Moringa oleifera Lam. Mutat Res/Genet Toxicol Environ Mutagen 440(2):181–188. https://doi.org/10.1016/S1383-5718(99)00025-X
3. Zhao T, Sun Q, Marques M, Witcher M, Zhao T, Sun Q, Marques M, Witcher M (2015) Anticancer properties of Phyllanthus emblica (Indian Gooseberry), anticancer properties of Phyllanthus emblica (Indian Gooseberry). Oxidative Med Cell Longev 2015(June):e950890. https://doi.org/10.1155/2015/950890
4. Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA (1966) Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata 1,2. J Am Chem Soc 88(16):3888–3890. https://doi.org/10.1021/ja00968a057
5. Liebmann JE, Cook JA, Lipschultz C, Teague D, Fisher J, Mitchell JB (1993) Cytotoxic studies of paclitaxel (Taxol) in human tumour cell lines. Br J Cancer 68(6):1104–1109
6. Anwar F, Latif S, Ashraf M, Gilani AH (2007) Moringa oleifera: a food plant with multiple medicinal uses. Phytother Res 21(1):17–25. https://doi.org/10.1002/ptr.2023
7. Kumar VV, Hussain Z, Verma M (2014) Antiinflammatory and antinociceptive activity of Moringa oleifera. J Biomed Pharm Res 3(1):14–21
8. Ndhlala AR, Mulaudzi R, Ncube B, Abdelgadir HA, du Plooy CP, Van Staden J (2014) Antioxidant, antimicrobial and phytochemical variations in thirteen Moringa oleifera Lam. cultivars. Molecules 19(7):10480–10494. https://doi.org/10.3390/molecules190710480
9. Amaglo NK, Deng J, Bennett R, Rosa E, Domínguez-Perles R (2014) Phytochemical content of distinct Moringa oleifera L, tissues (Leaves, Stems, and Petioles) as affected by shoot biomass (Re-Growth) age. J Bioprocess Eng Biorefinery 3(3):232–241. https://doi.org/10.1166/jbeb.2014.1101
10. Botstein D, Chervitz SA, Michael Cherry J (1997) Yeast as a model organism. Science (New York, NY) 277(5330):1259–1260
11. Yang X, Summerhurst DK, Koval SF, Ficker C, Smith ML, Bernards MA (2001) Isolation of an antimicrobial compound from impatiens Balsamina L. using bioassay-guided fractionation. Phytother Res 15(8):676–680. https://doi.org/10.1002/ptr.906
12. Wang L, Weller CL (2006) Recent advances in extraction of nutraceuticals from plants. Trends Food Sci Technol 17(6):300–312. https://doi.org/10.1016/j.tifs.2005.12.004
13. Raaman N (2006) Phytochemical techniques. New India Publishing, New India
14. Harborne JB (2012) Phytochemical methods: a guide to modern techniques of plant analysis. Springer Science & Business Media, New York
15. Kerem Z, German-Shashoua H, Yarden O (2005) Microwave-assisted extraction of bioactive saponins from chickpea (Cicer Arietinum L). J Sci Food Agric 85(3):406–412. https://doi.org/10.1002/jsfa.1989
16. Verma A, Laakso I, Seppänen-Laakso T, Huhtikangas A, Riekkola M-L (2007) A simplified procedure for indole alkaloid extraction from Catharanthus roseus combined with a semi-synthetic production process for vinblastine. Molecules 12(7):1307–1315. https://doi.org/10.3390/12071307
17. Sánchez NS, Königsberg M (2006) Using yeast to easily determine mitochondrial functionality with 1-(4,5-dimethylthiazol-2-Yl)-3,5-diphenyltetrazolium bromide (MTT) assay. Biochem Mol Biol Educ 34(3):209–212. https://doi.org/10.1002/bmb.2006.49403403209
18. Levitz SM, Diamond RD (1985) A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. J Infect Dis 152(5):938–945
19. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63
20. Villarini M, Moretti M, Pasquini R, Scassellati-Sforzolini G, Fatigoni C, Marcarelli M, Monarca S, Rodríguez AV (1998) In vitro genotoxic effects of the insecticide deltamethrin in human peripheral blood leukocytes: DNA damage ('comet' Assay) in relation to the induction of sister-chromatid exchanges and micronuclei. Toxicology 130(2–3):129–139. https://doi.org/10.1016/S0300-483X(98)00097-3

21. Charoensin S (2014) Antioxidant and anticancer activities of Moringaoleifera leaves. J Med Plants Res 8(7):318–325
22. Khalafalla MM, Abdellatef E, Dafalla HM, Nassrallah AA, Aboul-Enein KM, Lightfoot DA, El-Deeb FE, El-Shemy HA (2010) Active principle from Moringaoleifera Lam leaves effective against two leukemias and a hepatocarcinoma. Afr J Biotechnol 9(49):8467–8471
23. Parvathy MVS, Umamaheshwari A (2007) Cytotoxic effect of Moringaoleifera leaf extracts on human multiple myeloma cell lines. Trends Med Res 2(1):44–50
24. Simon JA, Bedalov A (2004) Yeast as a model system for anticancer drug discovery. Nat Rev Cancer 4(6):481–487. https://doi.org/10.1038/nrc1372
25. Lee J-H, Yeon J-H, Kim H, Roh W, Chae J, Park H-O, Kim D-M (2012) The natural anticancer agent plumbagin induces potent cytotoxicity in MCF-7 human breast cancer cells by inhibiting a PI-5 kinase for ROS generation. PLoS One 7(9):e45023. https://doi.org/10.1371/journal.pone.0045023

Hydroxy Fatty Acid from *Camelina sativa* Seed Oil for Industrial Application

10

Neha Sharma, Lekha Charan Meher, Mitesh Mittal, and Sanjai Kumar Dwivedi

Abstract

Renewable bio-based feedstocks are viable alternative to crude petroleum for fuel and chemical demands. Moreover, mineral oil-based lubricants used in industries and transport sector are associated with environmental concerns. *Camelina sativa* is an oilseed crop, and the oil may be a potential feedstock for fuel and lubricant base stocks. The present study revealed that the Camelina oil contains 82.3% of unsaturated fatty acids (monounsaturated fatty acid 33.6% and polyunsaturated fatty acid 48.7%). The physicochemical properties of Camelina oil reveal the presence of long chain fatty acid and high unsaturation. The oil may be chemically modified for lubricant applications and eco-friendly. The article describes the modification of Camelina oil to hydroxy fatty acid by the process of splitting, separation of saturated fatty acid so that the unsaturated fatty acid having iodine value 160gI_2/100 g is further epoxidized to obtain hydroxy fatty acids. The hydroxy functionality introduced into the Camelina fatty acid serves as a potential renewable material for synthesis of bio-lubricant base stocks.

Keywords

Bio-lubricant · *Camelina sativa* seed oil · Hydroxy fatty acid · Iodine value

N. Sharma (✉) · L. C. Meher · M. Mittal
Defence Institute of Bio-energy Research, Project Site Secunderabad, Secunderabad, Telangana, India
e-mail: meher@darl.drdo.in

S. K. Dwivedi
Defence Institute of Bio-Energy Research, Haldwani, Uttarakhand, India
e-mail: director@darl.drdo.in

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_10

10.1 Introduction

In the past three decades, world energy demand is increasing continuously with increase in industrialization. The total world energy consumption in 2012 was 1.384×10^{19} million tons of oil equivalent (Mtoe) and is estimated to reach 1.586×10^{19} Mtoe and 2.055×10^{19} Mtoe in 2020 and 2040, respectively, i.e. almost a 48% increase from 2012 to 2040 [1].

The crude petroleum serves as raw materials for lubricants, polymers and personal care products. The lubricants from petroleum are being widely used in machineries and industries. The conventional mineral lubricants are usually environmentally unacceptable because of their low biodegradability and toxicity and lead to contaminate the whole environment [2]. The bio-based products are environment friendly to replace petrochemicals, and it is a strategic approach to national security [3]. Therefore, more attention has been focused to reduce the dependency from fossils source by synthesis bio-based chemicals from renewable and sustainable resources that should reduce the environmental impact.

Vegetable oils are considered to potential bio-lubricant-based stock to replace conventional mineral oil-based lubricating oil and synthetic esters. Vegetable oil-based lubricant is used due to its enhanced biodegradability; superior, low toxicity; and low temperature properties. It has been studied that castor and lesquerella fatty acid estolide esters were formed by reacting with different types of saturated, unsaturated and branched fatty acids with low temperature properties [4]. Castor oil consists 12 hydroxy fatty acids; these properties of castor fatty acid have application as lubricant base stocks such as estolide esters and their derivatives [5]. In this context, various studies have been carried out for synthesis of precursor for lubricant base stock.

Camelina sativa is an oilseed crop, and the oil may be a potential feedstock for lubricant base stocks. The physicochemical properties of Camelina oil reveal the presence of long chain fatty acid and 82.3% of high unsaturation. The present study describes the modification of Camelina oil to hydroxy fatty acid by the process of splitting followed by isolation of unsaturated fatty acid which is further epoxidized to obtain hydroxy fatty acids with multiple –OH groups that may serve as a potential renewable material for synthesis of bio-lubricant base stocks.

10.2 Materials and Methods

10.2.1 Extraction and Analysis of Camelina Oil

The dried seeds were powdered, and the oil was isolated by Soxhlet extraction method using petroleum ether (60–80 °C) as solvent. In each extraction experiment, 50–60 g of crushed *Camelina sativa* seed was taken in a thimble. The extract was collected and the oil was obtained after removal of solvent under vacuum. The oil was characterized for its physicochemical properties as per IS-548:1976, Part-1

method of Bureau of Indian Standards, and the fatty acid composition was carried out by gas chromatographic analysis as per method IS-548:1976, Part-3.

10.2.2 Splitting of Fatty Acid from Camelina Oil

Saponification of Camelina oil with potassium hydroxide was followed by phosphoric acid treatment to obtain free fatty acids. The organic layer was washed with water till the wash water was neutral, followed by drying with anhydrous sodium sulphate. The acid value and iodine value were estimated. During typical experiments, 100 g Camelina oil was mixed with 80 ml water, 60 ml ethanol and 30 g KOH, followed by addition of phosphoric acid to lower pH up to 4.0 and stirred for a few minutes, then washed with water to remove phosphoric acid and the remaining reaction mixture. The fatty acids were dried under vacuum.

10.2.3 Isolation of Unsaturated Fatty Acid by Urea Inclusion Complex

During the urea inclusion complex method [6], 40 g of free fatty acid was added to 400 ml methanol in 1 l conical flask boiled at 65 °C under water bath. 30 g of urea was added. It was allowed to cool in freeze at 7–8 °C overnight. The urea complexes were separated by filtration and the unsaturated fatty acids were isolated.

10.2.4 Epoxidation of Unsaturated Fatty Acid

The epoxidation was carried out in the laboratory with 30 g unsaturated free fatty acid under ice bath taken in a 150 ml round-bottom flask, and 5.8 ml of formic acid was added to it followed by gradual addition of 30.9 ml hydrogen peroxide (30% solution) with stirring and cooling. The flask containing the reactants was heated up to 40–45 °C in a water bath with stirring at 600 rpm for 6 h. After the reaction was complete, the mixture was cooled down to room temperature, washed with distilled water. The epoxidized fatty acid was dried under vacuum to obtain colourless transparent, viscous liquid. The oxirane oxygen content (OOC) of epoxidized Camelina oil was determined as per AOCS Official Method Cd 9-57, 1993, iodine value of epoxidized Camelina oil was estimated according to IS :548 1964 (Part I) using Wijis solution.

10.2.5 Ring Opening of Epoxidized Fatty Acid to Hydroxy Fatty Acid

The acid-catalysed ring opening was studied in the laboratory. In typical experiment 50 g of epoxidized fatty acid was taken in a 250 ml flat-bottom flask with addition

of 2% H_2SO_4 with stirring. The reaction was conducted at below 100 °C using an oil bath and stirring at 600 rpm for 4 h. The hydroxyl value of hydroxy fatty acid physicochemical properties was measured according to using IS-548:1976 Part-1 method of Bureau of Indian Standards.

10.3 Results and Discussion

10.3.1 Extraction and Analysis of Camelina Oil

Camelina seed contained 28–31 wt% of oil. The iodine value of Camelina oil is 140gI_2/100 g. The physicochemical properties of Camelina oil are listed in Table 10.1. The oil was found to be yellowish clear liquid. The saponification value is 180 mg KOH/g which indicated long chain of fatty acids in it. The iodine value of the Camelina oil is high, i.e. 140 g gI_2/100 g, which indicates that the oil is rich in unsaturated fatty acid. The fatty acid distribution in Camelina oil is listed in Table 10.2. The fatty acid composition of Camelina oil contains 82.3% of unsaturated fatty acids (monounsaturated fatty acid 33.6% and polyunsaturated fatty acid 48.7%) which indicates oil rich in unsaturation.

10.3.2 Splitting of Fatty Acid of Camelina Oil

The aim of splitting the triglycerides is to obtain the free fatty acid. Free fatty acids were obtained by saponification of Camelina oil with potassium hydroxide followed by acidification with phosphoric acid. The iodine value of free fatty acid of Camelina oil was 143 gI_2/100g, and its acid value was 212 mg KOH/g.

10.3.3 Urea Inclusion Complex Formation for Isolation of Unsaturated Fatty Acid

Saturated fatty acid was precipitated out by urea inclusion complex method and separated from the oil, obtaining unsaturation fatty acids. The iodine value of unsaturated fatty acid was 162 gI_2/100g which revealed the presence of high unsaturation in the oil, and its acid value was 212 mgKOH/g.

Table 10.1 Physicochemical properties of Camelina oil

S.No	Properties	Results
1.	Appearance	Yellowish colour, clear liquid
2.	Acid value (mg KOH/g)	2.7
3.	Saponification value (mg KOH/g)	180
4.	Iodine value (gI_2/100g)	140

Table 10.2 Fatty acid composition of Camelina oil

S.No.	Fatty acid	Weight%
1.	Myristic acid ($C_{14:0}$)	0.2
2.	Palmitic acid ($C_{16:0}$)	7.3
3.	Stearic acid ($C_{18:0}$)	3.1
4.	Oleic acid ($C_{18:1}$)	14.0
5.	Linoleic acid ($C_{18:2}$)	22.2
6.	Linolenic acid ($C_{18:3}$)	23.9
7.	Arachidic acid ($C_{20:0}$)	3.2
8.	Gadoleic acid ($C_{20:1}$)	14.8
9.	Ecosadienoic acid ($C_{20:2}$)	1.8
10.	Ecosatrienoic acid ($C_{20:3}$)	0.8
11.	Behenic acid ($C_{22:0}$)	0.8
12.	Eruic acid ($C_{22:1}$)	4.8
13.	Unidentified	3.1

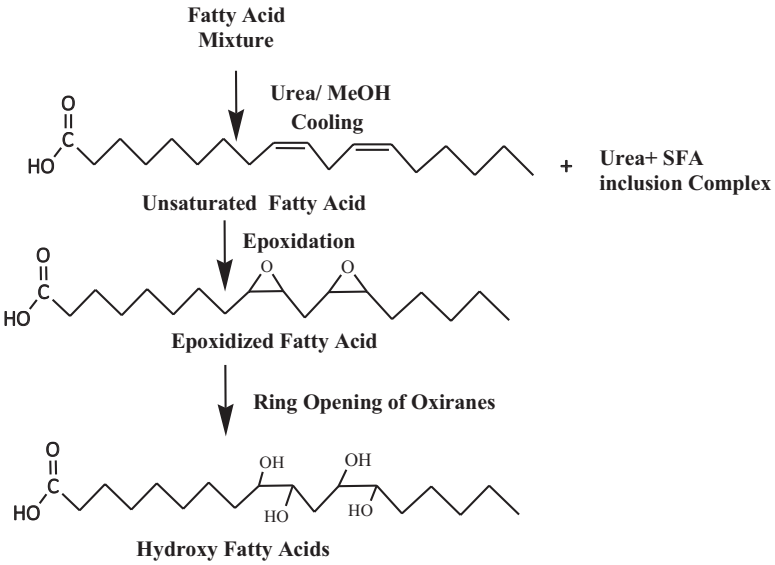


Fig. 10.1 The process of preparation of hydroxy fatty acid from Camelina oil fatty acid

10.3.4 Epoxidation of Unsaturated Fatty Acid and Ring Opening of Oxirane

Epoxidation is an important step where the fatty acid rich in C=C unsaturation forms oxirane ring which was followed by ring opening reaction to form hydroxy fatty acid. The conversion of C=C bond to oxirane and further hydroxy groups was introduced to form hydroxy fatty acid as shown in Fig. 10.1.

Table 10.3 Physicochemical properties of free fatty acid, unsaturated fatty acid, epoxidized fatty acid and hydroxy fatty acid

S.No	Product	Properties			
		Iodine value (gI_2/100g)	Acid value (mgKOH/g)	Oxirane oxygen content (wt. %)	Hydroxyl value (mgKOH/g)
1.					
2.	Free fatty acid	143	198	–	–
3.	Unsaturated fatty acid	162	198	–	–
4.	Epoxidized fatty acid	9.49	178	8.11	–
5.	Hydroxy fatty acid	08	162.48	0.28	289

In typical experiment the epoxidation is carried out with double bond to formic acid molar ratio 1:0.75 and double bond to hydrogen peroxide ratio 1:1.5 at 46.7 °C for 6 h. The percentage of oxirane oxygen content is 8.1 which shows the extent of epoxidation is >88% conversion of unsaturated moiety. The iodine value decreases from 162 to 9.49 gI_2/100g which further revealed >94% conversion during epoxidation. The ring opening of epoxidized fatty acids was carried out with sulfuric acid at below 100 °C for 3 h. The obtained hydroxy fatty acid having iodine value 8gI_2/100g and oxirane oxygen content 0.28% indicated the presence of low unsaturated in it. The physicochemical properties of free fatty acid, unsaturated fatty acid, epoxidized fatty acid and hydroxy fatty acids are listed in Table 10.3.

The hydroxyl value of the hydroxy fatty acid is 289 mg KOH/g which is higher than the hydroxyl value of castor oil (160–168 mg KOH/g) indicating that the Camelina oil is converted to hydroxy fatty acid with multiple –OH functionality groups.

10.4 Conclusion

The Camelina oil may be chemically modified to derive industrially important oleochemicals. The present article described the method to derive hydroxy fatty acid which is having –OH functional group. The castor oil with its ricinoleic acid (12-hydroxy fatty acid) finds various industrial applications, i.e. for lubricant base stock. The camelina-based hydroxy fatty acid with higher hydroxyl value is best suited as precursor to derive estolide with free –OH group to impart better lubricity.

Acknowledgements The authors express their gratitude to DRDO, Ministry of Defence, Government of India, for providing financial assistance to Ms. Neha Sharma in the form of Senior Research Fellowship. The authors are also grateful to Dr. J. Vollmann, BOKU-University of Natural Resources and Applied Life Sciences, Vienna, Austria, for providing the nucleus seed of *Camelina* cv. Calena.

References

1. World energy demand and economic outlook Date of access: 28/05/2017, International Energy Outlook 2016 (IEO2016) Chapter 1. https://www.eia.gov/outlooks/ieo/pdf/world.pdf
2. Soni S, Agarwal M (2014) Lubricants from renewable energy sources–a review. Green Chem Lett Rev 7(4):359–382
3. Rani S, Joy ML, Nair KP (2015) Evaluation of physiochemical and tribological properties of rice bran oil–biodegradable and potential base stoke for industrial lubricants. Ind Crops Prod 65:328–333
4. Cermak SC, Brandon KB, Isbell TA (2006) Synthesis and physical properties of estolides from lesquerella and castor fatty acid esters. Ind Crops Prod 23:54–64
5. Potula SB, Korlipara VP, Bhamidipati VSKR, Krishnasamy S, Rachapudi BNP (2014) Castor oil fatty acid based estolide esters and their derivatives as potential lubricant base stocks USP patent no US8,742,150 B2
6. Hayes DG, Bengtsson YC, Van Alstine JM, Setterwall F (1998) Urea complexation for the rapid, ecologically responsible fractionation of fatty acids from seed oil. J Am Oil Chem Soc 75(10):1403–1409

Comparison of Different Planting Methods to Determine the Precision of Phenotyping Wheat in Field Experiments

11

Davinder Sharma, Jagadish Rane, Rajender Singh, Vijay Kumar Gupta, and Ratan Tiwari

Abstract

Lack of uniformity of plant stand while conducting a field experiment can substantially contribute to errors in the prediction of association between plant phenotype and genotype. Among the several factors that can contribute to experimental errors, inconsistent seed depth and plant spacing often occur due to lack of precision when seeds are sown by hand or seed drills. Hence, we compare three planting methods, novel dibbling, seed drill, and hand sowing, to determine the most efficient method for precision phenotyping in field. We showed the advantage of the new methods over conventional methods of sowing, viz., seed drill and by hand. Compared with conventional methods, the new method improved the consistency in plant spacing substantially as indicated by reduction in standard deviation at least by three times. The desired seed depth (6.5 cm) and plant spacing (10 cm intra- and 20 cm inter-row spacing) could be maintained with greater precision in dibbling method than in seed drill or hand sowing method. The reduction in error and the least coefficient of variation (CV%) for the plant traits measured in the new method relative to other methods indicated possibility of enhancing precision in phenotyping responses of wheat plants under field condition.

D. Sharma
Presenting author, ICAR-Indian Institute of Wheat & Barley Research, Karnal, Haryana, India

J. Rane
School of Drought Stress, ICAR-National Institute of Abiotic Stress Management, Baramati, Maharashtra, India

R. Singh · R. Tiwari (✉)
ICAR-Indian Institute of Wheat & Barley Research, Karnal, Haryana, India

V. K. Gupta
Department of Biochemistry, Kurukshetra University, Kurukshetra, Haryana, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_11

Keywords
Precision phenotyping · Planting methods · Plant stand · Coefficient of variation

11.1 Introduction

Reducing experimental error is one of the major challenges while phenotyping a large number of genotypes for genetic variations under field conditions. Nongenetic variations in field crop experiments result from extraneous variations. Lack of uniformity of plant stand while conducting a field experiment can substantially contribute to errors in the prediction of association between plant phenotype and genotype. Among the several factors that can contribute to plant stand, inconsistent seed depth and plant spacing often occur due to lack of precision while planting. Any error during the planting can leave a permanent effect on the plant performance throughout their life cycle [1]. The microclimate may be altered considerably in the field by uneven space and depth of planting which can contribute to nongenetic variations and hence affect the precision of phenotyping. Individual development was influenced directly by the level of crowding [2]. Further, variability in plant space and emergence can cause crowding stress, which can result in a reduction in the capture and utilization of resources by plants [3]. The lack of precision in phenotyping limits our ability to dissect the genetics of quantitative traits. Since conventional methods, including hand sowing and seed drills, often fail to place the seeds at consistent depth or space, we compared these planting methods with dibbling method to determine the most efficient method for precision phenotyping in field experiments.

11.2 Material and Methods

The experiments were conducted at the Indian Institute of Wheat and Barley Research (IIWBR) (29°42′ N latitude and 77°2′E longitudes at an altitude of 250 m above the mean sea level) in the Indo-Gangetic Plain in northwestern India, with mildly alkaline soil (typic ustochrept) during the crop seasons 2012–2013 and 2013–2014. Experiments were laid out in a randomized complete block design with four replications using a split plot arrangement where the planting methods composed the main plots and genotypes of the subplots. The experiment was conducted with four wheat (*Triticum aestivum* L.) genotypes, viz., DBW 88, DBW 96, DBW 97, and DBW 123. In both the crop seasons, experiments were planted in the third week of November. Agronomic practices were followed as recommended for irrigated timely sown conditions. Three planting methods used:

1. *Seed drill method (SDM):* Seeds were sown at 96 seeds/plot in four lanes with the help of seed drill (precision plot drill; equipment code, ER-03; CIAE, Bhopal, Hyderabad). Drill was set to drop seeds at the interval of 10 cm and at the depth of 6.5 cm.

2. *Hand sowing method (HSM):* Furrows in the soil were made with a marked hoe, to the predetermined depth, 6.5 cm. Seeds in each row were dropped by hand and subsequently covered with surrounding soil. Seeds were sown at 96 seeds/subplot in four lanes.
3. *Dibbling tool method (DBM)* [4, 5]: Dibbling area (11.7 m × 6.5 m) was divided into 16 subplots (2.3 m × 1 m) with path (0.5 m) on either side with the help of threads. Dibbler was set to create cavities of 6.5 cm deep with inter- and intra-row spacing of 10 and 20 cm, respectively. With one seed placed in each cavity, each subplot initially had 288 seeds. Two weeks after sowing, two of three plants were uprooted to finally keep one plant/spot and 96 plants of a genotype in each subplot.

11.2.1 Data Measurements and Analysis

Depth of seeding was measured during the morning hours of the next day of sowing by removing the soil very carefully with the help of a narrow strip of metal to locate the seed, and then scale was used to measure the depth from the soil surface. Three weeks after sowing, the variability in plant spacing was measured by using a scale. Leaf chlorophyll status (SPAD) was measured (GS 73; [6]) with a chlorophyll meter (Minolta SPAD-502, Osaka, Japan). Productive tillers (PT) as tillers bearing spike were recorded at the time of harvest. The postharvest measurements included thousand grain weight (TGW) and yield per plant (GY). During the collection of data, border effects were eliminated by sampling plants at the center of each subplot. All the data were analyzed using the SAS statistical software program, PROC MIXED (SAS version 9.3, SAS Institute Inc., Cary, NC, USA) with a MODEL statement containing the ALPHA = 0.05 and DDFM = Satterthwaite option. Replication was treated as a RANDOM effect, but planting method (PM) and genotype (GEN) were treated as fixed effects. An estimate statement was used to derive means for the main effects and their interaction. The LSMEANS statement included the ADJUST = TUKEY option for using the Tukey range test (adjusted for multiple comparisons) to evaluate a ranked (high to low) ordering of main effects means for significant differences. Combined analysis was performed due to homogeneous experimental error and non-significant interaction in year × planting method × genotypes.

11.3 Result and Discussion

The standard deviation (SD) of means was used to determine the variability in seed depth and space between the plants for each of the planting methods, and results revealed that the SD was much higher for seed drill method (SDM) and hand sowing method (HSM) as compared to dibbling method (DBM) (Fig. 11.1). Thus, the desired seed depth (6.5 cm) and plant spacing (10 cm intra- and 20 cm inter-row spacing) could be maintained with greater precision in DBM than in SDM or

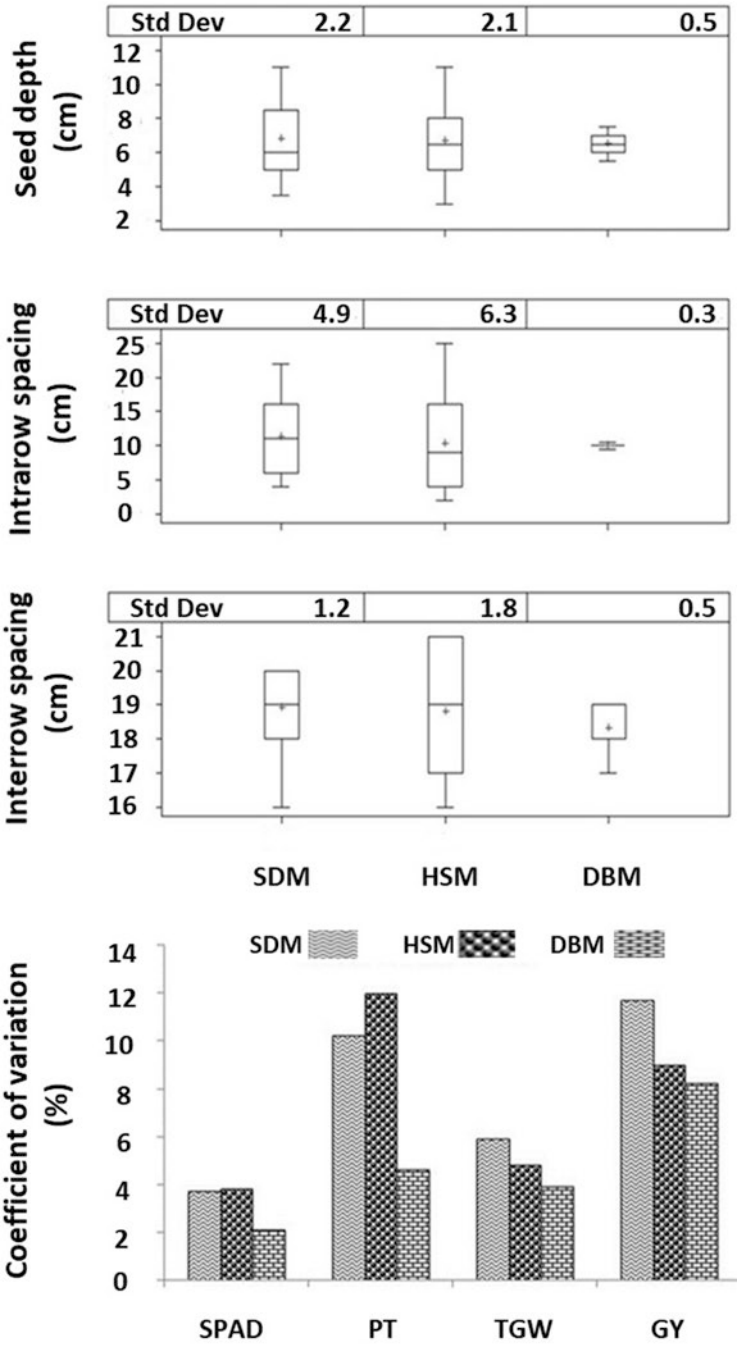


Fig. 11.1 Plot depicting deviations in seed depth and plant spacing obtained with three different planting methods and graph showing the coefficient of variation (CV%) for the investigated traits under different planting methods. *SDM* seed drill method, *HSM* hand sowing method, and *DBM* dibbling method

HSM. Combined variance analysis of all the traits over the years showed that differences in effects of planting methods were significant for SPAD, PT, and GY except TGW. However, planting method × genotype interaction was significant for SPAD and GY (Table 11.1). Compared with conventional methods, the new method improved the precision in phenotyping as indicated by reduction in percent of error variance (Table 11.2) and coefficient of variation (CV) at least by one-half and two times, respectively (Fig. 11.1). Further, the differential responses of genotypes represented by measured traits were more conspicuous and consistent across the years in those plants which were sown by DBM than by SDM or HSM. High-quality genotypic and phenotypic data are essential to dissect the genetic architecture of complex traits. While the power of current genotyping approaches enables high-density genotyping of entire genomes, field phenotyping is the bottleneck.

Hence, the present study was aimed to find an efficient method that can enhance our precision to differentiate genotypic responses by minimizing experimental errors arising from the inconsistent crop establishment. The results obtained from this experiment clearly revealed that factors contributing to the initial crop establishment were effectively addressed by the novel dibbling method for planting that takes into consideration the variation in plant responses due to aboveground factors such as solar radiation [7] and belowground factors such as nutrient and soil moisture [8] which influence plant growth and development. Although there was no significant response in plant growth under different planting methods used, variation within the genotype was much greater in SDM or HSM than in DBM. We attribute it to the consistent plant spacing in DBM that resulted in uniformity in the interception of available solar radiation, water use, and nutrient uptake which collectively contributed to the homogenous crop stand as revealed by different parameters relative to the same observed in other methods. In DBM, the observed seed depth and plant spacing closely matched the desired values (Fig. 11.1) because of equidistant cavities of same depth created by the dibbler.

Additionally, three seeds sown at one locus but in separate cavities completely eliminated the possibility of gaps that might have been formed due to lack of germination at the place. Seed drill might get out of level due to lack of perfection in the leveling of the field, inconsistent speed, or jerks due to which seed placement at variable depth could occur. Uneven seed placement by hand or seed drill often create bunching and gaps in the field. In addition, lack of germination at some locus added further variation in plant spacing. Moreover, hand sowing method was least precise as there was no perfect control over placement of seeds. Further, the lack of homogeneity among neighboring plants could be due to seedling emergence which

Table 11.1 Source of variation, degree of freedom for the F-tests, and probability values obtained in a PROC MIXED analysis of variance of agronomic traits for planting method (PM) and genotype (GEN) main effects and their interaction for combined 2 years

Source of variation	num df	den df	SPAD	PT	TGW	GY
PM	2	6	0.0108	<0.0015	0.2038	<0.0081
GEN	3	75	<0.0001	<0.0001	<0.0001	<0.0001
PM*GEN	6	75	0.0092	0.5602	0.1196	0.0002

Table 11.2 Error as percent of total sum of squares in different planting methods followed in the experiment for various traits studied

Traits	SPAD	PT	TGW	GY
SDM	31.3	55.7	38.3	54.8
HSM	23.2	53.5	19.7	20.8
DBM	9.6	29.6	14.2	13.2

Error% = (error sum of squares/total sum of squares) × 100
SDM seed drill method, *HSM* hand sowing method, *DBM* dibbling method, *SPAD* leaf chlorophyll status, *PT* productive tillers, *TGW* thousand grain weight, *GY* grain yield

occurs over a period of several days due to variability in soil resistance and soil moisture or due to uneven compaction of soil by human feet or tractor wheels [1] which could be avoided in DBM. The precision in sowing by DBM was reflected in higher efficiency in differentiating the genotypes based on their phenotypes. DBM could help in identifying the differences in SPAD among all the genotypes, while the other two methods were not efficient. Canopy structure is the major determinant of the quantity and the distribution of radiation and consequently of SPAD. Continuous and spatially homogeneous canopy establishment in DBM favors precision in measurement, while discontinuous and spatially heterogeneous canopy in HSM or SDM made it difficult to distinguish genotypes on the basis of this trait. [9] reported that the uniform plant spacing could greatly enhance precision in radiation-based phenotyping. The differential responses of genotypes could also be seen for traits other than SPAD in those plants which were sown with DBM.

In addition, varietal responses are more conspicuous and consistent across the years in DBM while highly inconsistent in HSM and SDM. This was mainly due to reduction in error (Table 11.1) as indicated by the substantially less coefficient of variation (CV%) for each trait in the DBM as compared to other methods (Fig. 11.1). Experimental error is the difference between a genotype treated alike in replicated experiment and is the primary basis for making a decision whether an observed difference is real or just due to chance. The CV can be used to indicate the stability of phenotypes in field experiments. Experiments with low CV and with reduced extraneous variations improve the ability to detect differences. The ability to detect genetic differences increases as the experimental error decreases. DBM minimizes the extraneous variations which ultimately contribute to greater precision in phenotyping. It is suggested that this method can enhance our capacity to identify genes and gene marker for crop improvement through phenotyping under field conditions.

Acknowledgments We gratefully acknowledge the financial support from ICAR for the Network Project on Transgenic in Crop: Functional Genomics in Wheat.

References

1. Nielsen RL (1991) Stand establishment variability in Corn. Purdue University AGRY-91-01
2. Lauer J (1994) Should I be planting my corn at a 30-inch row spacing? Wisconsin crop manager. Crop Agron 1(6):311–314
3. Tollenaar M, Deen W, Echarte L, Liu W (2006) Effect of crowding stress on dry matter accumulation and harvest index in maize. Agron J 98(4):930–937
4. Sharma D, Singh R, Rane J, Gupta VK, Mamrutha HM, Tiwari R (2016) Mapping quantitative trait loci associated with grain filling duration and grain number under terminal heat stress in bread wheat (Triticum aestivum L.). Plant Breed 135(5):538–545
5. Sharma D, Tiwari R, Gupta VK, Rane J, Singh R (2018) Genotype and ambient temperature during growth can determine the quality of starch from wheat. J Cereal Sci 79:240–246
6. Zadoks JC, Chang TT, Konzak CF (1974) A decimal code for growth stages of cereals. Weed Res 14(6):415–421
7. Sinclair TR, Muchow RC (1999) Radiation use efficiency. Adv Agron 65:125–265
8. Casper BB, Jackson RB (1997) Plant competition underground. Annu Rev Ecol Evol Syst 28:545–570
9. Blackmer TM, Schepers JS, Vigil MF (1993) Chlorophyll meter readings in corn as affected by plant spacing. Commun Soil Sci Plant Anal 24(17–18):2507–2516

12

Effect of Extraction Temperature and Different Carrier Agents on Physicochemical and Antioxidant Properties of Spray-Dried *Murraya koenigii* (Linn.) Leaf Extract

Vandana Sablania, Sowriappan John Don Bosco, and Shubham Rohilla

Abstract

The work evaluated the effect of extraction temperature on *Murraya koenigii* L. extract, prepared at an extraction temperature of 80 °C and 90 °C. The prepared *Murraya koenigii* L. leaf extract was encapsulated with different carrier agents such as guar gum, pectin, and whey protein isolates at a concentration of 1%, 5%, and 10% respectively. Spray drying was done at an inlet temperature of 130 °C with constant feed flow rate (8 rpm) at a pressure of 0.4 kg/cm^2 and outlet temperature of 80 °C. The encapsulated powders were analyzed for their physicochemical properties such as moisture content, water activity, bulk density, tapped density, flow properties, antioxidant activity, and total phenolic content. The extract encapsulated with pectin and whey protein isolates did not show significant difference on DPPH activity at varied extraction temperature, whereas the extract encapsulated with guar gum showed more activity at an extraction temperature of 80 °C. The extract encapsulated with whey protein isolates showed more total phenolic content at an extraction temperature 90 °C. Therefore, it can be considered as a good source of active phytochemicals. Thus from this study, encapsulated *Murraya koenigii* L. extract could be considered as an important source of antioxidant and anti-inflammatory in human cells.

Keywords

Murraya koenigii L. · Spray drying · Antioxidant activity · Total phenolic content

V. Sablania · S. J. D. Bosco (✉) · S. Rohilla
Department of Food Science and Technology, Pondicherry University, Puducherry, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_12

12.1 Introduction

In current status, there is an increase in demand of herbal products due to their higher antioxidant activities, which have the potential to protect the body against free radical damage and degenerative diseases. Several studies were done to identify new compounds capable to inactivate free radicals generated by metabolic pathways in human tissues and cells. *Murraya koenigii* L. leaf is a much demanded spice commodity in South India and all over the world. It belongs to the Rutaceae family, used for its characteristic color and flavor [1]. Around 14 species of *Murraya* are found all over the world in which *Murraya koenigii* and *Murraya paniculata* are the most popularly used variety in India. *Murraya koenigii* leaf is known for its aromatic, spicy, bitter taste and acrid, cooling, and strong fragrance. It is described to have antidiabetic, antioxidant, antidysenteric, anticarcinogenic, stimulant, hypoglycemic, and antimicrobial activities due to the presence of tocopherol, β-carotene, and lutein [2]. There is an increase in demand of naturally present antioxidants for the treatment of oxidative stress-related diseases. Plants extract are usually available in the form of liquid and viscous solution which cannot be stored for a longer period of time in liquid form. Hence the drying of these extracts could be a better alternative to increase the shelf life of the liquid feed without much altering in its chemical composition. Therefore, the drying techniques such as spay drying and freezed drying can be used for the drying of extracts. Any product in dried form shows significant merits over conventional liquid extract, including chemical, physicochemical, and microbiological stability, easy to transport, less space for storage, and the use of powders for its pharmaceutical applications [3]. Several researchers use spray-drying technique for encapsulation of any herbal extract with their efficacy and better stability in powder form. With this scenario *Murraya koenigii* L. leaf extract was prepared at varied extraction temperature and encapsulated with carrier agents such as whey protein isolates, pectin, and guar gum in order to produce dried powder with maximum retention of antioxidant activity and total phenolic content.

12.2 Materials and Methods

12.2.1 Extract preparation

Fresh curry leaves were purchased from the local market of Pondicherry, washed under running tap water, and then leaves were taken out from the stalks and kept for drying in tray dryer at 60 °C for 4 h (Ezidri Ultra FD1000). The tray-dried leaves were ground into a grinder (Kenstar Karishma), and then 100 g of dried powder was mixed in 1000 mL of distilled water and kept in water bath at a temperature of 80 °C and 90 °C for 1 h for the extraction of bioactive extract. After 1 h it was filtered through muslin cloth, and again 500 mL of distilled water was added into the remaining residues and kept in water bath at same temperature for another 30 min. After 30 min the extract was filtered through muslin cloth and mixed with the previously prepared extract.

12.2.2 Encapsulation of Curry Leaf Extract with Different Carrier Agents

The prepared curry leaf extract was blended with different carrier agents such as whey protein isolates (10%), guar gum (1%), and pectin (5%) and then kept overnight at 4 °C for complete hydration of carrier agents into the extract. Many studies have been conducted to optimize the operational conditions for the spray-dried juice and extract powders. High inlet temperature leads to degradation of phenolic compounds; therefore, the inlet and outlet temperature were kept constant at 130 °C and 80 °C, respectively. The feed solution was fed into pilot plant spray dryer with a co-current flow rate of 8 rpm and pressure 0.4 kg/cm^2 for atomization. The process conditions were optimized by taking into account the experimental studies and previously conducted studies to enable the maximum powder yield with more antioxidant activity. The powder was collected in pre-weighed glass bottles and refrigerated until the analysis to be done.

12.3 Physicochemical Analysis of Spray-Dried Powder

12.3.1 Powder Yield

The spray-dried powder yield was calculated by using the formula given by Santhalakshmy et al. [4].

12.3.2 Moisture Content

Moisture content was determined by the method given in AOAC manuals [5].

12.3.3 Water Activity

The water activity of the powder was measured by using the electronic instrument (AquaLab Series 4TE, Decagon Devices, Inc., Pullman, Washington, USA).

12.3.4 Bulk and Tapped density

Bulk density and tapped density of spray-dried powders were measured by using the method given by Tonon et al. [6] and Goula et al. [7].

12.3.5 Total Phenolic Content

Total phenolic content in spray-dried samples were determined by Folin-Ciocalteu assay given by Shah et al. [8] with slight modifications.

12.3.6 DPPH Radical Scavenging Activity

The free radical scavenging activity was determined by the method given by Shah et al. [8] with slight modification.

12.4 Statistical Analysis

The data interpretation was accomplished by SPSS software SPSS 20.0 (IBM Corporation, Armonk, New York). The one-way analysis of variance (ANOVA) was done by using duplicate values and mean values which were separated by using Duncan's multiple range test ($p \leq 0.05$). All the data were expressed as the mean ± standard deviation.

12.5 Results and Discussion

12.5.1 Powder Yield

The powder yield of encapsulated extract with different carrier agents is shown in Table 12.1. The powder yield was observed to be increased with increase in extraction temperature. The maximum yield of the powder is obtained with whey protein isolates (6.70–9.15%) followed by pectin (2.90–5.25%) at an extraction temperature of 80 °C and 90 °C, whereas guar gum showed a decrease in yield of powder with increased extraction temperature (0.70–0.50%). Whey protein isolates are considered to be a strong surfactant which specially migrates to the droplet air interface. Immediate reaction of hot air with extract in the presence of high concentration of protein content developed a film on particle and resulted in higher powder yield as compared to other carrier agents. Whereas in the case of guar gum, increase in extraction temperature leads to decrease in powder yield which could be due to the sticky behavior of guar gum inside the spray dryer chamber and resulted in reduced powder yield [9].

12.5.2 Moisture Content

The moisture content of the spray-dried powder is found to be in the range between 3.57% and 5.84% (Table 12.1). Significant difference was observed with increase in extraction temperature of the curry leaf powder. There was an increase in moisture content with increased extraction temperature from 80 to 90 °C. Spray-dried

Table 12.1 Physicochemical analysis of encapsulated curry leaf extracts using different carrier agents

	Whey protein isolate		Pectin		Guar gum	
Parameters	80 °C	90 °C	80 °C	90 °C	80 °C	90 °C
Yield (%)	6.70	9.15	2.90	5.25	0.70	0.50
Moisture (%)	3.57 ± 0.16^{c}	4.18 ± 0.28^{b}	3.80 ± 0.21^{c}	4.60 ± 0.50^{a}	4.82 ± 0.11^{b}	5.84 ± 0.56^{a}
Water activity	0.33 ± 0.01^{ab}	0.35 ± 0.01^{a}	0.28 ± 0.02^{c}	0.34 ± 0.06^{b}	0.31 ± 0.02^{b}	0.34 ± 0.04^{a}
Bulk density (g/mL)	0.18 ± 0.07^{d}	0.14 ± 0.03^{e}	0.30 ± 0.00^{a}	0.25 ± 0.09^{b}	0.20 ± 0.00^{c}	0.13 ± 0.02^{e}
Tapped density (g/mL)	0.22 ± 0.12^{c}	0.24 ± 0.04^{c}	0.38 ± 0.05^{a}	0.41 ± 0.00^{a}	0.30 ± 0.00^{b}	0.19 ± 0.05^{d}
Carr index	$36.27 \pm 0.07^{a,b}$	$35.81 \pm 0.31^{a,b}$	25.47 ± 5.52^{d}	38.43 ± 2.20^{a}	$33.99 \pm 0.00^{b,c}$	32.13 ± 0.51^{c}
Hausner ratio	1.56 ± 0.07^{b}	1.55 ± 0.07^{b}	1.37 ± 0.00^{d}	1.62 ± 0.05^{a}	$1.51 \pm 0.00^{b,c}$	1.47 ± 0.01^{c}
TPC (mgGAE/g)	46.62 ± 4.11^{b}	71.16 ± 8.25^{a}	19.01 ± 1.95^{c}	47.23 ± 4.43^{b}	51.14 ± 1.40^{b}	52.91 ± 0.43^{b}

All these values are mean ± standard deviation of duplicate readings. In each row superscripts show significant difference at $P \leq 0.05$

powder obtained with whey protein isolates showed less moisture content in comparison to other carrier agents which could be due to its high concentration. Increase in concentration of carrier agent results in decrease in moisture content reported by Abadio et al. [10]. Increase in extraction temperature leads to release more substance from the dried powder. Constant carrier agent spontaneously increases the moisture content with increase in extraction temperature. In general, less concentration of carrier agents and low inlet temperature could also be a reason for increase in moisture content of the obtained spray-dried powder. The same results were also observed by Goula et al. [7].

12.5.3 Water Activity

Water activity is the free water availability in food, which is considered to be a better medium for the growth of microorganism and make the food unstable which leads to the deterioration of the quality of food. Water activity of spray-dried powder was found to be in the range between 0.28 and 0.35 (Table 12.1). Obtained spray-dried powders showed water activity below 0.6 is considered to be appropriate for powder's stability. Hence, water activity between 0.2 and 0.3 minimizes the microbial propagation and oxidative and enzymatic activity [11]. Water activity was found to be increased with increase in extraction temperature due to increase in moisture content of spray-dried powder, in which the intermolecular bonds stabilize the solution by increasing its viscosity and leads to retention of more water molecules in its matrix [12].

12.5.4 Bulk and Tapped Density

Encapsulated curry leaf extract showed significant effect on bulk density at different extraction temperature (Table 12.1). It was found to be in the range between 0.13 and 0.30 g/mL. Bulk density was found to be decreased significantly with increase in moisture content. Goula et al. [7] suggested that high bulk density is linked with low moisture content which shows that there is an inverse relationship between the residual moisture and bulk density. Powder encapsulated with pectin showed higher value of bulk density, whereas powder obtained with whey protein isolates showed lowest bulk density. Ferrari et al. [13] reported that high molecular weight of carrier agent leads to less entrapment of particles and ultimately increases the bulk density of the powder. It was observed that, stickier the particles of an agglomerate, the more interspaces will be formed between them, and bulk density of powder decreases. Tapped density was found to be higher in comparison to bulk density which could be due to change in the volume, compaction of the particles, and transitions from loosely packed particles to a more compact form, which increases the cohesive forces among particles. There was no significant difference found on tapped density of spray-dried powder at varied extraction temperature with different carrier agents. Lower bulk density results in more accumulation of air into the powder and leads to increase the possibility of the powder to oxidize and reduce the storage stability [4].

12.5.5 Carr Index and Hausner Ratio

Flowability is an important parameter for any of the powder material, and it was expressed as the Carr index, whereas cohesiveness was expressed as a Hausner Ratio. The higher the value of Carr index, the lower will be the flowability [14]. Hausner ratio shows an interparticle friction between the particles and indicates the compaction properties of materials. From Table 12.1, the Hausner value of the spray-dried powder found to be more than 1.25 resulted in the formation of agglomerates. Increased extraction temperature significantly leads to decrease in Hausner ratio. Cohesiveness determines the flow properties and consistency of powders. Low value of cohesiveness results in better flowability of powder. Chemical composition of powder such as protein, fat, moisture, and dietary fiber content also affects the flowability of powders. The Carr index of the obtained spray-dried powder found to be in the range between 25.47 and 38.43 resulted in reduced flowability which could be due to the particle morphology and its interaction. The flowability of powder was found to be increased with increase in extraction temperature, but there was no significant effect of carrier agents reported on flow properties.

12.5.6 Total Phenolic Content

Total phenolic content (TPC) of encapsulated *Murraya koenigii* leaf extract is shown in Table 12.1. It showed a significant effect of extraction temperature and different carrier agents on phenolic content of encapsulated powder. Total phenolic content is found to be increased with increase in extraction temperature. This indicates that the extraction temperature is the key factor for the extraction of bioactive compounds and leads to increase in total phenolic content. Total phenolic content of powder obtained with whey protein isolates ranged from 46.62 to 71.16 mg gallic acid equivalent (GAE)/g followed by guar gum 51.14–52.91 mg GAE/g and then pectin 19.01–47.23 mg GAE/g. Powder obtained with WPI showed highest total phenolic content which is due to its high emulsification property. Suhag and Nanda [15] reported that retention of phenolic compounds was higher in honey powder encapsulated with WPC, whereas Flores et al. [16] also observed that blueberry pomace extract showed more anthocyanin stability when encapsulated with whey protein isolate.

12.5.7 DPPH Scavenging Radical Activity

A DPPH assay was conducted to assess the scavenging effect of encapsulated powder. While interacting with the DPPH free radical, the antioxidants transfer an electron to DPPH and neutralize the free radicals [17], due to which the extract color changed from purple to golden yellow. Figure 12.1 shows the scavenging effect of *Murraya koenigii* L. leaf extract encapsulated with different carrier agents at varied extraction temperature. The scavenging activity of encapsulated powder obtained

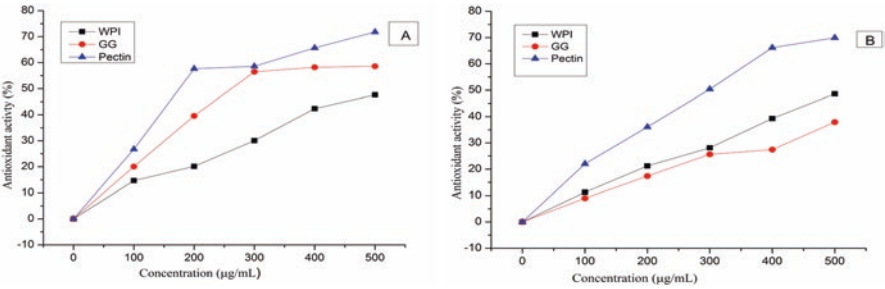


Fig. 12.1 Antioxidant activity of encapsulated powder extracted at an extraction temperature of (**a**) 80 °C and (**b**) 90 °C

with pectin is found to be more in comparison to other carrier agents which may be due to the formation of gel networks which stabilizes the aqueous phase, by increasing the viscosity [12].

Therefore, pectin is considered to anticipate the wall matrix component in a homogenous liquid mixture. With increase in extraction temperature from 80 °C to 90 °C leads to decrease in scavenging activity. Extraction temperature of *Murraya koenigii* leaves showed significant difference on scavenging activity of encapsulated powders which means temperature is the key factor leading to a higher extraction of bioactive compounds. The chemical structure of antioxidants decides their integral reactivity toward free radicals. Scavenging activity of pectin is found to be 71.77%, whereas guar gum and WPI showed 58.59% and 47.60%, respectively, at concentration of 500 µg/mL. The scavenging activity was not found to be correlated with the total phenolic content. Cho et al. [18] also observed that the antioxidant activity of the extracts from *Enteromorpha prolifera* is not directly correlated with their TPC ($r^2 = 0.12$).

12.6 Conclusion

The study was done to produce spray-dried powder of *Murraya koenigii* leaf extract which was extracted at varied extraction temperature. The extract was encapsulated with different carrier agents. The physicochemical parameters were found to be desirable at an extraction temperature of 80 °C, whereas the antioxidant properties were found to be more effective at an extraction temperature of 90 °C. Extract encapsulated with pectin showed more antioxidant activity, whereas extract encapsulated with whey protein isolates showed more total phenolic content. Therefore, the retention of bioactive compounds would be more effective if blends of carrier agents will be used for the encapsulation of *Murraya koenigii* leaf extract.

Acknowledgment We acknowledge the Department of Food Science and Technology, Pondicherry University, Pondicherry, for providing facilities, and are also grateful to UGC for providing Junior Research Fellowship.

References

1. Singh AP (2011) LC-MS-MS characterization of curry leaf flavonols and antioxidant activity. Food Chem 127:80–85
2. Ningappa MB, Dinesha R, Srinivas L (2008) Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (*Murraya koenigii* L.) extracts. Food Chem 106:720–728
3. Fernandes MRV, Azzolini AECS, Martinez MLL, Souza CRF, Lucisano-Valim YM, Oliveira WP (2014) Assessment of antioxidant activity of spray dried extracts of Psidium guajava leaves by DPPH and chemiluminescence inhibition in human neutrophils. BioMed Res Int Article ID 382891, 10 pages
4. Santhalakshmy S, Bosco SJD, Francis S, Sabeena M (2015) Effect of inlet temperature on physicochemical properties of spray-dried jamun fruit juice powder. Powder Technol 274:37–43
5. Association of Official Analytical Chemists (2006) Official methods of analysis of the Association of Official Analytical Chemists, 18th edn. AOAC Press, Gaithersburg
6. Tonon VR, Brabet C, Hubinger M (2008) Influence of process conditions on the physicochemical properties of acai powder produced by spray drying. J Food Eng 88:411–418
7. Goula AM, Adamopoulos KG, Kazakis NA (2004) Influence of spray drying conditions on tomato powder properties. Dry Technol 22(5):1129–1151
8. Shah MA, Bosco SJD, Mir SA (2015) Effect of Moringa oleifera leaf extract on the physicochemical properties of modified atmosphere packaged raw beef. Food Packag Shelf Life 3:31–38
9. Şahin Nadeem H, Torun M, Ozdemir F (2011) Spray drying of the mountain tea (*Sideritis stricta*) water extract by using different hydrocolloid carriers. LWT-Food Sci Technol 44:1626–1635
10. Abadio FDB, Domingues AM, Borges SV, Oliveira VM (2004) Physical properties of powdered pineapple (*Ananas comosus*) juice: effect of malt dextrin concentration and atomization speed. J Food Eng 64(3):285–287
11. Rao MA, Rizvi SSH, Datta AK (2005) Engineering properties of foods, 3rd edn. CRC Press, Boca Raton
12. Paraskevopoulou A, Kiosseoglou V (2013) Interfacial properties of biopolymers, emulsions, and emulsifiers. In: Handbook of biopolymer-based materials: from blends and composites to gels and complex networks. Wiley, Weinheim, pp 717–740
13. Ferrari CC, Germer SPM, Alvim ID, Vissotto FZ, de Aguirre JM (2012) Influence of carrier agents on the physicochemical properties of blackberry powder produced by spray drying. Int J Food Sci Technol 47:1237–1245
14. Lebrun P, Krier F, Mantanus J, Grohganz H, Yang M, Rozet E, Boulanger B, Evrard B, Rantanen J, Hubert P (2012) Design space approach in the optimization of the spray-drying process. Eur J Pharm Biopharm 80:226–234
15. Suhag Y, Nanda V (2015) Evaluation of different carrier agents with respect to physicochemical, functional and morphological characteristics of spray dried nutritionally rich honey powder. J Food Process Preserv. ISSN 1745−4549
16. Flores FP, Singh RK, Kerr WL, Pegg RB, Kong F (2014) Total phenolics content and antioxidant capacities of microencapsulated blueberry anthocyanins during in vitro digestion. Food Chem 153:272–278
17. Naik GH, Priyadarsini KI, Satav JG, Banavalikar MM, Sohoni DP, Biyani MK, Mohana H (2003) Comparative antioxidant activity of individual herbal components used in ayurvedic medicine. Phytochemistry 63:97–104
18. Cho M, Lee HS, Kang IJ, Won MH, You S (2011) Antioxidant properties of extract and fractions from *Enteromorpha prolifera*, a type of green seaweed. Food Chem 127:999–1006

Isolation and Characterization of Microbial Asparaginase to Mitigate Acrylamide Formation in Food

13

Mausumi Ray, Sunita Adhikari (Nee Pramanik), and Pradyut Kundu

Abstract

Asparaginase is an enzyme which is used in food processing industry and also used as a medicine. It is used to treat acute lymphoblastic leukemia, acute myeloid leukemia and non-Hodgkin's lymphoma. In food manufacturing it is used to decrease the acrylamide which is occurring in some starch-based foods during baking, frying and roasting. Acrylamide has carcinogenic effect on animals and human. The free amino acid asparagine reacts with sugars like glucose and fructose during Maillard reaction under high temperature and low moisture condition. To reduce acrylamide in food products, bacterial LA (L-asparaginase) is used. LA catalyzes the conversion reaction of L-asparagine to L-aspartic acid and ammonia. In present investigation, characterization of an extracellular LA from an isolated *Bacillus* sp. strain M6 was carried out in batch scale fermentation process. The effect of pH, temperature and incubation time was measured and the highest asparaginase activity (47 IU/ml) was achieved at pH 7.0, temperature 30 °C.

Keywords

Acrylamide · L-asparaginase · *Bacillus* sp

M. Ray (✉) · S. Adhikari (Nee Pramanik)
Department of Food Technology & Biochemical Engineering, Jadavpur University, Kolkata, West Bengal, India

P. Kundu
Department of Food Processing Technology, A. P. C Ray Polytechnic, Kolkata, West Bengal, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_13

13.1 Introduction

Acrylamide is a chemical compound which has carcinogenic effects on human as well as on animals [1]. In 2008 a research work was conducted on acrylamide and it was found that different types of human cancer such as ovarian, endometrial, breast and kidney cancer are linked with high exposure to acrylamide [2, 3]. Acrylamide is linked with asparagine and Maillard reaction in foods [4–8]. Many processes are used to reduce the formation of acrylamide in foodstuffs. Acrylamide formation during baking and frying depends on moisture content and baking temperature/time. Longer baking times are required to minimize the formation of acrylamide if final products are prepared to the same final moisture but with less color development [9, 10]. The acrylamide load can be reduced by substituting ammonium bicarbonate with inverted sugar and raising agents or by spraying with glycine or by adding inorganic salts and organic acids [11, 12]. Alteration of the sensorial properties like flavor, texture, browning [13] and formation of other undesirable compounds [14] may lead to less consumer acceptance. The changes may even affect the technology of the process like reduction of yeast fermentation properties in bread [15].

It has been also found that asparagine is the major ingredient for the formation of acrylamide in cereal food products. Increased amount of asparagine in various cereals can enhance the production of acrylamide. To reduce acrylamide in different baked and fried products, the free asparagine content needs to be reduced by the enzyme asparaginase that hydrolyzes asparagine to aspartic acid and ammonia. Over 90% of acrylamide content is reduced when asparaginase-treated mashed potato, potato flakes, rye flour, and wheat flour are incubated [8, 11, 16].

The objective of the present work is to isolate a potent bacterial strain capable of producing asparaginase enzyme and optimize various process parameters for maximum production of the desired enzyme.

13.2 Materials and Methods

13.2.1 Isolation of a Potent Bacterial Strain

For the isolation of a potent bacterial strain, 10 g of soil was transferred to conical flask containing 100 ml of sterile modified M9 medium having the following composition (g/L): KH_2PO_4, 3.0; Na_2HPO_4, 6.0; NaCl, 0.5; $MgSO_4$,$7H_2O$, 0.12; and $CaCl_2$, $2H_2O$, 0.001. The medium was enriched with 1% asparagine. Then the conical flask was kept in incubator for 24 h. The diluted soil sample was added to the modified M9 agar medium enriched with 1% asparagine and 0.005% phenol red. Plates were incubated at 37 °C for 24 h. The asparaginase enzyme production was accompanied by an increase in pH of the medium; as a result there will be formation of pink zone around the colonies that help in identification of asparaginase production. The selected colonies were isolated and maintained on nutrient agar slant at 4 °C.

13.2.2 Enzyme Assay

Asparaginase enzyme assay was performed for each isolated colonies to select the most potent strain using UV–visible spectrophotometer. During estimation ammonia was produced due to the action of asparaginase enzyme on the substrate asparagine. The ammonia produced was proportional to the amount of the enzyme present and it was detected using Nessler's reagent. The reaction mixture contained 0.5 ml of 0.08 mM l-asparagine, 1.0 ml of 0.1 M acetate buffer (pH 5.0) and 0.5 ml of enzyme solution. With the addition of 0.5 ml of 15 % trichloroacetic acid solution after 30 min of incubation, the reaction was terminated. One unit (IU) of LA is the amount of enzyme that produces 1 μM of NH_3 per minute under the optimum assay conditions.

13.2.3 Optimization of Process Parameters

13.2.3.1 Optimization of pH

The selected strain of the organism was grown at different pH i.e., 4, 5, 6, 7 and 8 at 30 °C for 24 h. After completion of 24 h the fermented broth was centrifuged and the clear supernatant was taken for the enzymatic assay.

13.2.3.2 Optimization of Temperature

The selected strain of the organism was grown at different temperatures, i.e., 25, 30, 35 and 40 °C for 24 h. After completion of 24 h, the fermented broth was centrifuged, and the clear supernatant was taken for the enzymatic assay.

13.2.4 Morphological Study of the Isolated Strain

The morphological, cultural and biochemical characteristics of the isolated strain were studied according to *Bergey's Manual of Determinative Bacteriology* [17].

13.3 Results and Discussion

13.3.1 Screening of Bacterial Strains for LA Activity

Fifteen different bacterial strains were isolated from soil samples. After screening of LA production capability one particular strain (M6) was selected as it was the most potent strain.

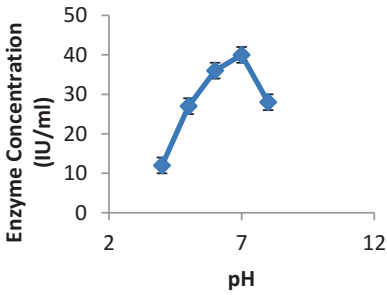


Fig. 13.1 Optimization of pH for maximum enzyme production by the isolated strain

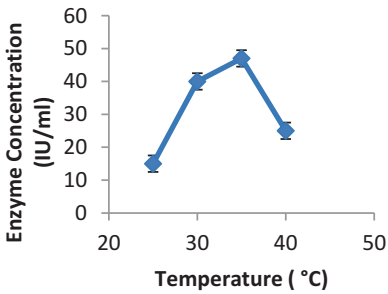


Fig. 13.2 Optimization of temperature for maximum enzyme production by the isolated strain

13.3.2 Morphological Study of the Isolated Strain

The isolate was aerobic, gram positive and rod shaped and showed sub-terminal endospore on spore staining. Positive growth of the strain was observed in the medium that contained glucose, arabinose, galactose, lactose and sucrose. The strain had the capability to hydrolyze starch, casein and can use citrate but could not produce hydrogen sulfide (H_2S). Positive urease and catalase activity had been shown by the isolated strain. According to the morphological and biochemical characteristics of the strain M6 was identified as *Bacillus sp.* M6.

13.3.3 Optimization of Process Parameters

The production of LA by the isolated *Bacillus* sp. M6 was found maximum at pH 7.0. The asparaginase activity at this optimum pH was 40 IU/ml. To study the effect of temperature on the enzyme production by the isolated *Bacillus* sp. M6 pH was fixed to 7.0 but the incubation temperature was varied. It was found that at 35 °C maximum enzyme production was obtained and it was 47 IU/ml (Figs. 13.1 and 13.2).

13.4 Conclusion

In the present study 15 organisms were isolated from soil sample capable of producing L-asparaginase enzyme. After screening the strain marked as M6 was selected as the most potent one. The morphological and biochemical test of the strain was performed and it was identified as *Bacillus* sp. M6. The strain was capable to produce 47 IU/ml of the desired enzyme at pH 7.0 and temperature 35 °C. The other environmental conditions for maximum enzyme production by the isolated strain can be further optimized. The enzyme can be produced in large quantity, purified and applied to raw materials before application of high-temperature treatment like baking or deep-oil frying to reduce its asparagine content. As a result lesser quantity of acrylamide will be formed.

References

1. Abdel FY, Olama ZA (2002) l-Asparaginase production by *Pseudomonas aeruginosa* in solid-state culture: evaluation and optimization of culture conditions using factorial designs. Process Biochem 38:1665–1668
2. Adinarayana K, Ellaiah P, Srinivasulu B, Devi RB, Adinarayana G (2003) Response surface methodological approach to optimize the nutritional parameters for neomycin production by Streptomyces marinensis under solid state fermentation. Process Biochem 38:1565–1572
3. Aghaiypour K, Wlodowes A, Lubkowski J (2001) Structural basis for the activity and substrate specificity of *Erwinia chrysanthemi* l-asparaginase. Biochemistry 40:5655–5664
4. Amrein TM, Schonbachler B, Rohner F, Lukac H, Schneider H, Keiser A (2004) Potential for acrylamide formation in potatoes: data from the 2003 harvest. Eur Food Res Technol 219:572–578
5. Basha SN, Rekha R, Komala M, Ruby S (2009) Production of extracellular antileukaemic enzyme l-asparaginase from marine actinomycetes by solid state and submerged fermentation: purification and characterization. Trop J Pharm Res 8:353–360
6. Borkotaky B, Bezbaruah RL (2002) Production and properties of asparaginase from a new Erwiniasp. Folia Microbiol 47:473–476
7. Bradford MA (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem 72:248–254
8. Zyzak DV, Sanders SA, Stojanovic M, Tallmadge DH (2003) Acrylamide formation mechanism in heated foods. J Agric Food Chem 51:4782–4787
9. Amrein TM, Andres L, Manzard GG, Amado R (2006) Investigations on the promoting effect of ammonium hydrogen carbonate on the formation of acrylamide in model systems. J Agric Food Chem 54:10253–10261
10. Gordon K (2006) Review of acrylamide mitigation in biscuits, crackers and crisp bread on behalf of the CAOBISCO acry-lamide research project group, Brussels
11. Amrein TM, Schonbachler B, Escher F, Amado R (2004) Acrylamide in gingerbread: critical factors for formation and possible ways for reduction. J Agric Food Chem 52:4282–428812
12. Gökmen V, Şenyuva HZ (2007) Acrylamide formation is prevented by divalent cations during the Maillard reaction. Food Chem 103:196–203
13. Gökmen V, Açar ÖÇ, Köksel H, Acar J (2007) Effects of dough formula and baking conditions on acrylamide and hydroxyl methyl furfural formation in cookies. Food Chem 104:1136–1142
14. Gokmen V, Senyuva HZ (2007) Effects of some cations on the formation of acrylamide and furfurals in glucose-asparagine model system. Eur Food Res Technol 225:815–820

15. CIAA, Acrylamide Toolbox. Confederation of Food and Drink Industries of the EEC, February 2009, revision 12.
16. Ciesarová Z, Kiss E, Kolek E (2006) Study of factors affecting acrylamide levels in model systems. Czech J Food Sci 24:133–137
17. Bergey DH, Buchanan RE, Gibbons NE (1974) American Society for Microbiology, 8th edn

14 Small Colony Variant Selection, Biofilm Induction, and Interspecies Interactions of Ocular Clinical *Pseudomonas aeruginosa*

Sadhana Sagar and Shilpa Deshpande Kaistha

Abstract

Pseudomonas aeruginosa, an opportunistic pathogen, is frequently isolated from ocular infections. In this study, antibiotic resistance and biofilm formation ability of ocular isolate, *Ps. aeruginosa* AV2 is characterized. *Ps. aeruginosa* AV2 is shown to be a potent multiple drug resistant, biofilm former using the adherence, Congo red binding, and acyl homoserine lactone production assays. The extracellular products of the isolate show strong antimicrobial activity against interspecies ocular isolates *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus cereus*, and *Enterobacter aerogenes*. Antimicrobial activity was also found against reference strains *S. aureus* ATCC 25923 and *S. aureus* ATCC 33529. The active compounds in the cell-free extract (CFE) were characterized to be stable at 55 °C and pH 7.0 and non-proteinaceous in nature. ATR-FTIR spectroscopy peaks show the presence of amines, phenolic, and lactone groups. In the presence of the CFE, *S. aureus*, *E. coli*, and *Ps. aeruginosa* exhibit approximately twofold induction in biofilm type of growth. While the CFE has no effect on interspecies mature biofilms, it showed induction of intraspecies mature biofilm. The results suggest that although *Ps. aeruginosa* secondary metabolites may be characterized as possessing antimicrobial activity, they may in fact increase intra- and interspecies cells to adhere to substrates and form biofilms. Inter-/intraspecies

S. Sagar
Agriculture Microbiology, Rani Lakshmi Bai Central Agricultural University, Jhansi, Uttar Pradesh, India

S. D. Kaistha (✉)
Department of Microbiology, Institute of Biosciences & Biotechnology, Chhatrapati Shahu Ji Maharaj University, Kanpur, Uttar Pradesh, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_14

microbial infections are common in the eye. Hence, polymicrobial interactions at infectious site may lead to development of microbial populations such as biofilms that are refractory to antimicrobial treatments.

Keywords
Biofilm · Antimicrobial activity · *Pseudomonas aeruginosa* cell-free extract

14.1 Introduction

Ps. aeruginosa is a gram-negative ubiquitous opportunistic pathogen isolated typically from the eye, skin, wound, burn, catheter, and cystic fibrosis infections. The inhibitory activity of *Ps. aeruginosa* through its extracellular products on co-species has been the subject of several reports [1–3]. A plethora of primary and secondary metabolites, antibiotics, signaling molecules, nutrients, and enzymes regulate the interactions and growth of surrounding communities. Interactions between intra- and interspecies organisms occur with the help of diffusible products which are produced at different stages of microbial growth. The responses to such signals can be varied including synergism and antagonism. *Ps. aeruginosa* is a producer of several antimicrobial compounds such as hydrogen cyanide, pyocyanin, phenazines, quinolones, etc. [3, 4]. There are, however, also reports of the persistence of multispecies with *Pseudomonas*. Hence, the isolation, for example, of *S. aureus*, a gram-positive cocci, frequently co-isolated with *Ps. aeruginosa* despite its sensitivity to *Pseudomonas* exotoxins [5, 6]. Interspecies interactions of *Ps. aeruginosa* have severe clinical implications as microbial consortia are being frequently isolated from infections which were considered to be primarily *Ps. aeruginosa* infections [7]. Clinical infections due to microbial consortia are likely to be develop increasing resistance to high generation antimicrobial agents by horizontal gene transfer [8].

Microorganisms may exist as free living or in the form of communities called biofilms [8, 9]. Biofilm form of growth is a survival strategy for several microorganisms under conditions of stress [8]. Antimicrobial settings often induce transcriptional changes in growth forms leading to the development of biofilm communities which insulate the encapsulated organisms. In many infectious and ecological settings, microbial consortia compete with each other in an exopolymeric matrix of the biofilms [8, 9]. Within biofilms, cell-cell communication occurs using diffusible chemical signaling messengers or quorum sensing signals that can affect gene expression profiles as well as metabolic changes within the biofilm [9]. Biofilm formation as a mechanism responsible for the persistence of interspecies with *Ps. aeruginosa* within clinical settings is therefore explored in this study.

Interspecies interactions have been studied previously with *Ps. aeruginosa* NCNC6750 and *Ps. aeruginosa* PA01 [3]. There are few reports of the multispecies interactions with freshly isolated clinical *Pseudomonas* [2]. The nature of such interactions within clinical isolates remains poorly reported. In the present study, the interspecies interactions with clinical isolate from ocular infection have been

explored. A clinical isolate *Ps. aeruginosa* AV2 was isolated from an ocular infection and its virulence traits characterized. Further, the effect of *Ps. aeruginosa* AV2 and its extracellular product on microbial growth, both as planktonic and biofilms, was studied on multiple cultures such as *S. aureus* and *E. coli*.

14.2 Materials and Methods

14.2.1 Bacteria

Ps. aeruginosa AV2, *B. cereus*, *M.luteus*, *E.aerogenes*, *E. coli*, and *S. aureus* are clinical isolates from ocular infections obtained from Department of Microbiology, CSJM University, Kanpur. The cultures were maintained on Tryptone Soya Media at 37 °C. *Ps. aeruginosa* PA01 and *Chromobacterium violaceum* ATCC 12472 are a kind gift from Prof I. Ahmad, AMU, Aligarh, and Prof R McLean, Texas State University, TX, USA. *Ps. aeruginosa* ATCC 15442, *S. aureus* ATCC 25893 (MSSA), and *S. aureus* ATCC 33592 (MRSA) were purchased from American Type Culture Collection (Hi Media, India) and all the cultures were maintained on Tryptone Soya (TS) Media at 37 °C.

14.2.2 Characterization of Cell-Free Extract

Log phase culture of *Ps. aeruginosa* AV2 was extracted at different time points post infection, centrifuged, filtered, and supernatant used as cell-free extract (CFE). The composition of the CFE was determined by measurement of exopolysaccharide and protein content using phenol-sulfuric acid method [10] and Folin-Lowry method, respectively [10]. Varying treatments of pH (3.0, 5.0, 7.0, and 9.0) and temperature (37 °C, 55 °C, 70 °C, and 95 °C for 30 min) and proteinase K treatment (0.1 mg/ml for 20 min) of the cell-free extract were adjusted prior to setting up antimicrobial zone of inhibition assay. Spectra for ATR-FTIR spectroscopy were obtained with a Bruker Vertex 70 ATR-FTIR spectrometer. For each spectrum, eight interferograms were collected at 4 cm^{-1} resolution and 4000–600 cm^{-1} wave-number range.

14.2.3 *C. violaceum* Bioassay

The bioassay was performed as per the modified protocol of McLean et al. [11]**.** Log phase culture of *C. violaceum* ATCC12472 was spread on TS agar. 10 µl of *Ps. aeruginosa* PA01 and *Ps. aeruginosa* AV2 was added to the bored wells in the agar. Zone of inhibition of growth of *C. violaceum* and zone of no pigmentation were recorded.

14.2.4 Antimicrobial activity

Antimicrobial activity was determined using the colony biofilm, spot culture method as well as agar well diffusion in a zone of inhibition assay. Overnight grown test cultures were spread on tryptone soya peptone agar and dried for 10 min. Subsequently log phase culture of *Ps. aeruginosa* AV2 was spot inoculated on the agar, air-dried and incubated for 24 h at 37 °C. Cell-free extract was used in agar well diffusion assay for determination of antimicrobial activity against different cultures. Zone of inhibition was measured in mm.

Antimicrobial activity was measured in arbitrary units (AU). It was defined as the maximum dilution which produced a clearly visible antibacterial zone. The reciprocal of the dilution gave the titer of antibacterial activity in AU per ml. It is defined as reciprocal of the highest dilution resulting in a clear zone of growth inhibition.

14.2.5 Biofilm Formation and Eradication Assay

Static biofilm formation assay was used as per modified protocol O'Toole and Kotler [12]. Isolates were grown in 1.5 ml polyethylene tubes containing 500 µl of TS broth (TSB) and 96-well microtiter plates containing 200 µl of TSB for different time point at 37 °C for 24 h. For biofilm eradication assays, 24 h mature biofilm was washed prior to treatment with the cell-free extract (1:1). After 24 h of incubation, biofilms were washed with sterile saline. Cultures were removed and planktonic growth measured spectrophotometrically at A630. Static surface with biofilms was washed with sterile saline. Adherent bacteria were stained with 1%w/v crystal violet for 20 min. Stained adherent bacteria were washed and detached using 200 µl of dimethyl sulfoxide and solubilized biofilms measured using microtiter plate reader (Thermo Scientific, USA) at A630. Results are mean of three experiments done in triplicates.

14.2.6 Motility

Motility was determined by swimming plate, swarming plate, and twitch plate assay as described previously [13].

14.2.7 Exopolysaccharide Determination

Exopolysaccharide measurement for biofilms was performed by phenol-sulfuric method [14] as well as Congo red binding assay [15].

14.2.8 Hydrophobicity

Microbial hydrophobicity assay was performed as described by Rosenberg et al. [16].

14.2.9 Acyl Homoserine Lactone

The production of acyl homoserine lactone was checked as per modified protocol [17].

14.2.10 Antibiotic Sensitivity

Antibiotic susceptibility tests for *Ps. aeruginosa* AV2 and *Ps. aeruginosa* PA01 isolates were performed by disk diffusion method (Hi Media) on Mueller Hinton Agar (HiMedia, India) as per CLSI nomenclature [18].The antibiotics tested include ampicillin (Ap, 10 μg), cephalothin (Ch, 30 μg), colistin sulfate (Cl, 10 μg), gentamicin (G, 10 μg), streptomycin (S, 10 μg), and tetracycline (T, 30 μg).

14.2.11 SCV induction

Double agar overlay method was used wherein upper layer of TSA containing 160 AU/ml of CFE was prepared. *S. aureus* ATCC 25983 was spread on the plate and growth measured up to 48 h of incubation at 37 °C for the emergence of SCV. In additionally, 24 h *S. aureus* mature biofilm was treated with 160 AU/ml of CFE for 24 h with incubation at 37 °C. Titers of *S. aureus* were determined by dilution plating of biofilm cells on TSA in the absence or presence of CFE as per double agar overlay method assay. Frequency of SCV generation was calculated by determining the ratio of SCV to normal phenotype cells.

Statistics Statistical analysis was done using student's t test. One way ANOVA was used for comparison of means. All experiments were repeated at least thrice in triplicates. $p \leq 0.05$ was considered as biologically significant.

14.3 Results

14.3.1 Bacterial Characterization

Ps. aeruginosa AV2 was isolated from intraocular lens-associated microbial keratitis infection and characterized using standard microbiological and biochemical tests as per Bergey's determinative bacteriology [19]. The isolate was also characterized for its ability to form biofilm in static biofilm assay [12] and antibiotic susceptibility using disk diffusion method as per CLSI nomenclature [18]. Table 14.1 shows biofilm characterization of the isolate on the basis of static biofilm assay; swarming,

twitching, and swimming motility; production of acyl homoserine lactone; and EPS production. *Ps. aeruginosa* PA01 was used as standard reference strain for biofilm formation. *Ps. aeruginosa* AV2 is a moderate biofilm former and shows resistance to ampicillin, cephalothin, colistin sulfate, co-trimoxazole, gentamicin, streptomycin, and tetracycline. Isolate shows strong swimming, swarming, and twitching motility. Exopolysaccharide production and acyl homoserine lactone production were comparable to standard reference strain for biofilm studies, *Ps. aeruginosa* PA01.

14.3.2 Antimicrobial Activity and CFE Characterization

The antimicrobial activity of the isolate was serendipitously discovered during a motility assay; large zones of inhibition were seen to a contaminating clinical isolate *B. cereus*. Cell-free extracts (CFE) were obtained at different times post incubation (6–72 h) to determine the kinetics of the production of the antimicrobial metabolite using zone of inhibition assay. Figure 14.1 shows the kinetics for growth of *Ps. aeruginosa* AV2 and its antimicrobial metabolite production against *B. cereus*. The antimicrobial product was a secondary metabolite which shows maximum activity at 48 hrs post inoculation. Further, spot culture assay was carried out with *Ps. aeruginosa* AV2 against *B. cereus*, *M.luteus*, *S. aureus* ATCC 25983 (MSSA), *S. aureus* ATCC 33529 (MRSA), *E. aerogenes*, *E. coli*, and *Ps. aeruginosa* ATCC 15442 (Fig. 14.2). 48 h cell-free extract was obtained and used in agar

Table 14.1 Biofilm characteristics and antibiogram of *Ps. aeruginosa* AV2 and *Ps. aeruginosa* PA01

Characteristics	*Ps. aeruginosa* AV2	*Ps. aeruginosa* PA01
Antibiogram[a]	$Ap^RCh^RCl^RCo^ROf^R$ $G^RS^RT^R$	$Ap^RCh^RCl^RCo^ROf^R$ $G^RS^RT^R$
Biofilm formation assay[b]		
Polyethylene	1.305 ± 0.54	1.576 ± 0.275
Polystyrene	0.296 ± 0.091	0.188 ± 0.011
Hydrophobicity[c]	11.94 ± 5.25%	39.4 ± 5.2%
Exopolysaccharide production[d] $_{(A540)}$	0.5 ± 0.177	0.37 ± 0.2
Congo red binding assay[e] $_{(A630)}$	0.392 ± 0.005	0.54 ± 0.8
Acyl homoserine lactone[f] production	0.412 ± 0.055	0.491 ± 0.085
Motility (Zone in mm)		
Swimming	31.0 ± 12	34.33 ± 10.27
Swarming	25.25 ± 0.6	47 ± 6.68
Twitching	12.25 ± 1.2	25.66 ± 14.05

[a]Ap, ampicillin (10 μg); Ch, cephalothin (10 μg); Cl, colistin sulfate (10 μg); Co, co-trimoxazole (25 μg); G, gentamicin (10 μg); S, streptomycin (10 μg); T, tetracycline (30 μg), [b]Biofilm formation assay [18], [c]hydrophobicity assay [23], [d]exopolysaccharide production [4], [e]Congo red binding assay [9], [f]acylhomoserine lactone assay [25],[e] motility[22] were performed by using equal cells of OD = 0.5 from both the isolates.
Results are mean of triplicates and represent one of three experiments

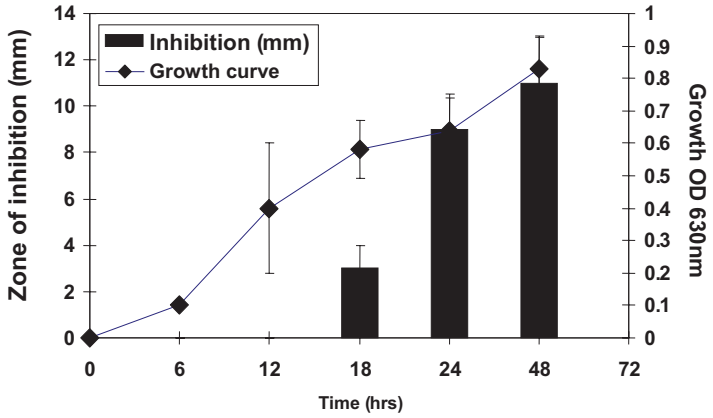


Fig. 14.1 Growth kinetics of *Ps. aeruginosa* AV2 and CFE antimicrobial activity, shown as zone of inhibition in mm (mean ±SD), against *Bacillus cereus* isolate at different time points

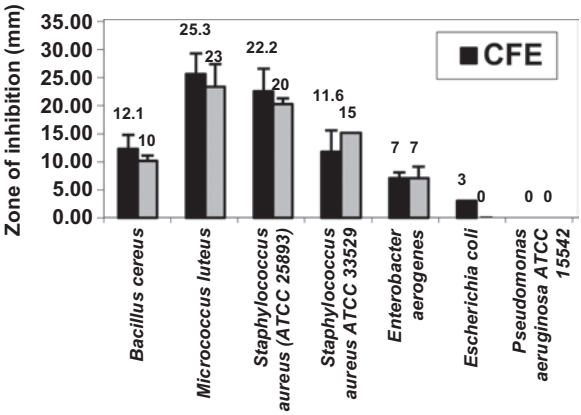


Fig. 14.2 Antimicrobial activity of *Ps. aeruginosa* AV2 in spot culture assay using cells and zone of inhibition assay using cell-free extract (CFE). Data shown as zone of inhibition in mm (mean ± SD) represent means of three experiments

diffusion assay to determine the range of activity against gram-positive and gram-negative bacteria (Fig. 14.2). *Ps. aeruginosa* AV2 and its CFE were found to inhibit growth of *S. aureus* ATCC 25893 (MSSA), *S. aureus* ATCC 33592 (MRSA), *B. cereus*, and *E.aerogenes* but had no effect on gram-negative *E. coli* and *Ps. aeruginosa* ATCC 15442. Maximal antimicrobial activity of the extracellular product was targeted at gram-positive bacteria such as *S. aureus* and *B. cereus* at 160 AU/ml.

The physicochemical characteristics of the cell-free extract were determined by various treatments followed by checking its activity against quality control reference organism *S. aureus* ATCC 25893 in a zone of inhibition assay. Antimicrobial activity of the cell-free extract was found to be acid stable from pH (5.0–7.0) but alkali unstable (pH 9.0) and temperature stable at 55 °C for 30 min but inactivated beyond 75 °C for 10 min. Activity was unaffected by proteinase K treatment (1 mg/ml), suggesting primarily non-proteinaceous nature (Fig. 14.3). Exopolysaccharide

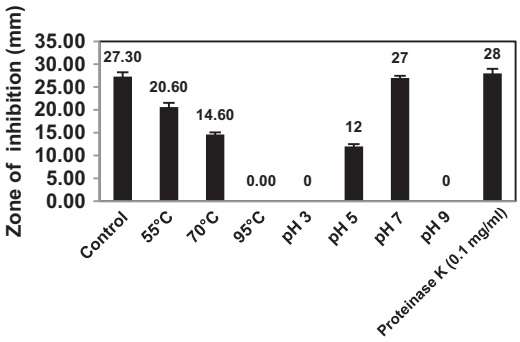


Fig. 14.3 Physicochemical characteristics of the *Ps. aeruginosa* AV2 CFE following treatments at various temperatures (37 °C, 55 °C,70 °C, 90 °C), pH (pH 3.0, 5.0, 7.0, 9.0), and proteinase K (0.1 mg/ml) treatment. Data shown as zone of inhibition in mm (mean ±SD) represent means of three experiments

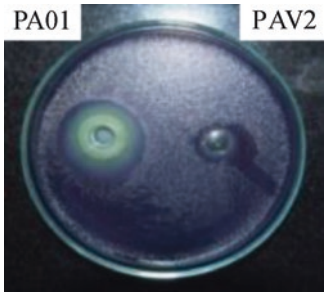
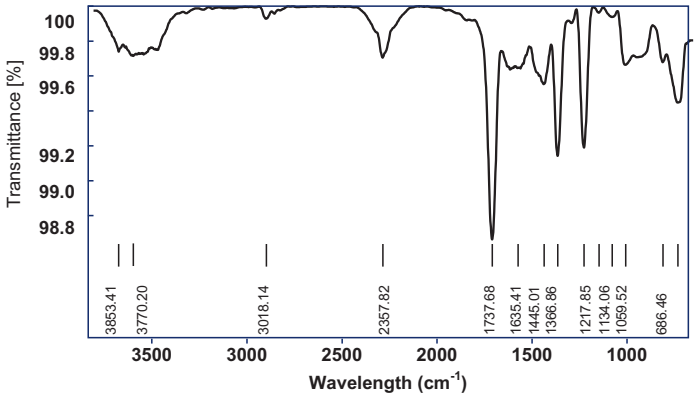


Fig. 14.4 Effect of *Ps. aeruginosa* AV2 (PAV2) and *Ps. aeruginosa* PA01 (PA01) in violaceum bioassay using *Chromobacterium violaceum* ATCC 12472

content of the CFE was determined by the phenol-sulfuric method [14] and found to be 1.5 mg/ml, whereas protein content was found to be 6.4 mg/ml [10]. The effect of the CFE on quorum sensing was tested using *C. violaceum* ATCC 12472 in agar well diffusion bioassay. However, CFE was found to inhibit growth of *C. violaceum* ATCC 12472 and not affect pigment formation (Fig. 14.4). *Ps. aeruginosa* PA01 was used as positive control as a producer of C-4 and 3 oxo-C12 AHL [20]. Hence, activity is primarily antimicrobial and does not affect production of dodecanoyl-homoserine lactone, quorum sensing molecule for which violaceum production is bioassayed.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was used to determine the molecular structure of the cell-free extract. Figure 14.5 shows the characteristic absorption spectra of the peaks obtained for the CFE. The three main peaks at 1737 cm^{-1}, 1366 cm^{-1}, and 1217 cm^{-1} correspond to lactones or primary amines, carbohydrate groups inclusive of OH/CH groups, and C–O stretching and O–H deformation of carboxyl, phenols, and aromatic ethers [20].



Peaks (cm^{-1})	Characteristics
3550-3853	(-OH) hydroxyl stretch intermolecular stretch for water
2357	NH or CO stretching vibrations
1737	Primary amines and associated amides, lactones
1445	CH2 bending vibration involving methyl, methylene and methoxy groups
1217	C-O stretching and O-H deformation of carboxyls, phenols, and aromatic ethers CO Amide III
1366	CH and OH deformational vibrations (carbohydrates)
983-1060	C=CH2
686-771	-CH=CH2 (trans)

Fig. 14.5 ATR-FTIR spectroscopy of *Ps. aeruginosa* AV2 CFE. Table lineate the characteristics of the various peaks found in the CFE spectra

14.3.3 Effect on Biofilm Formation and Biofilm Eradication

The 48 h CFE was used to study its effect on biofilm formation of *S.aureus* (MSSA and MRSA), *Ps. aeruginosa*, and *E. coli* strains. Figure 14.4 shows that while no effect was observed on planktonic growth of both the *Staphylococcal* isolates, biofilm formation was induced at biologically significant levels. In *Ps. aeruginosa,* biofilm induction was found to be over 50% with a decrease in planktonic cells, while in *E.coli* cells significant induction was observed in growth of planktonic as well as biofilm growth (Fig. 14.6).

The effect of the supernatant was also studied on preformed biofilms. No effect was observed in the planktonic and biofilm forming cells for the *Staphylococcal* cells. *E. coli*-associated planktonic cells were mitigated with no effect on biofilm formation (Fig. 14.7). Presence of the CFE induced biofilm formation in preformed *Ps. aeruginosa,* biofilm likely due to presence of quorum sensing lactones. Acyl homoserine lactone assay for the CFE extract suggests that the biological active component responsible for biofilm induction is likely to be homoserine lactone (Table 14.1). Although the CFE showed antimicrobial properties in agar diffusion assay as well as mitigation of planktonic cells in liquid culture, it was shown to induce biofilm formation particularly for all gram-positive and gram-negative isolates tested with no effect on preformed biofilm cells.

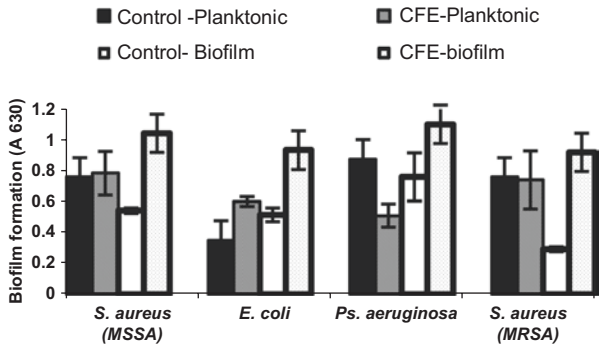


Fig. 14.6 Effect of *Ps. aeruginosa* AV2 CFE on planktonic growth and biofilm in a biofilm formation assay

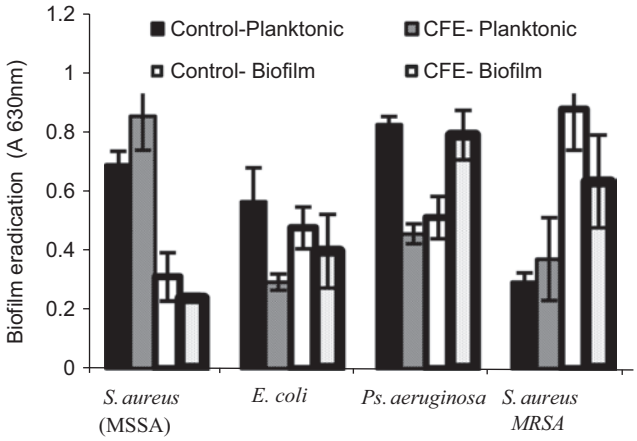


Fig. 14.7 Effect of *Ps. aeruginosa* AV2 CFE on 24hr mature biofilm of different cultures

14.3.4 Selection of *S. aureus* single colony variants (SCV)

In order to determine the cause of enhanced biofilm following treatment with CFE, *Ps. aeruginosa* AV2 CFE were used in SCV assay for *S. aureus* ATCC 25983 as described in material and method. Interestingly, small colony variants (SCV) for *S. aureus* appeared following 48 h of incubation (Fig. 14.8). When the SCV were subsequently plated on fresh TSA, they regained their morphological size suggesting reversible phenotype switch. The *S. aureus* SCVs were characterized by small colony size, reduced growth rate, and pigmentation. *S. aureus* biofilm cells treated with CFE for 24 h were plated on TSA in the presence and absence of CFE.

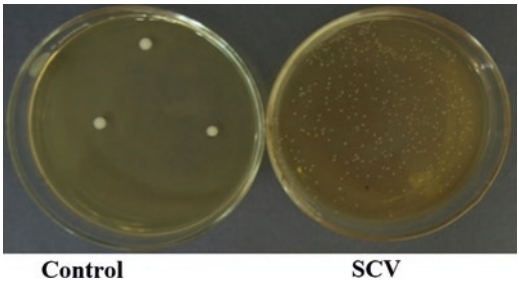


Fig. 14.8 Induction of *S. aureus* small colony variants by *Ps. aeruginosa* AV2 CFE

Figure 14.8 shows the SCV in the presence of CFE in comparison to control untreated cells. The frequency of the SCV generation in biofilms was found to be 1.26×10^{-4}. Hence, CFE can select *S. aureus* SCV in biofilm mode as well double agar plate assay.

14.4 Discussion

In this study we isolated a biofilm forming clinical isolate of *Ps. aeruginosa* AV2 and compared the effect of its extracellular supernatant on planktonic and biofilm modes of growth of co-cultures. Antimicrobial activity at 48 h post-logarithmic growth was detected against predominantly gram-positive clinical ocular isolates such as *S. aureus*, *M. luteus*, *B. cereus*, and *E. aerogenes* but not to *E. coli* and *Ps. aeruginosa* ATCC 15542. The cell-free extract responsible for the antimicrobial activity was found to be non-proteinaceous and heat stable up to 55 °C. ATR-FTIR spectroscopy analysis revealed strong peak at 1737 cm^{-1}which correlate to structure containing lactones group [20].

Multispecies biofilms comprising of *Ps. aeruginosa* and *S. aureus* or *E. coli* have been reported from clinical samples [4–7, 10, 21]. *Ps. aeruginosa* secretes a number of extracellular products such as pyocyanin, hydrogen cyanide, or quinolone N oxides that can act against normal microflora as well as host cells [4]. Toxicity against *S. aureus* by *Ps. aeruginosa* has been reported to target specifically the electron transport chain [1, 22]. Whereas in a similar co-culture experiment *Ps. aeruginosa* conditioned supernatant gave rise to synergistic effect on *E coli* growth [23]. In this study, we report that cell-free supernatant of *Ps. aeruginosa* is capable of inhibiting growth of co-cultures in zone of inhibition assay but inducing biofilm formation without affecting mature biofilm. More than 50 quinolone products from *Ps. aeruginosa* are produced having significant antibiotic activity against gram-positive organisms [6, 23]. Las A protease from *Pseudomonas* also shows notable antistaphylococcal activity [6]. Utilization of *Staphylococcus* as source of iron has also been reported in isolates from cystic fibrosis [24].

Stress environments can induce cells to form biofilms [9]. Low concentrations of antibiotics can induce biofilm formation in many pathogenic settings [25, 26]. The mechanisms for such activity are not very well understood. *Ps. aeruginosa* AV2 supernatant which showed antimicrobial activity in a zone of inhibition assay showed

significant induced biofilm formation in all isolates studied. Hence, secondary metabolite such as antibiotic may work to trigger gene regulation inducing biofilm formation, while quorum sensing lactones may induce formation of gram-negative biofilms. The antimicrobial activity produced by the *Pseudomonas* herein showed no effect or dispersal of mature biofilms. Extracellular polysaccharides produced by the *pel* and *psl* system were found to disrupt *S. epidermis* biofilms independent of its bactericidal activity evidenced with mutant studies [3]. Yet, presence of *Staphylococcus* in infectious settings suggests that subpopulation of *Staphylococcus* can persist and coexist. In the present study, SCV developed within the zone of inhibition following exposure to *Ps. aeruginosa* AV2 CFE or secreted products from colony biofilm. Small colony variants as a survival strategy by *S. aureus* in the presence on *Ps. aeruginosa* supernatant has been reported recently [21].

Once a biofilm is formed, it is extremely difficult to eradicate. The effectiveness of *Ps. aeruginosa* exotoxins on neighboring species in conditions of biofilms may hence be far more limiting compared to when it is in free living conditions. The role of quorum sensing-dependent extracellular products in competing with other species is likely to differ compared to metabolites produced as planktonic cells and require further investigations. Hence the extracellular products of *Ps. aeruginosa* may have differential effect on cultures depending on their growth status [2].

In light of differential behavior of antimicrobial compounds in the case of planktonic as well as biofilm forming cells, it is imperative to not depend only on zone of inhibition assay or liquid assays for antimicrobial activity but also study effects of the compounds on biofilm forming cells. *Ps. aeruginosa* and *S. aureus* multispecies biofilms have been identified in a number of infectious diseases such as cystic fibrosis, otitis media, etc. The role of *Ps. aeruginosa* extracellular products in enhancing the biofilm formation in multispecies biofilms may be a responsible for the refractory nature of such microbial growths.

Acknowledgment Financial support from Department of Science and Technology and Department of Atomic Energy, Government of India, is gratefully acknowledged. We are grateful for ATR-FTIR facility used at Indian Institute of Technology, Kanpur.

References

1. Goldsworthy MJH (2008) Gene expression of *Pseudomonas aeruginosa* and MRSA within a catheter-associated urinary tract infection biofilm model. Biosci Hor 1:28–37
2. Pihl M, Chávez de Paz LE, Schmidtchen A, Svensäter G, Davies JR (2010) Effects of clinical isolates of *Pseudomonas aeruginosa* on *Staphylococcus epidermidis* biofilm formation. FEMS Immunol Med Microbiol 59(3):504–512
3. Qin Z, Yang L, Qu D, Molin S, Tolker-Nielsen T (2009) *Pseudomonas aeruginosa* extracellular products inhibit staphylococcal growth, and disrupt established biofilms produced by *Staphylococcus epidermidis*. Microbiology 7:2148–2156
4. Machan ZA, Pitt TL, White W, Watson D, Taylor GW, Cole PJ, Wilson R (1991) Interaction between *Pseudomonas aeruginosa* and *Staphylococcus aureus*: description of an antistaphylococcal substance. J Med Microbiol 34(4):213–217
5. Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI (2006) Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 103:19890–19895

6. Kessler E, Safrin M, Olson JC, Ohman DE (1993) Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. J Biol Chem 10:7503–7508
7. Klepac-Ceraj V, Lemon KP, Martin TR, Allgaier M, Kembel SW, Knapp AA, Lory S, Brodie EL, Lynch SV, Bohannan BJ, Green JL, Maurer BA, Kolter R (2010) Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and *Pseudomonas aeruginosa*. Environ Microbiol 12(5):1293–1303
8. Anderson GG, O'Toole GA (2008) Innate and induced resistance mechanisms of bacterial biofilms. In: Romeo T (ed) Bacterial biofilms. Springer, Heidelberg, pp 85–105
9. López D, Vlamakis H, Kolter R (2010) Biofilms. Cold Spring Harb Perspect Biol 12(7):398
10. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1951) Colorimetric method for determination of sugars and related substances. Nature 28:167–174
11. McLean RJC, Pierson LS III, Fuqua C (2004) A simple screening protocol for the identification of quorum signal antagonists. J Microbiol Meth 58(3):351–360
12. O'Toole GA, Kotler R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens*WCS365 proceed via multiple, convergent signaling pathways: a genetic analysis. Mol Microbiol 28:449–461
13. Rashid MH, Kornberg A (2000) Inorganic polyphosphate is needed for swimming, swarming and twitching motilities of *Pseudomonas aeruginosa*. PNAS 97(9):4885–4890
14. Kay WW, Phipps BM, Ishiguro EE, Trust TJ (1985) Porphyrin binding by the surface array virulence protein of *Aeromonassalmonicida*. J Bacteriol 164:1332–1336
15. Abate G, Mshana RN, Miorner H (1998) Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in Mycobacterium tuberculosis. Int J Tuberc Lung Dis 2:1011–1016
16. Rosenberg M (1984a) Bacterial adherence to hydrocarbon: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol Lett 22:289–295
17. Yang Y, Lee T, Kim JH, Kim EJ, Joo H, Lee C, Kim B (2006) High-throughput detection method of quorum sensing molecules by colorimetry and its applications. Anal Chem 356:297–299
18. Clinical Laboratory Standards Institute (2008) Performance standards for antimicrobial disc susceptibility tests CLSI 28(1)
19. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams & Wilkins, Baltimore
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein estimation with Folin Phenol reagents. J Biol Chem 193:265–275
21. Nyquist RA (1990) Infrared group frequency assignments aided by solvent studies. Appl Spectrosc 44(4):594–599
22. Biswas L, Biswas R, Schlag M, Bertram R, Götz F (2009) Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*. Appl Environ Microbiol 75(21):6910–6912
23. Voggu L, Schlag S, Biswas R, Rosenstein R, Rausch C, Götz F (2006) Microevolution of cytochrome bd oxidase in *Staphylococci* and its implication in resistance to respiratory toxins released by *Pseudomonas*. J Bacteriol 188(23):8079–8086
24. Lopes SP, Machado I, Pereira MO (2011) Role of planktonic and sessile extracellular metabolic byproducts on *Pseudomonas aeruginosa* and *Escherichia coli* intra and interspecies relationships. J Indian Microbiol Biotechnol 38(1):133–140
25. Mashburn LM, Jett AM, Akins DR, Whiteley M (2005) *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during in vivo coculture. J Bacteriol 187(2):554–566
26. Hoffman LR, D'Argenio DA, MJ MC, Zhang Z, Jones RA, Miller SI (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436:1171–1175

Comparative Analysis of Phytochemicals of Healthy and Symptomatic *Clerodendrum inerme*

15

Sonal, Sharmita Gupta, Yati Prabha, and S. K. Soni

Abstract

Clerodendrum, a flowering plant, belongs to family Lamiaceae. The aim of present study was to analyse phytochemicals from the leaves of healthy and viral symptomatic *C. inerme* plants. Leaves having chlorotic spots, yellow-green patches, leaves curling, etc. were due to virus infection as seen in electron microscopy. Extraction was done using solvents chloroform, acetone, methanol, ethanol and water. For analysis of phytochemicals, each extract was subjected to qualitative test for identification of various constituents like alkaloids, steroids, flavonoids, coumarins, quinones, flavanones, terpenoids, tannins and phenolic compounds and proteins. After analysing all constituents, it has been confirmed that symptomatic leaves of *C. inerme* have less amount of phytochemicals as compared to healthy leaves of *C. inerme*. The major purpose of this study was to make a comparative account of the phytochemical constituents of healthy and symptomatic *Clerodendrum inerme*.

Keywords

C. inerme · Healthy · Symptomatic · Phytochemicals

15.1 Introduction

The medicinal plants are beneficial for healing and curing of diseases of human, because of phytochemical constituents present in them. Plants are found to contain chemical constituents, which are used as natural drugs to treat common fungal and bacterial infections. Medicinal plants are frequently used in different combinations

Sonal · S. Gupta (✉) · Y. Prabha · S. K. Soni
Botany Department, D.E.I., Agra, Uttar Pradesh, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_15

of medicines in India because of least side effect and cost which makes it suitable for therapeutic applications in tribal medicine. About 25 percent of all new medicines are indirectly or directly derived from higher monocot and dicot plants [5]. Paste and leaf extract are used in treatment of malaria, inflammation, itching skin diseases and infected wounds. The genus *Clerodendrum* entails more than 450 species of tropical and sub-tropical zones of the world, which involves herbs, shrubs and trees. *C. inerme* is used for the purpose of landscaping or hedge plants in roads, streets, parks, etc. It is an evergreen shrub of 1–1.8 m tall having smooth and woody stems. Leaves are green, smooth and simple and have venation pinnate, entire margins and opposite leaves with shiny dorsal surface. Plants have attractive flowers in white colour which are arranged in clusters [2]. Stamens are four, usually in pairs of two of variable length and thrusting beyond the mouth of red to yellow petals [1]. In the present study, viral symptomatic and healthy leaves of *C. inerme* were screened for the presence of phytochemicals. Phytochemical constituents were extracted using various solvents, like chloroform, ethanol, methanol, acetone and water. Extracts were used for identification of various constituents like proteins, alkaloids, flavonoids, steroids, coumarins, flavanones, quinones, terpenoids and tannins and phenolic compounds.

15.2 Materials and Methods

15.2.1 Collection of Plant Material

Healthy and symptomatic leaves of *C. inerme* were collected from hedge grown outside of the botany department, faculty of science, DEI, Dayalbagh, Agra (UP) (Figs. 15.1 and 15.2). Healthy and symptomatic leaves of *C. inerme* were cleaned with tap water and dried in shade. After 7 days, leaves were grounded in

Fig. 15.1 Image of *C. inerme* plant showing healthy leaves

Fig. 15.2 Image of *C. inerme* plants showing chlorotic spots, leaf curling, etc

mortar-pestle. Grounded leaf material of healthy and symptomatic plants was stored in airtight containers, separately.

15.2.2 Extraction of Phytochemicals

To 100 ml conical flasks, each containing 50 ml of solvents, namely, chloroform, ethanol, methanol, acetone and water, 1 gm of dried plant material was added. These flasks were stored at room temperature for 72 h. Prepared extracts were preserved in cold at 4 °C till further application.

15.2.3 Phytochemical Evaluation

Extracts obtained from healthy and symptomatic *C. inerme* plants were assessed for presence of various constituents like alkaloids, steroids, terpenoids, flavonoids, phenol and tannins, quinones, flavanones, coumarins, etc.

15.2.3.1 Test for Proteins

To 1 ml of plant extract, 2–3 drops of ninhydrin reagent were appended. Purple colour appeared which indicated the presence of proteins [4].

15.2.3.2 Test for Alkaloids

Two ml of extract was added in a test tube along with 5–6 drops of Wagner's reagent. Formation of reddish-brown precipitate confirmed the presence of alkaloids [4].

15.2.3.3 Test for Steroids

One ml of crude extract with 2 ml of chloroform was taken. To this 2 ml of concentrated sulphuric acid and acetic acid was added. The presence of steroids was confirmed, when greenish colour developed [6].

15.2.3.4 Test for Terpenoids

Two ml of extract was taken in a test tube. One ml of chloroform and sulphuric acid each was added to it. Appearance of greyish colour ratified the presence of terpenoids [6].

15.2.3.5 Test for Flavonoids

Two percent of sodium hydroxide was added to 2 ml of plant extract. Visualization of yellow colour which later becomes colourless on addition of 5–6 drops of diluted acid showed the presence of flavonoids [6].

15.2.3.6 Test for Phenol and Tannins

Two percent of ferric chloride was added to 2 ml of plant extract. Development of blue green or black colour confirmed the presence of tannins and phenols [6].

15.2.3.7 Test for Quinones

Take 1 ml of extract in a test tube; 1 ml of concentrated sulphuric acid was added. Red colour indicated the presence of quinones [3].

15.2.3.8 Test for Flavanones

To the substance, concentrated sulphuric acid was added; orange to crimson red colour confirms the presence of flavanones [3].

15.2.3.9 Test for Coumarins

To 1 ml of extract, 1 ml of ten percent sodium hydroxide was added. Visualization of yellow colour ratified the presence of coumarins [3].

15.3 Results

Results of phytochemical analysis are summarised in Tables 15.1 and 15.2.

Table 15.1 Preliminary phytochemical analysis of healthy and symptomatic *C. inerme* leaf extracts in solvents (aqueous, acetone and chloroform)

		Water		Acetone		Chloroform	
Sr. no.	Phytoconstituents	Healthy	Symptomatic	Healthy	Symptomatic	Healthy	Symptomatic
1.	Proteins	+++	++	+++	+	+++	–
2.	Steroids	–	–	+++	–	+++	+
3.	Terpenoids	–	–	–	–	+++	+
4.	Flavonoids	++	+	++	++	++	+
5.	Phenol and tannins	–	–	–	–	+++	+++
6.	Quinones	++	+	+	+	+++	++
7.	Flavanones	++	++	++	++	+++	+
8.	Coumarins	–	–	–	–	–	–
9.	Alkaloids	+++	++	–	–	+++	–

Table 15.2 Phytochemical analysis of healthy and symptomatic *C. inerme* leaf extracts in solvents (ethanol and methanol)

Sr. no.	Phytoconstituents	Ethanol		Methanol	
		Healthy	Symptomatic	Healthy	Symptomatic
1.	Proteins	–	–	–	–
2.	Steroids	+	+	+	–
3.	Terpenoids	+	–	–	–
4.	Flavonoids	+++	+	++	+
5.	Phenol and tannins	+	+	–	–
6.	Quinones	+	–	–	–
7.	Flavanones	–	–	++	+
8.	Coumarins	–	–	–	–
9.	Alkaloids	+	+	++	+

+ indicates average colour presence, ++ indicates good colour, +++ indicates very good colour

Visual colour observations were made during phytochemical analysis.

15.4 Conclusion

The results revealed the presence of medicinally important constituents in the extract of healthy leaves of *C. inerme*. Chloroform, water and acetone were found most suitable for the extraction of phytoconstitutents from healthy leaves of *C. inerme*, whereas extracts from symptomatic leaves were showing least results. Studies show the presence of phytochemicals, which are important physiologically as well as medicinally in the treatment of different ailments. Therefore these extracts from *C. inerme* could be seen as a good source for useful drugs.

References

1. Chakraborthy GS, Mazumdar A, Singh S, Verma P (2013) *Clerodendrum inerme*: a review. Pharmacophore 4(6):230–232
2. Chethana GS, Harivenkatesh KR, Gopinath SM (2013) Preliminary phytochemical analysis of *Clerodendrum inerme*. Int Res J of Pharm 4(5):208–209
3. Firdouse S, Parwez A (2011) Phytochemical investigation of extract of *Amorphophallus campanulatus* tubers. Int J of Phytomed 3:32–35
4. Misra CS, Pratyush K, LipinDev MS, James J, ThaliyilVeettil AK, Thankamani VA (2011) Comparative study on phytochemical screening and antibacterial activity of roots of *Alstonia scholaris* with the roots, leaves and stem bark. Int J Res Phyto Pharm 2:77–82
5. Venkatanarasimman B, Rajeswari T, Padmapriya B (2012) Preliminary phytochemical screening of crude leaf extract of *Clerodendrum Philippinum* Schauer. Int J Inst Pharm LSci 2(2):133–138
6. Yadav RNS, Munin A (2011) Phytochemical analysis of some medicinal plants. J Phytomed 3(12):10–14

16 Synthesis of Silver Nanoparticle of Aqueous Extract of *Allium Fistulosum* and Its Efficiency Against Bacterial Contaminants from Industrial Waste Water and Its Photocatalytic Potential

Uma Ramaswamy and Vicky Mani

Abstract

In this study eco-friendly synthesis and characterization of silver nanoparticle using aqueous extract of whole plant *Allium fistulosum* Linn and its antibacterial efficiency in waste water and photocatalytic potential were studied. Green synthesis of silver nanoparticle (AgNP) was performed by treating the 5% aqueous plant extract with 1 mM concentration of silver nitrate at 60 °C. AgNPs were characterized by using UV-visible spectroscopy, scanning electron microscopy and Fourier transform infrared spectroscopy. Silver surface plasmon resonance (SPR) occurred at 432 nm for 1 mM AgNP. The silver nanoparticle size ranges from 66 to 86 nm. Silver nanoparticle of *Allium fistulosum* treated with industrial waste water with different time durations (12, 24 and 72 h). In 72 h AgNP exhibited antibacterial efficiency against pathogenic bacteria *Proteus vulgaris*, *Shigella dysenteriae* and *Escherichia coli* isolated from industrial waste water. Synthesized silver nanoparticle has potent photocatalytic dye degradation for safranin and methyl orange at 43.1% and 34.6%, respectively.

Keywords

Silver nanoparicle · *Allium fistulosum* · Dyes · Antibacterial

U. Ramaswamy (✉)
Department of Biochemistry, Dwaraka Doss Goverdhan Doss Vaishnav College, Chennai, Tamilnadu, India

V. Mani
Department of Biochemistry, University of Madras, Chennai, Tamilnadu, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_16

16.1 Introduction

Nanomaterials are becoming a part of our daily life, but properties and behavior of material at the nanoscale differ significantly when compared to microscale. Nanotechnology provide the solutions to many medical, social and environment problems. Metals such as gold, silver, copper and zinc are widely used in the synthesis of nanoparticles; silver has the unique property to fight against the pathogens and it has been used in herbal medicine *Allium fistulosum* L. is a member of Amaryllidaceae family that comes under the genus *Allium* and it is a perennial onion commonly called as spring onion, in Hindi as "hara pyaz," in tamil as "thal vangayam." The roots and bulbs have been used for the treatment of febrile disease, headache, abdominal pain, diarrhea, snakebite, ocular disorders and habitual abortion, as well as having antifungal and antibacterial effects [4]. Recently numerous studies have indicated that *A. fistulosum* has antifungal activity [3, 6]. The study focuses on the synthesis of silver nanoparticle of aqueous extract of Allium fistulosum and its efficiency against bacterial contaminants from industrial effluent and its photocatalytic potential.

16.2 Materials and Methods

16.2.1 Collection and Authentication of Allium fistulosum

Allium fistulosum plant was collected from Avadi market, Chennai and it was authenticated by Dr. S. Jayaraman, Director of Plant Anatomy Research Centre, West Tambaram, Chennai (Reg. no: PARC/2017/3436).

16.2.2 Aqueous Extract of the Allium fistulosum

50 g of fresh whole plant *Allium fistulosum was* crushed finely and mixed in 100 ml of deionized water mixture boiled for 8 min [1]. Cool the extract it was filtered with Whatman number 1 filter paper and the extract was made up to 100 ml with distilled water. The extracts were stored at 4 °C and used for silver nanosynthesis.

16.2.3 Preparation of Silver Nitrate

One millimolar (1 mM) silver nitrate solution ($AgNO_3$) – 0.084 g of silver nitrate – was weighed and dissolved in 500 ml of distilled water and stored in brown bottle.

Biosynthesis of Silver Nanoparticles 10 ml of *A. fistulosum* extract was mixed to 90 ml of prepared 1 mM $AgNO_3$ solution with constant stirring at 60°C for 20 min.

16.2.4 Characterization of Silver Nanoparticles

UV-Visible Spectroscopy 2 ml of the reaction mixture was taken and the optical density was measured from a wavelength of 200–800 nm using the Shimadzu UV-visible spectrophotometer.

16.2.5 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis of silver nanoparticles was carried out by potassium bromide pellet method to identify the possible biomolecules responsible for the reduction of silver ions using Bruker-Alpha spectrometer.

16.2.6 Scanning Electron Microscopy (SEM)

SEM analysis was carried out to determine the size and shape of the biosynthesized nanoparticles of *A. fistulosum* using Hitachi gu SU 6600.

16.2.7 Photocatalytic Degradation of Dye

Photocatalytic degradation of dye was estimated by colorimetric method. Typically 10 mg of safranin and methyl orange dyes were added to 1000 mL of double-distilled water used as stock solution. About 1 mg of biosynthesized silver nanoparticles was added to 100 mL of safranin and methyl orange dye solution. The dye solution along with silver nanoparticles was mixed in a cyclomixer for 15 min. The solution was kept in sunlight from morning to evening. At regular intervals of time, 3 ml of the solution was withdrawn and taken optical density using UV-visible spectrophotometer at wavelength 450 and 520 nm for methyl orange and safranin, respectively.

$$\text{Dye degradation}\left(\%\right) = \text{A} - \text{B} / \text{A} \times 100$$

where A is the initial concentration of dye solution and B is the concentration of dye solution exposed to sunlight [5].

16.2.8 Antibacterial Effect of Silver Nanoparticles of *A. fistulosum*

To detect the presence of bacteria in industrial waste water was determined by pour plate method [2]. 100 ml of industrial waste water (sample B) was taken in two flasks namely, BT and BC where BC served as control for sample B and BT served as sample B treated with 10mg of silver nanoparticles of *A. fistulosum* and it was incubated for 12, 24 and 72 h. After incubation 100 µl of samples (BC and BT) were poured in each 20 ml of nutrient agar plate kept for incubation at 37 °C for 12, 24,

and 72 h. After incubation the colonies of bacteria were analyzed. Based on the morphology and color of the colonies in selective media (MacConkey agar and Salmonella-Shigella agar (SS)) for specific bacterial species (Klebsiella, Escherichia coli, Proteus, Shigella) were chosen and done pour plate method. The colonies were counted for both control and test plate.

16.3 Results and Discussions

UV-Visible Spectrum Analysis Optimum intensity of UV-visible spectra peak or SPR (surface plasmon resonance) band centered at 432 nm for 10 ml of the aqueous extract of *A. fistulosum* treated with 1 mM silver nitrate (Fig. 16.1).

FTIR Analysis FTIR spectrum of AgNPs of *A. fistulosum* extract shows bend at 3318 cm^{-1}, 1637, 1015 cm^{-1} corresponds to OH stretches, H-bonded alcohols and phenol, NH bend, primary amines, N-O asymmetric stretch nitro compounds, C-O stretch alcohols, carboxylic acids, esters and ether, respectively (Figs. 16.2 and 16.3). FTIR spectrum of AgNPs suggested that the particle was surrounded by different organic molecules such as alcohols, ketones, carboxylic acid and terpenoids.

SEM Analysis The AgNP formed were spherical in shape, agglomerated, and polydispersed. The AgNP diameter varied from 66 to 86 nm (Fig. 16.4).

Photocatalytic Degradation of Dyes Absorption peaks of methyl orange and safranin dyes at 450 and 520 nm for methyl orange and safranin dyes were decreased initially with the increase of the exposure time. This clearly indicates the photocata-

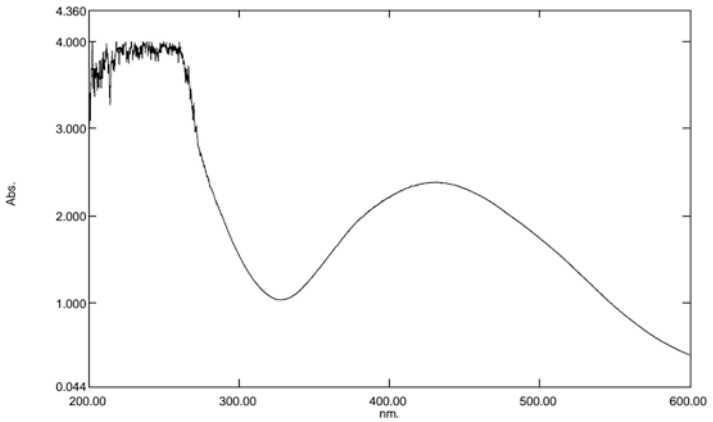


Fig. 16.1 UV-visible spectra of aqueous extract of *A. fistulosum* with 1 mM silver nitrate

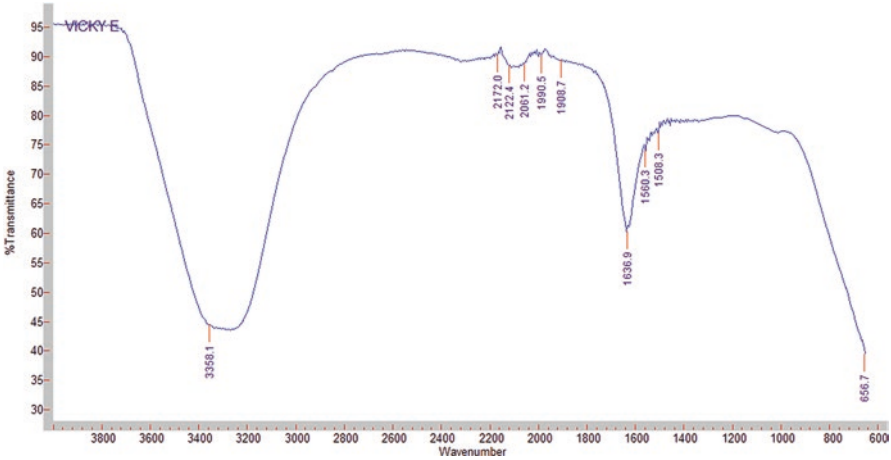


Fig. 16.2 FTIR analysis of aqueous of *Allium fistulosum*

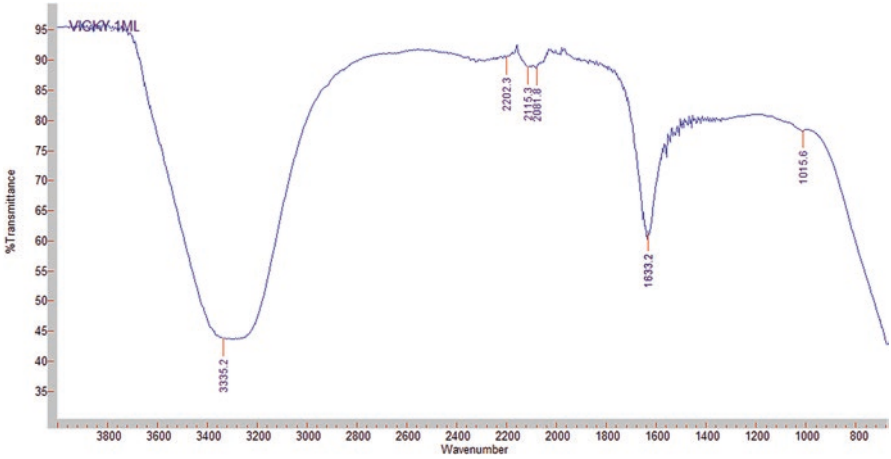


Fig. 16.3 FTIR analysis of silver nanoparticle of *Allium fistulosum* (1 mM)

lytic degradation of methyl orange and safranin dye. Dye degradation efficiency of AgNP of *A. fistulosum* was 34.6% and 43.1% for methyl orange and safranin, respectively (Fig. 16.5).

Effect of AgNP of *A. fistulosum* on Bacteria of Domestic and Industrial Waste Water

Findings of the study revealed that in AgNP-treated industrial waste water (BT) colonies of *Shigella* and *Proteus* were less than control plate (BC) in 24 h incubation. In 72 h there was no growth of *E. coli*, *Shigella* and *Proteus* in BT plate

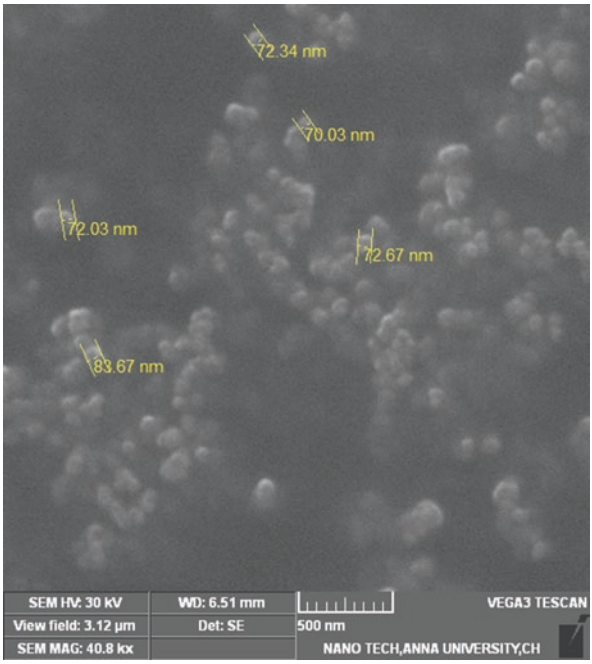


Fig. 16.4 SEM analysis of silver nanoparticle of *Allium fistulosum*

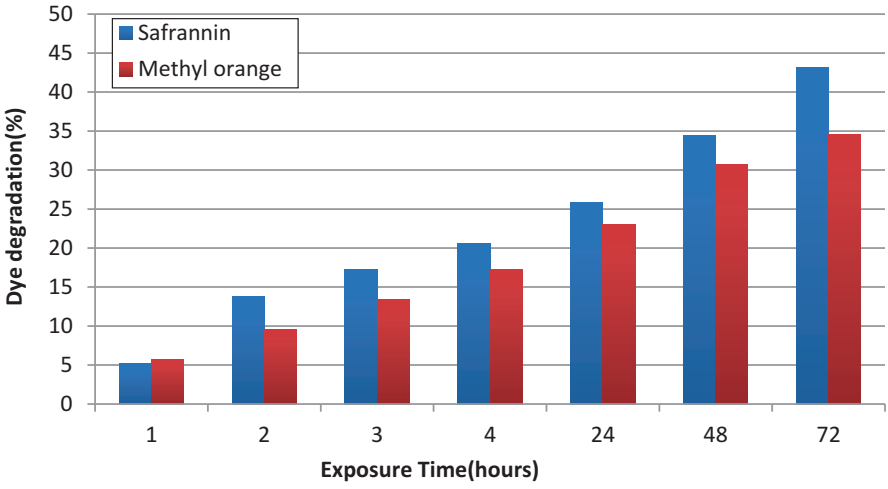


Fig. 16.5 Photocatalytic degradation of methyl orange and safranin by the AgNPs of *A. fistulosum*

Table 16.1 Mean values of total bacterial count of industrial waste water in (a) MacConkey and (b) SS agar medium

Time (h)	*E. coli*		*Klebsiella*		Time (h)	*Shigella*		*Proteus*	
	BC	BT	BC	BT		BC	BT	BC	BT
12 h	800	650	850	700	12 h	490	300	350	200
24 h	810	150	860	600	24 h	500	150	360	150
72 h	815	NIL	879	380	72 h	500	NIL	368	NIL

compared to BC plate of both MacConkey and SS agar (Table 16.1). This result shows that silver nanoparticle has the good ability to inhibit *E. coli*, *Proteus* and *Shigella* in industrial waste water.

16.4 Conclusion

In this study a simple, eco-friendly, pollutant-free and economic biological procedure has been developed to synthesize the AgNPs of *Allium fistulosum*. The silver nanoparticles were characterized by UV-visible spectra, FTIR spectra and scanning electron microscopy. Silver nanoparticles of *Allium fistulosum* exhibited potent photocatalytic activity against methyl orange and safranin. Silver nanoparticles of *Allium fistulosum* exhibited potent antibactericidal effect on *E. coli*, *Proteus* and *Shigella* on industrial waste water.

References

1. Benjamin G (2011) IPCBEE, vol 5. IACSIT Press, Singapore
2. Dosoky R, Kotb S, Farghali M (2015) Efficiency of silver nanoparticles against bacterial contaminants isolated from surface and ground water in Egypt. J Adv Vet Anim Res 2(2):175–184
3. Sang S, Lao A, Wang Y, Chin CK, Rosen RT, Ho CT (2002) Antifungal constituents from the seeds of Allium Fistulosum L. J Agric Food Chem 50:6318–6321
4. Shogakukan (1985) Shanghai science and technology publisher and shougakukan, Tokyo. Dict Chin Drugs 3:1599–1600
5. Vanaja M, Paulkumar K, Baburaja M, Rajeshkumar S, Gnanajobitha G, Malarkodi C, Sivakavinesan M, Annadurai G (2014) Degradation of methylene blue using biologically synthesized silver nanoparticles. Bioinorg Chem Appl, Article ID 742346
6. Yin MC, Tsao SM (1999) Inhibitory effect of seven Allium plants upon three Aspergillus species. Int J Food microbial. 49:49–56

Exploration of Biocontrol and Growth-Promoting Activity of Bacterial Strains Isolated from the Sugarcane Crop

17

Beenu Shastri, Anil Kumar, and Rajesh Kumar

Abstract

Naturally occurring bacteria were isolated from the internal tissues of stalks as well as from roots of sugarcane crop and from the rhizospheric soil. The highest numbers of bacterial populations were isolated from the rhizospheric zone. Isolated bacterial strains were subjected to antagonistic activity in vitro against *Colletotrichum falcatum* fungus causing red rot disease in sugarcane crop. Most of the isolated bacteria showed antagonistic activity against *C. falcatum* in vitro. Isolated antifungal isolates were identified morphologically and biochemically. Further, the potential strains were examined for various plant growth promoting traits and hydrolytic enzymes production. Bacteria isolated from rhizospheric as well as from endophytic zone of sugarcane crop showed the inhibition of red rot pathogen as well showed the in vitro plant growth promotory traits. Thus, isolates help in biocontrol of red rot as well as can be used for increment of sugarcane yield.

Keywords

Sugarcane · Endophytes · Rhizospheric bacteria · *Colletotrichum falcatum*

B. Shastri · A. Kumar · R. Kumar (✉)
Rhizosphere Biology Laboratory, Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

R. Kundu, R. Narula (eds.), *Advances in Plant & Microbial Biotechnology*,
https://doi.org/10.1007/978-981-13-6321-4_17

17.1 Introduction

Saccharum spp. (sugarcane) is an important agro-industrial crop cultivated in various regions (tropical and sub-tropical) of the world. Their valuable product includes high concentrations of sucrose, or sugar, in the stem and more recently used in ethanol production, which is an important renewable biofuel source. Brazil is the largest producer for sugarcane, and its area under production has expanded from 5.81 million hectare (M ha) in 2005 to 10.4 million ha in 2014 growing at an average annual rate of 6.79% [1]. Sugarcane is cultivated in many regions of India with total area covering of 5.01 M ha [1]. Sugar consumption is largest in India with an annual consumption of about 19 million MT (metric tons) [2]. Therefore, to sustain the increasing population and maintain the productivity as well as production, this area has become a major focus of research.

Various abiotic and biotic factors, including a wide range of fungal and bacterial diseases, are adversely affecting the growth and performance of sugarcane in the field. Red rot is one of the most dreadful diseases of sugarcane, caused by the fungus *Colletotrichum falcatum*. It not only causes severe loss in yield and quality but also reduces the quality of juice [3, 4]. It can reduce cane weight by up to 29% and loss in sugar recovery by 31% [5].

Current control strategies for red rot disease involve the use of resistant varieties and fungicide applications, but their efficacy is limited; therefore, novel and environmentally sound strategies to control disease of sugarcane are needed. Biocontrol agents provide an excellent alternative to chemical pesticides and biofertilizer. *Pseudomonas*, *Bacillus*, *Burkholderia*, *Agrobacterium*, *Streptomyces*, etc. are commonly genera used as biocontrol agents.

17.2 Methodology

For sample collection or isolation, red rot susceptible healthy sugarcane variety Co1148 and the rhizospheric soil were collected from the Indian Institute of Sugarcane Research (IISR) farms, Lucknow. Endophytic bacteria and rhizospheric bacteria were isolated from the internal tissues of roots and stalks of sugarcane variety Co1148 and from the rhizospheric soil using the protocol of Viswanathan et al. [6]. Isolated bacteria were then studied for dual plate assay for antagonism on potato dextrose agar (PDA) plates against red rot fungus, i.e., *Colletotrichum falcatum*, and were incubated for 7–10 days at 28 °C. The percentage inhibition was measured by the formula $[(C-T)/C] \times 100$, where C is the radial colony growth of fungal pathogen in control and T is the radial colony growth in dual plate assay [6].

Morphological (gram staining and motility) and biochemical characterization (IMViC, catalase, oxidase, urease, H_2S) of the isolated antifungal bacterial isolates was performed as per the Bergey's manual of Systematic Bacteriology [7].

17.2.1 In Vitro Evaluation of Plant Growth-Promoting Activities

Quantification of indoleacetic acid (IAA) was performed according to Gordon and Weber [8]. Solubilization of calcium phosphate was done according to Pikovskaya [9] on Pikovskaya agar medium. The confirmation of siderophore production was performed according to Schwyn and Neilands [10] universal method, with chrome azurol S (CAS) agar plate assay. Ammonia production was evaluated, after adding Nessler's reagent to a peptone broth containing bacterial culture after 72 h of incubation, and yellowish brown color was recorded [11]. For hydrocyanic acid (HCN), the Bakker and Schippers [12] method was followed in nutrient agar medium, supplemented with 4.4 g/L of glycine by observing the color change of filter paper placed on lid of plates.

17.2.2 Screening for Hydrolytic Activity

Chitinase activity was detected on colloidal chitin agar medium with 0.5% colloidal chitin, according to method of Berger and Reynold [13]. Cellulase activity was performed on Czapek's mineral salt medium containing 1% CMC [14]. The amylolytic activity was detected on starch agar medium containing 1% soluble starch [15]. The caseinase enzyme activity of culture was examined on skimmed milk agar medium showing halo zone around the colonies [16].

17.3 Results and Discussion

Rhizospheric soil gave the highest number of bacterial isolates (20) as compared to endophytic bacteria (8) isolated from sugarcane variety Co1148. Thus, total 28 bacteria were isolated collectively. Dual plate assay showed that out of the total 28 bacterial isolates, 14 bacterial isolates showed in vitro antagonism against *Colletotrichum falcatum*. Percentage inhibition showed that out of 14 bacteria, 4 bacterial isolates (So-6, So-9, So-22, and R-17) were showing inhibition more than 50% against *C. falcatum* in vitro (Table 17.1).

Table 17.1 Antagonism of selected isolates to *Colletotrichum falcatum* on PDA

S. No	Isolates	*C. falcatum* mycelia growth (mm) on tenth day	% Inhibition
1.	Control	74	–
2.	So-6	32	57
3.	So-9	28	62
4.	So-22	25	66
5.	R-17	30.3	59

Table 17.2 Identification and characterization of isolates

Isolates	So-6	So-9	So-22	R-17
Gram nature	+	–	–	–
Motility	–	–	–	–
Catalase	+	+	+	+
Indole	–	–	–	–
Citrate	–	+	–	+
H_2S production	–	–	–	–
Urease	–	–	–	–
MR	–	–	–	–
VP	–	–	–	–
Oxidase	–	–	–	–
IAA	+	+	+	+
HCN	–	–	–	–
Ammonia	+	+	+	+
PSB	+	–	–	+
Siderophore	–	+	–	–
Chitinase	–	–	–	–
Amylase	–	+	–	+
Cellulase	+	–	–	–
Caseinase	–	+	+	+

17.3.1 Identification of Antifungal Isolates

Four bacterial isolates (So-6, So-9, So-22, and R-17) were identified morphologically as well as biochemically (Table 17.2).

17.3.2 In Vitro Screening of Plant Growth Promoting Activities

All the four isolates that showed inhibition above 50% (So-6, So-9, So22, and R-17) were showing positive results for IAA production (Table 17.2). Phosphate solubilization is a very important trait of plant growth promotion as microorganisms solubilize insoluble phosphate by producing phosphatase enzyme and making it available to the plants. Isolates So-6 and R-17 were showing positive result for phosphate solubilization. In our study, all the four isolates were able to produce ammonia. Fravel [17] suggested that ammonia production by microorganisms is also related for inhibitory action on fungal pathogen. None of the bacteria showed positive result for HCN. Siderophore is an iron-chelating secondary metabolite produced by several microorganisms that bind insoluble Fe^{3+} and make it available for its own growth and also for plants. Less availability of iron results in the growth inhibition of phytopathogens which happen due to chelating iron by siderophore and hence enhance the growth of plant. Thus, it promotes plant growth in an indirect way. In the present study, only isolate So-9 were showing positive results for siderophore.

17.3.3 Hydrolytic Enzyme Activity

There are several mechanisms by which microorganisms bring about control of plant diseases. Lytic enzymes such as amylases, cellulases, caseinase and chitinase have the ability to degrade structural fungal cell walls therefore they are related to hyperparasitic activities [18, 19]. In the present study none isolates showed chitinase activity. Isolates So-9 and R-17 showed positive results for amylase production and only isolates So-6 showed positive results for cellulase. Isolates So-9, So-22 and R-17 gave positive test for caseinase production (Table 17.2).

17.4 Conclusion

The bacteria isolated in this work have the possibility to be used in the control of red rot disease because of the presence of various lytic enzymes and plant growth promotory traits as discussed above. Isolates So-6, So-9, and So-22 have been isolated from the rhizospheric soil, and isolates R-17 have been isolated from root region of sugarcane variety. These findings showed that endophytic as well as rhizospheric bacteria act as a biocontrol agent which promotes the growth of plants indirectly by protecting them from phytopathogens by means of inhibiting the growth of pathogenic organisms.

Acknowledgments The authors acknowledge Dr. Ram Ji Lal, Prinicipal Scientist (now retired) and Dr. Dinesh Singh (Principal Scientist), IISR, Lucknow for providing various assistance.

References

1. Food and Agricultural Organization (FAO) of United Nations: Economic and Social Department: The Statistical Division (2014)
2. Vishwakarma SK, Kumar P, Nigam A et al (2013) Pokkah Boeng: an emerging disease of sugarcane. J Plant Pathol Microbiol 4:170
3. Satyavir S (2003) Red rot of sugarcane – current Scenario. Indian Phytopathol 56:245–254
4. Duttamajumder SK (2008) Red rot of sugarcane. Indian Institute of Sugarcane Research, Lucknow
5. Hussnain Z, Afghan S (2006) Impact of major cane diseases on sugarcane yield and sugar recovery, Annual report. Shakarganj Sugar Research Institute, Jhang
6. Viswanathan R, Rajitha R, Sundar RA, Ramamoorthy V (2003) Isolation and Identification of Endophytic Bacterial Strains from Sugarcane Stalks and Their *In Vitro* Antagonism against the Red Rot Pathogen. Sugartech 5(l & 2):25–29
7. Krieg NR, Holt JG (1984) Bergey's manual of determinative bacteriology, vol 1. The Williams and Wilkins Co, Baltimore
8. Gordon SA, Weber RP (1951) Colorimetric estimation of indole-acetic acid. Plant Physiol 26:192–195
9. Pikovskaya RI (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiol 17:362–370
10. Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of Siderophore. Anal Biochem 160:47–56

11. Dye DW (1962) The inadequacy of the usual determinative tests for identification of *Xanthomonas* sp. New Zeal J Sci 5:393–416
12. Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp-mediated plant growth-stimulation. Soil Biol Biochem 19:451–457
13. Berger LR, Reynolds DM (1958) The chitinase system of a strain of *Streptomyces griseus*. Biochim Biophys Acta 29:522–534
14. Farkaš V, Lišková M, Biely P (1985) Novel media for detection of microbial producers of cellulase and xylanase. FEMS Microbiol Lett *28*(2):137–140
15. Mishra S, Behera N (2008) Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. Afr J Biotechnol 7(18):3326–3331
16. Berg G, Roskot N, Steidle A et al (2002) Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl J Environ Microbiol 68(7):3328–3338
17. Fravel DR (1988) Role of antibiosis in the biocontrol of plant diseases. Annu Rev Phytopathol. 26:75–91
18. Kim PI, Chung KC (2004) Production of an antifungal protein for control of *Colletotrichum lagenarium* by *Bacillus amyloliquefaciens*. FEMS Microbiol Lett 234:177–183
19. Oppenheim AB, Chet I (1992) Cloned chitinase in fungal plant-pathogen control strategies. Trends Biotechnol 10:392–394