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ROLE OF ANTIMICROBIAL AGENTS IN SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF PADDY MALT MASH TO ETHANOL BY MIXED CULTURES OF SACCHAROMYCES CEREVISIAE PH03 AND ZYMOMONAS MOBILIS ZM4

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SUMMARY

Among various antimicrobial plant extracts, chemicals and antibiotics used for simultaneous saccharification and fermentation, penicillin G prevented contamination and did not inhibit amylase activity and growth of the synergistic co-cultures Saccharomyces cerevisiae PH03 and Zymomonas mobilis ZM4 during a 7-day fermentation of paddy malt (25.0%) mash (18.0% dextrose equivalent) to ethanol at 30°C and pH 5.5. The treatment yielded 10.1% (v/v) ethanol from the mash which was significantly more than that of the boiled and fermented mash (9.3% v/v) and equal to that of the mash boiled and fermented (10 2% v/v) after added amylases treatment. Most of the other compounds (kanamycin, streptomycin, polymyxin, tetracycline) had growth inhibitory effect especially on Z. mobilis.

INTRODUCTION

In the conversion of starch to ethanol, treatment with added amylases is costly. Attempts to convert starch directly to ethanol by amylolytic and ethanol producing yeasts (Frelot et al., 1982; Casey et al., 1984; Amin et al., 1985; Malfait et al., 1986) have not been very successful, although Endomycopsis fibuligera was somewhat promising (Vijayasarathy Reddy and Basappa, 1993). Malting of grains though generated innate amylases, their activity was found to be very low especially in malted paddy (Kneen, 1944; Renu and Basappa, 1994) and generally required additional amylases for efficient conversion of starch to fermentable sugars. Hence, attempts to simultaneously saccharify and ferment the unsterile malted paddy mash to ethanol with the aid of antimicrobial agents by just utilizing the innate enzymes have been made and the results are presented in this paper

MATERIALS AND METHODS

Materials

Saccharomyces cerevisiae PH03 used in the studies is an isolate obtained from paddy husk as described earlier (Renu and Basappa, 1994),

and Zymomonas mobilis ZM4 was procured from the Laboratoire de Chimie Bacterienne, Marseille, France. The yeast culture was maintained on Wickerham's agar and the bacterium on glucose-yeast extract agar (Sreekumar and Basappa, 1991). Some of the widely grown and abundantly available medicinal plant (Chopra et al., 1956) parts such as the pod of Cassia fistula, the pepo of Cucurbita pepo, the leaves of Achyranthus aspera and Aloe indica were collected from the locally available sources. The hydraulically pressed (Carver Laboratory Hydraulic Press, USA) juices of these were Seitz-filtered to obtain sterile juices for use as antimicrobial agents. A selected coarse variety (Jaya) of paddy (Oryza sativa L.) was used in all the studies. All the antibiotics such as penicillin G, kanamycin, streptomycin, polymyxin and tetracycline were purchased from Sigma Chemical Co. (USA). And both potassium metabisulfite and sodium benzoate used were of analytical grade (BDH). Bacterial -amylase and fungal glucoamylase were obtained from Anil Starch Company (Ahmedabad, India).

Methods

Malting and mashing of paddy was carried out as per the method of Renu and Basappa (1994). Treatment of the mash with additional α-amylase and glucoamylase was performed by the method of Srikanta et al. (1987).

Twenty ml of paddy malt mash containing 1.0% sugar was taken in 50 ml Erlenmeyer conical flask and sterilized at 121°C for 20 min, cooled, inoculated with yeast or bacterial culture and incubated at 30°C under stationary conditions for 24 h. Five ml each of these cultures was inoculated into 100 ml portions of mash containing 18% sugar (dextrose equivalent) (pH 5.5). Each ml of yeast inoculum contained 9.0×10^9 cells and 2.5×10^9 in case of bacterium. The flasks were incubated stationary at 30°C for 7 days, as these conditions were found to be optimum.

Known concentrations of filter (Millipore, USA) sterilized antibiotic or antimicrobial agents were added to the above media before inoculation of the mixed cultures.

The centrifuged (8,000 g for 15 min) supernatant was assayed for ethanol by gas chromatography (15A, Shimadzu, Kyoto) using Poropak Q-column and flame ionization detector. The free sugars extracted from the mash with 70% w/v ethanol (Paramahans and Tharanathan, 1980) were estimated by Shaffer and Somogyi (1933) method. The extract was further hydrolysed by 1.0M HCl (AOAC,1970) and the dextrose equivalent was estimated (Shaffer and Somogyi, 1933). The amylase activity was estimated by the method of Manning and Campbell (1961). The standard deviations and the analysis of variance were calculated by the procedure described by Steel and Torrie (1980).

The growth of yeast and bacteria (Z.mobilis) during fermentation with antimicrobial agents was determined by serial dilution plate technique using Wickerham's agar and glucose yeast extract agar (Sreekumar and Basappa, 1991) respectively. Bacterial contamination was determined by the use of nutrient agar. The colony forming units (cfu) were counted after incubating the plates at 30°C for 2 days and expressed as log 10 cfu/ml.

Sensory evaluation of the distilled liquor was carried out by a panel of 5 members mainly to test the acceptability or otherwise of the product.

RESULTS AND DISCUSSION

Malted paddy contained very low activities of amylase (Renu and

Basappa, 1994; Kneen, 1944) as compared to malted barley or malted wheat (Kneen, 1944). The amylase activity in the paddy malt (25.0% in water) mash (18% w/v dextrose equivalent), was stable up to 80°C, but boiling for 30 min or autoclaving inactivated it (Renu and Basappa, 1994). Addition of α-amylase and glucoamylase during mashing though improved the conversion of starch to fermentable sugars, it was still far from completion and required long period for hydrolysis (data not presented) besides being cost intensive. Therefore, simultaneous saccharification and fermentation of paddy malt mash at optimum conditions of pH (5.5), temperature (30°C) and mixed cultures of S.cereivisiae PH03 and Z.mobilis having synergistic effect (Renu and Basappa, 1994) in producing higher yield of ethanol over a period of 7 days was contemplated, in order to utilise the innate enzyme for obtaining a desirable quality of end product (ethanol) with unique flavour and aroma. Since this procedure though encouraging, posed problems of frequent contamination due to prolonged fermentation period of 7 days, it was decided to use antimicrobial agents that can prevent contamination and at the same time do not inhibit either the fermenting organisms or the amylase activity.

Plant extracts known to possess antimicrobial properties in Ayurvedic medicine were used and their performance is presented in Table 1. It has been observed that as the concentration of extract increased in the fermenting mash, the ethanol production decreased in spite of the significant decrease in bacterial contamination. This is mainly due to the inhibitory effect of the extract specifically on Z.mobilis as evidenced by its decrease in $\log_{10}$ cfu/ml during fermentation. However, the inhibitory effect of these extracts on the yeast S.cerevisiae PH03 was very minimal. In case of control where the mash was boiled for 30 minutes, the ethanol concentration was higher, although there was incomplete conversion of starch to sugars, due to heat inactivation of amylase. Use of additional amylases and boiling the mash yielded however more ethanol (10.2% v/v) at shorter time than without (9.3% v/v). The activity of amylase in the mash was not affected by the plant extract, tested separately. The acceptability of the distilled product was also not affected significantly as evaluated by a panel of judges, although the samples derived from boiled mash and with the lowest concentration (0.1%) of plant extract were found to be somewhat superior.

The commonly used food preservatives like sodium benzoate and potassium metabisulfite incorporated (100-200 ppm) in the fermenting

Table 1. Effect of antimicrobial plant extracts on simultaneous saccharification and fermentation of paddy malt mash to ethanol

Extract (% w/v)	Log_{10} cfu/ml			Ethanol % (v/v)
	S. cerevisiae	*Z. mobilis*	Bacterial contamination	
Control	9.2±0.07	7.5±0.12	5.1±0.11	8.00±0.08
Cp (0.1)	10.5±0.08	9.5±0.11	2.0±0.07	8.90±0.16
Cp (0.5)	9.9±0.08	8.0±0.16	0.8±0.02	4.69±0.08
Cp (1.0)	10.0±0.13	5.0±0.12	0.1±0.01	3.34±0.16
Aa (0.1)	9.1±0.08	8.0±0.16	2.7±0.06	7.82±0.03
Aa (0.5)	8.5±0.16	6.1±0.09	0.9±0.03	7.50±0.17
Aa (1.0)	10.1±0.12	5.0±0.12	0.2±0.05	4.70±0.03
Cf (0.1)	9.5±0.11	8.9±0.12	2.9±0.09	7.90±0.02
Cf (0.5)	9.5±0.11	6.0±0.08	1.0±0.02	7.30±0.01
Cf (1.0)	9.8±0.12	4.0±0.16	0.1±0.04	5.00±0.06
Ai (0.1)	9.0±0.08	9.2±0.07	3.0±0.10	8.00±0.05
Ai (0.5)	8.9±0.07	8.0±0.16	1.1±0.05	7.80±0.03
Ai (1.0)	8.9±0.07	7.0±0.12	0.1±0.01	6.80±0.14
Control (mash boiled)	10.3±0.12	10.0±0.13	0.0	9.3±0.08*
Control: (mash boiled after treatment with amylases) **	10.2±0.16	10.3±0.12	0.0	10.2±0.12*

* The values were found to be significantly more than control at p 0.001

** 0.06% of α-amylase and 0.1% of glucoamylase

Cp = *Cucurbita pepo* Aa = *Achyranthus aspera*
Cf = *Cassia fistula* Ai = *Aloe indica*

mash did not yield the expected amount of ethanol (data not presented).

Since Z.mobilis was generally inhibited by the above antimicrobial agents, an experiment to include certain antibiotics that could be ineffective on this organism was carried out. The data presented in Table 2 show that, of all the antibiotics tried in fermenting mash, penicillin G appeared to be very effective in not only controlling the contamination but also allowing the growth of the synergistic co-cultures and the simultaneous amylase activity resulting in optimum ethanol yield (10.1% v/v). Higher concentrations of antibiotics except

Table 2: Effect of antibiotics on simultaneous saccharification and fermentation of paddy malt mash to ethanol

Antibiotics	Log_{10} cfu/ml				Ethanol % (v/v)	
	S.cereviciae		Z.mobilis			
	SD	SE	SD	SE	SD	SE
Control	9.0±0.17	0.056	7.6±0.08	0.046	7.9±0.11	0.063
Kanamycin (10 µg/ml)	9.0±0.15	0.086	7.0±0.20	0.115	5.9±0.08	0.046
Penicillin G (25 µg/ml)	10.5±0.16	0.092	10.0±0.20	0.115	10.1*±0.12	0.069
Streptomycin (10 µg/ml)	9.2±0.18	0.104	6.0±0.17	0.098	6.2±0.09	0.052
Polymyxin (10 µg/ml)	8.0±0.2	0.115	4.0±0.25	0.144	4.9±0.14	0.08
Tetracycline (10 µg/ml)	9.3±0.18	0.104	7.9±0.08	0.046	8.0±0.20	0.115

SD = Standard deviation ; SE = Standard error.
*The value was found to be significantly more than the control at p 0.05

penicillin G were more inhibitory especially to Z.mobilis. Microscopic examination also revealed absence of other contaminating organisms in Penicillin G treated cultures as compared to control. The log cfu/ml of both S.cerevisiae PH03 and Z.mobilis ZM4 were more in the penicillin G treatment than the control. It is known that Z mobilis being a Gram negative bacterium is resistant to penicillin G, and sensitive to broad spectrum antibiotics like tetracycline (Swings and de Ley, 1977). However, its sensitivity to the other antibiotics like kanamycin,

polymyxin and streptomycin indicates that the strain is different from the strains tested by Swings and De Ley (1977). Since penicillin is used very widely to cure certain infectious diseases, to prevent contamination even in commercial alcohol fermentation as well as in certain microbiological experiments, it may be recommended for use in the above fermentation, as the antibiotic is not distilled; and it can be easily biodegraded in the environment. The acceptability of the distilled product derived from penicillin G treated mash was superior to others and equal to the one derived from boiled mash. It is also economically feasible as it would cost only $0.05/l ethanol produced in the above process as compared to cost of amylases ($ 0.15/l) used in the conventional process of ethanol production from starchy substrates.

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