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ORIGINAL PAPER

Cellulase production by *Sinorhizobium meliloti* strain 224 using waste tobacco as substrate

Utilization of waste tobacco for cellulase production

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Abstract
This study has investigated the valorization of waste tobacco, as lignocellulosic biomass, for cellulase production by rhizobium belonging to genus *Sinorhizobium*. For the first time, *Sinorhizobium meliloti* strain 224 was used to produce cellulase (Avicelase and carboxymethyl cellulase) during the submerged and solid-state fermentation using tobacco waste as substrate. The effect of substrate chemical modification on enzymes production has been examined as well. The obtained optimal conditions for the maximum activity of both produced enzymes during submerged fermentation using response surface methodology were: 5 g/L of unmodified waste tobacco concentration, incubation time of 2 days and inoculum concentration of 9%. On the other hand, the use of 1 g of sodium hydroxide modified tobacco for the production of cellulase during solid-state fermentation with 10% inoculum, after 2 days of incubation at 28 °C, expressed the maximum Avicelase activity of 1.503 U/g and carboxymethyl cellulase activity of 1.615 U/g. In addition to its basic role in plant root colonization and the provision of nitrogen compounds, strain 224 can also be exploited to produce cellulases by bioconversion of plant waste.

Keywords Avicelase · Carboxymethyl cellulase · Lignocellulose · Response surface method · Rhizobium

Introduction

Cellulose is the most widespread water-insoluble polymer consisting of repeated units of β-D-glucopyranose interconnected by β-1,4 glycosidic bonds (Liu and Kokare 2017; Farinas 2018). Plant biomass contains a significant amount of cellulose because it is their major cell-wall polysaccharide. There are two ways to convert cellulose to glucose: chemical and enzymatic hydrolysis. The application of cellulases for cellulose hydrolysis is an environmentally friendly process, performed without by-products generation (Sukumaran et al. 2005; Juturu and Wu 2014).

Cellulases are inducible enzymes produced by a number of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Quintanilla et al. 2015; Liu and Kokare 2017). Fungal cellulases include all components of the cellulase enzyme complex. They have different specificity and mode of actions and include: endoglucanases (carboxymethylcellulase-CMCase), cellobiohydrolases (exoglucanases–Avicelase) and β-glucosidas. However, in most cases, bacteria do not have a complete cellulase system. The main activity is endoglucanase that does not hydrolyze crystalline cellulose (Kuhad et al. 2011; Kumar et al. 2012).

The biotechnological potential of cellulase production is in their application in various industries, such as pulp and paper industry, textile and bioethanol industry, wine and brewery industry, food industry, animal feed industry, agricultural and detergent industry (Kuhad et al. 2011; Adrio and Demain 2014). Microbiological production of cellulases may be performed by submerged fermentation (SmF) or solid-state fermentation (SSF) technology (Farinas 2018). Various lignocellulosic residues with low-cost values can be used as sources of carbon in those processes: straw, spent grains and pulse carcasses, rice

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or wheat openings, bagasse, waste in the paper industry (Sukumaran et al. 2005).

Tobacco solid waste is produced during the tobacco manufacturing process in a large quantity (Wang et al. 2005; Onorevoli et al. 2018). Nowadays, the usage of electronic cigarettes (smokeless cigarette which extract nicotine without tobacco combustion and smoke) is increasing. Tobacco residues from these types of cigarettes present a new useful carbon source with lower nicotine content, and it also can be further treated with bioconversion instead of depositing. There are few studies on the use of tobacco waste for the production of enzymes, but the genus *Sinorhizobium* (*Ensifer*) was not used for this purpose up to now (Narasimha et al. 2006; Akpinar et al. 2009; Sun et al. 2010).

Rhizobia are a unique group of soil bacteria that can establish symbiotic association forming root nodules with specific host legumes. They convert atmospheric nitrogen to ammonia inside the root nodule and have a beneficial effect on plant growth (Shahzad et al. 2012). So far, enzymatic potential, especially cellulolytic, of this genus was not investigated. Only, Chen et al. (2004) noted CMCase activity for *Sinorhizobium fredii* CCRC 15769. On the other side, *Sinorhizobium* was described in Bergey's manual as non-cellulolytic (could not utilize cellulose) (Kuykendall et al. 2015).

This work is the first study that deals with cellulolytic potential of a *S. meliloti* strain 224. The aim of present research was to optimize conditions for the cellulase production (Avicelase and CMCase) from the strain 224 using tobacco waste during SmF and SSF. The response surface methodology (RSM) was applied for enzymes production process optimization. A chemical modification of the lignocellulosic substrate was made, and its influence on the production of enzymes was tested. All qualitative and quantitative experiments in the present study were conducted in the period from January to May 2018 in the Department of microbiology of the Institute of Soil Science, Belgrade, Serbia.

Materials and methods

Microorganism and inoculum preparation

Sinorhizobium meliloti strain 224 is a part of the Collection of the Institute of Soil Science (ISS WDCM375-Collection of Bacteria, Institute of Soil Science, Department of Microbiology). The strain was identified to nearest species based on morphological characteristics, and sequence of the 16S rRNA encoding gene (gene accession numbers FR714444.1) was higher than 99.7% (Stajković-Srbinović et al. 2012). It was selected according to qualitative test of the growing of strain on carboxymethyl cellulose (CMC) agar plate. Semi-quantification of cellulolytic potential was done on CMC, Avicel and waste tobacco agar plates (per liter: 1 g of CMC/Avicel/tobacco waste, 3.0 g of yeast extract, 3.0 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4$ and 6.0 g of agar) (Mihajlovski et al. 2016). A loopful of bacteria, previously grown in liquid CMC medium (without agar, 24 h, 28 °C), were spot-plated on these agar plates. After incubation (4 days, 28 °C), plates were flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 mL distilled water) for 3 to 5 min.

The working culture of rhizobium was prepared in Erlenmeyer flasks containing yeast mannitol broth [YMB; per liter: 10.0 of mannitol/1.0 g of CMC, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4$, 0.1 g of NaCl, 0.2 g of $CaCO_3$ and 100 mL of fresh yeast extract (30.0 g/L)] in a rotary shaker (125 rpm, 2 days, 28 °C) (Vincent 1970).

Substrate raw material and its modification

The tobacco solid waste material was dried and ground to a particle size of 0.063–0.1 mm (unmodified substrate). Its modification was done with 1% H_2SO_4 or NaOH in ratio 1:5 (w:v). After 2 h at room temperature (25 °C), the solid phase was separated by a vacuum pump and washed with distillate water. The resulting modified substrate was dried for overnight in an oven at 105 °C (modified substrate).

Experimental design of cellulase production

In order to evaluate the influence of selected factors on the production of cellulase as well as process optimization and statistical analysis, software Design Expert 8 (Stat-Ease, Inc) that includes Box–Behnken design (BBD) was employed. The model was described with 17 experiments and two responses, Avicelase activity (Y_1, U/mL) and CMCase activity (Y_2, U/mL) according to three process parameters: tobacco waste concentration (A, g/L), incubation time (B, days) and inoculum concentration (C, %). The levels of chosen parameters, independent variables, are presented in Table 1. In these experiments, rhizobium that was growing in the CMC medium was used. Multiple regressions were used to analyze BBD data and fit to a second-order polynomial regression model. The model equation of response (Y) of three independent variables listed above is given in the following equation:

$$Y = \beta_0 + \sum_{i=1}^{z} \beta_i X_i + \sum_{i=1}^{z=1} \sum_{j=1}^{z} \beta_{ij} X_i X_j + \sum_{i=1}^{z} \beta_{ii} X_i^2 \quad (1)$$

where Y represents the predicted response, X_i refers to the coded levels of the independent variables, X_iX_j is the interaction effect, X_i^2 is the square effect, β_0 is a constant, β_i, β_{ii} and β_{ij} are the linear, quadratic and interactive regression coefficients, respectively, and z is the number of independent

Table 1 Independent variables and their levels employed in a Box–Behnken design for the optimization of cellulase production

Independent variable	Coded levels		
	−1	0	1
A: Waste tobacco[a] concentration (g/L)	1	3	5
B: Incubation time (days)	1	2	3
C: Inoculum concentration (%)	1	5	9

[a]Unmodified substrate

variables (process parameter) (Pavlović et al. 2013; Talebi et al. 2017). Analysis of variance (ANOVA) was employed to check the adequacy of developed regression models.

Submersed fermentation

Liquid fermentation was carried out in 100-mL Erlenmeyer flasks with a predetermined amount of unmodified tobacco waste in the YMB medium. (Mannitol was replaced by waste material.) After sterilization, a predetermined amount of inoculum (growth in presence of CMC) was added and incubated on a rotary shaker (125 rpm, 28 °C). After the predetermined incubation time, the samples were centrifuged (6000×*g* for 15 min) and the cell-free supernatant was further tested for cellulase activity. Modified tobacco substrates were used to produce cellulases, under obtained optimal conditions, to test the effect of modification on the production of this enzyme.

Solid-state fermentation

Unmodified and modified tobacco substrates were used as a low-cost substrate for the production of cellulases by *S. meliloti* 224 within the SSF. SSF was performed in 100-mL Erlenmeyer flasks with 1.0 g of sterile tobacco waste [distilled water was added before sterilization in ratio 1:3 (w:v)] and 5% or 10% of rhizobium culture which was growing in YMB or CMC (1 g/L) medium. After incubation (2 days, 28 °C), 10 mL of 0.1 M tri-sodium citrate buffer (pH 4.8) was added for enzyme extraction. The samples were filtrated, and the liquid aliquot was centrifuged and analyzed for Avicelase and CMCase activity.

Enzyme assay for Avicelase and CMCase

The activity of cellulase was measured by the reduction of 3,5-dinitrosalicylic acid in the presence of glucose released by enzymatic cellulose hydrolysis (Miller 1959). Avicelase and CMCase activities were determined using 0.5 mL of enzyme sample and 0.5 mL of 1% (w/v) Avicel or CMC solution in 0.1 M tri-sodium citrate buffer (pH 4.8). The mixture was incubated in a rotary shaker (125 rpm, 50 °C).

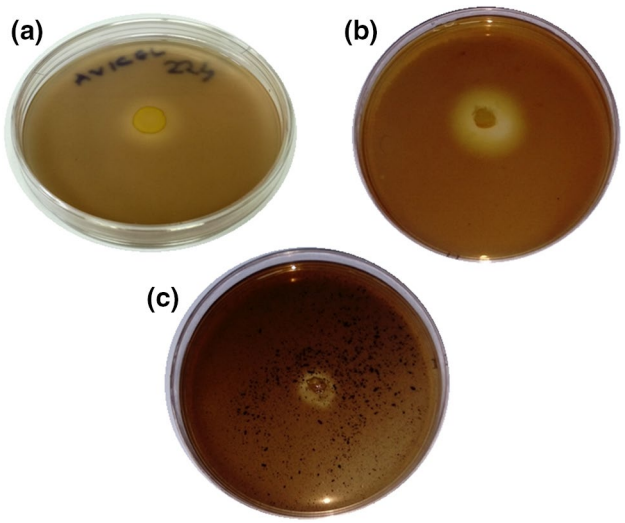

Fig. 1 Cellulolytic activity of the *Sinorhizobium meliloti* strain 224 on: Avicel (**a**), CMC (**b**) and waste tobacco (**c**) agar plates

After 30 min of incubation, 1 mL of DNS reagent was added and the reaction mixture boiled, cooled and diluted by adding 5 mL of distilled water. The absorbance was read on the UV/visible spectrophotometer (UV-160A, Shimadzu Corporation, Japan) at 540 nm against a blank (non-incubated enzyme). One unit of Avicelase or CMCase activity was defined as the amount of enzyme that released 1 µmol of glucose equivalents per minute.

Results and discussion

Cellulase qualitative test

Sinorhizobium meliloti strain 224 was growing on Avicel, CMC and waste tobacco agar plate for 4 days. The presence of halo zones around bacterial colonies on these plates produced by strain 224 indicates areas of hydrolysis of used substrates (Fig. 1).

Halo zones appeared on all agar plates with the appropriate substrate. This indicates that strain 224 can use both microcrystalline (Avicel) and amorphous (CMC) cellulose, as the sole carbon source, proving it to be a true cellulolytic bacterium. The hydrolysis of both Avicel and CMC by *S. meliloti* has not been reported yet in the literature. Just Hu and Lin (2003) reported positive CM-cellulase activity of the sonicated cell extracts of *S. fredi* CCRC15769 on double-layer plate with CMC, but not in the supernatant after bacterial growth in the broth as in the present study. In addition, strain 224 can hydrolyze waste tobacco (Fig. 1c) and the hydrolysis of this waste material by *S. melilot* was not previously mentioned in the literature.

Fitting the process parameters

In order to achieve the maximum activity of Avicelase and CMCase, the optimal combination of three independent variables according to the experimental design matrix derived from the BBD was performed (Table 2).

By applying a multiple regression analysis for experimental data, the relationship between the responses and the three examined factors is efficiently designed as a second-order response corresponding to the surface and it is shown by the two equations [Eqs. (2), (3)]:

$$Y_1 = 0.1156 + 0.0078 \times A - 0.0026 \times B + 0.0166 \times C + 0.0000 \times AB - 0.005 \times AC + 0.0053 \times BC - 0.0069 \times A^2 - 0.0177 \times B^2 - 0.0117 \times C^2 \quad (2)$$

$$Y_2 = 0.0796 + 0.01225 \times A + 0.00575 \times B + 0.0165 \times C - 0.001 \times AB + 0.007 \times AC + 0.002 \times BC - 0.0143 \times A^2 - 0.0263 \times B^2 + 0.0012 \times C^2 \quad (3)$$

where the Y_1 (Avicelase activity, U/mL) and Y_2 (CMCase activity, U/mL) are the responses, and the A (waste tobacco concentration, g/L), B (incubation time, days) and C (inoculum concentration, %) are the independent variables.

The quadratic model was found to be the most suitable model for both responses. The analysis of variance ANOVA to determine the significance of regression model for the responses was evaluated, and the results are presented in Table 3. The model F values of 30.28 (Y_1) and 91.81 (Y_2) corresponding to P value < 0.0001 imply the models are significant and accurate. In addition, the term of lack of fit is nonsignificant ($P > 0.05$) according to model assumption which explained that quadratic models were adequate for predicting the production of cellulases. The validity of the second-order model was further investigated by the correlation coefficient (R^2). It was 0.9750 for Avicelase activity and 0.9916 for CMCase activity and indicates a good correlation and fitting between the actual (experimental) and predicted values. Further, the adjusted multiple correlation coefficient ($R^2_{adj.}$) value was found to be 0.9428 and 0.9808 for Avicelase and CMCase activity, respectively. The predicted multiple correlation coefficients ($R^2_{pred.}$, 0.7775 for Avicelase and 0.8971 for CMCase activity) were close to the $R^2_{adj.}$ values. These result values showed that the model fitted the data well. The adequate precision values of 19.19 for Avicelase activity and 34.87 for CMCase activity indicated a desirable signal-to-noise ratio.

Independent factors are designated as significant under selected conditions according to the values of model terms Prob > F < 0.0500. Significant model terms for the response Avicelase activity are A, C, BC, A^2, B^2 and C^2, while for the response CMCase activity significant model terms are A, B, C, AC, A^2 and B^2 (Table 3).

Table 2 Values of examined variables and experimental values of Avicelase and CMCase activities in the Box–Behnken design

Run	Variables			Responses	
	A	B	C	Y_1	Y_2
1	1	1	5	0.090	0.022
2	5	1	5	0.099	0.047
3	1	3	5	0.083	0.033
4	5	3	5	0.092	0.054
5	1	2	1	0.073	0.046
6	5	2	1	0.085	0.058
7	1	2	9	0.099	0.061
8	5	2	9	0.131	0.101
9	3	1	1	0.078	0.031
10	3	3	1	0.064	0.041
11	3	1	9	0.098	0.064
12	3	3	9	0.105	0.072
13	3	2	5	0.116	0.047
14	3	2	5	0.113	0.050
15	3	2	5	0.114	0.052
16	3	2	5	0.120	0.048
17	3	2	5	0.115	0.051

A waste tobacco concentration, g/L; B incubation time, days; C inoculum concentration, %; Y_1 avicelase activity, U/mL; Y_2 CMCase activity, U/mL

Table 3 ANOVA for RSM parameters fitted to polynomial equation

Source	Response			
	Avicelase activity U/mL		CMCase activity U/mL	
	F value	P value Prob > F	F value	P value Prob > F
Model	30.28	< 0.0001[s]	91.81	< 0.0001[s]
A	24.92	0.0016[s]	126.94	< 0.0001[s]
B	2.86	0.1347	27.97	0.0011[s]
C	114.69	< 0.0001[s]	230.30	< 0.0001[s]
AB	0	1.0000	0.42	0.5362
AC	5.19	0.0568	20.73	0.0026[s]
BC	5.72	0.0481[s]	1.69	0.2345
A^2	10.47	0.0143[s]	91.04	< 0.0001[s]
B^2	68.23	< 0.0001[s]	307.96	< 0.0001[s]
C^2	29.77	0.0009[s]	0.64	0.4496
Lack of fit	4.83	0.0812[ns]	3.80	0.1150[ns]

A waste tobacco concentration, g/L; B incubation time, days; C inoculum concentration; %

[s]Significant "Prob > F" < 0.0500

[ns]Nonsignificant

Table 4 Model validation for cellulase production using unmodified tobacco waste during submersed fermentation

Run	Conditions			Response			
	A	*B*	*C*	Experimental		Predicted	
				Avicelase U/mL	CMCase U/mL	Avicelase U/mL	CMCase U/mL
1	4.78	2.06	8.66	0.132	0.103	0.127	0.101
2	4.50	2.59	9.00	0.120	0.990	0.122	0.989
3	2.34	2.12	9.00	0.119	0.896	0.116	0.900

A waste tobacco concentration, g/L; *B* incubation time, days; *C* inoculum concentration, %

Models validation

In the purpose of the model reproducibility, the validation was done by using the three points which were randomly selected from the numerical optimization results. The results were compared with the obtained (now predicted in Table 4) values from Table 2. The percentage errors between experimental and predicted values of Avicelase and CMCase activity during submersed fermentation using untreated tobacco waste were within the acceptable range (Table 4). In addition, results showed the same trends as predicted results. Thus, these results indicate that the model equation presented good agreement with the experimental result.

Effect of process variables on Avicelase activity

The maximum Avicelase activity of 0.131 U/mL was obtained under the following conditions: unmodified waste tobacco concentration of 5 g/L, 2 days of incubation time and inoculum concentration of 9% (Table 2). Avicelase activity was increased by increasing tobacco and inoculum concentrations, as well as increasing incubation time from 1 to 2 days and decreasing this parameter from 3 to 2 days (Fig. 2).

In this study, for the first time, Avicelase activity of the genus *Sinorhizobium* has been documented. Other studies which analyzed Avicelase activity of soil bacteria used equal or higher substrate concentrations (commercial or waste material) in order to achieve optimal Avicelase production (Abdel-Fattah et al. 2007; Martins et al. 2011; Oliveira et al. 2014). Shashidhar et al. (2018) investigated the influence of CMC concentration (2–20 g/L) on the Avicelase activity of enzymes from soil isolates *Serratia marcescens* WW4 and L4. Maximum activity was achieved with 5 g/L of CMC concentration. In addition to that, Acharya et al. (2008) obtained lower Avicelase activity from 0.0518 to 0.0804 U/mL produced by *Aspergillus niger* growing on a sawdust, for a concentration of 2.4% to 7.2% in a fermentation medium. Meanwhile, isolate CL5 (*Bacillus* sp.) achieved Avicelase activity of 0.108 U/mL during SmF at 37 °C with the presence of 3% of sawdust and about 10% of inoculum (Fauzi and Makky 2013). The RSM was used to obtain the maximum Avicelase activity of the natural isolate *Paenibacillus chitinolyticus* CKS1 using barley bran as low-cost substrate by Mihajlovski et al. (2017). Maximum Avicelase activity was 0.433 U/mL with applying 4% concentration of waste substrate during 3 days of incubation and 10% of inoculum concentration (Mihajlovski et al. 2017).

An increase in Avicelase activity with increasing inoculum concentrations, as is the case in this study, was also noted by Makky (2009). Lower inoculum concentration required longer time for the cells multiplication and utilization of the substrate to produce enzyme (Sun et al. 2010). Increasing inoculum concentration would allow rapid proliferation and biomass synthesis. Also, it can lead to a reduction in enzyme production, because after a certain limit due to exhaustion of nutrients, it could lead to a decrease in metabolic activity and cellulase production. The balance between the proliferating biomass and the available nutrient would give the optimum effect on which the synthesis of the enzymes would be maximal (Ramachandran et al. 2004; Sun et al. 2010).

The effect of different incubation times on the production of Avicelase is shown in Fig. 2. Maximum Avicelase activity over a period of 2 days, which was achieved for cellulase produced by strain 224, is the average time for reaching the maximum Avicelase activity, according to previous researches in this field. Incubation time ranged from 3 to 120 days, but it should also be noted that these cellulases are from different sources (Abdel-Fattah et al. 2007; Makky 2009; Oliveira et al. 2014; Mihajlovski et al. 2015, 2017).

Effect of process variables on CMCase activity

The maximum CMCase activity of 0.101 U/mL was obtained under the same conditions as maximum Avicelase activity: unmodified waste tobacco concentration of 5 g/L, 2 days of incubation time and inoculum concentration of 9% (Table 2). CMCase activity also increased when Avicelase activity increased, with increasing tobacco and inoculum concentrations, and decreased with incubation time of 1 to 2 and of 2 to 3 days (Fig. 3).

Among the nitrogen fixing bacteria, CMCase activities have been observed in *Sinorhizobium fredii*, *Bacillus*

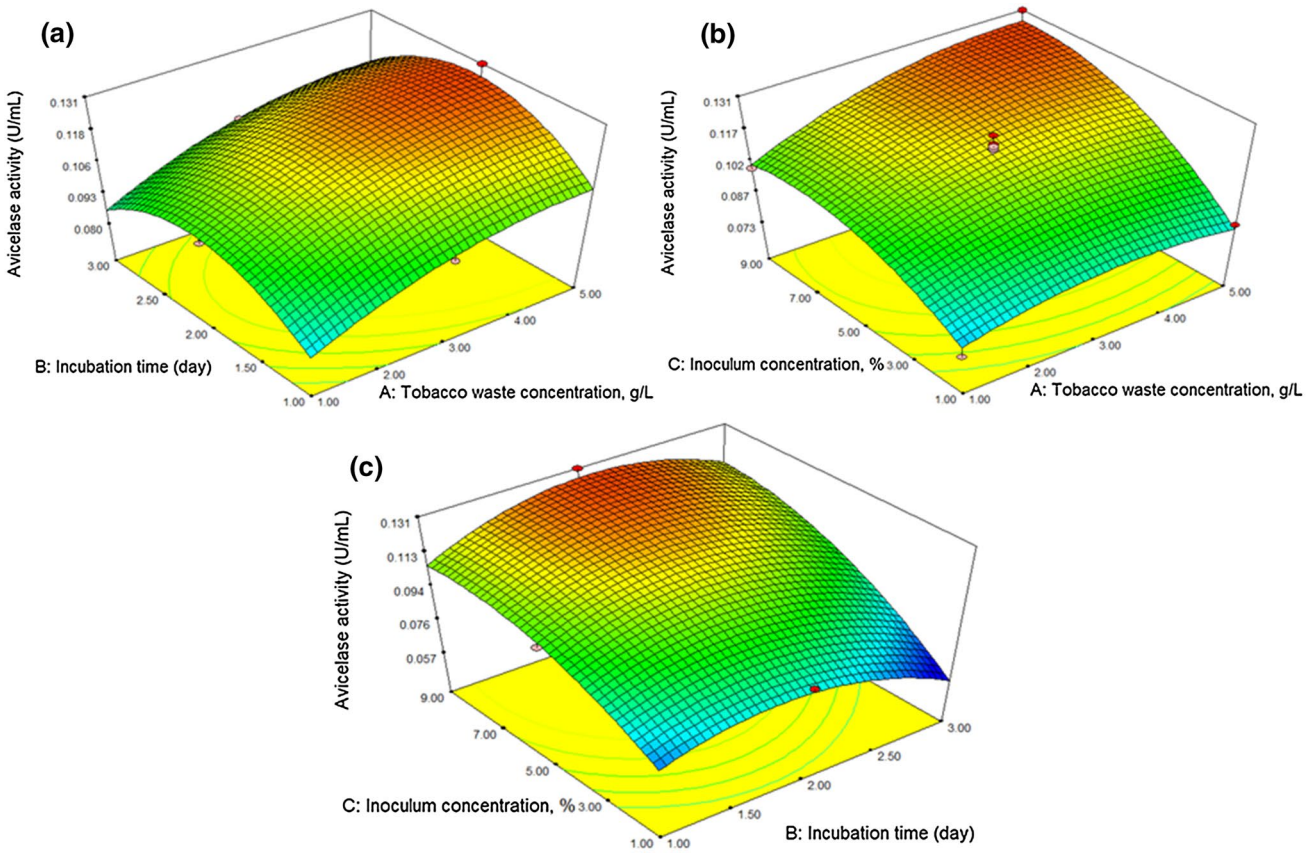

Fig. 2 Contour plot of combined effects of: *AB* (**a**), *BC* (**b**) and *AC* (**c**) on Avicelase activity U/mL. The actual values of other factors are: *C* (**a**, 9%); *A* (**b**, 5 g/L) and *B* (**c**, 2 days)

spharricus, *Bacillus circulans*, *Paenibacillus* sp., *Gluconacetobacter* (Emtiazi et al. 2007). Chen et al. (2004) demonstrated the presence of CMCase activity of *S. fredii*. However, the results cannot be compared with the results in this study, since the analysis of CMCase activity was performed from bacteria pellets which were resuspended in buffer (PCA; pH5.2) (Chen et al. 2004). In addition, the use of agro-industrial waste material as a substrate for the production of CMCase of the genus *Sinorhizobium* and their species *S. meliloti* was for the first time reported in the present study.

Emtiazi et al. (2007) and Pongsilp (2008) studied CMCase from *Paenibacillu*s sp. and *Rhizobium* sp., which were produced with activity of 0.2 U/mL and 3 U/mL and about 2 mU/mL using 10 g/L of CMC in the growing medium, respectively. They were only tested in this CMC concentration, and the obtained CMCase activities were both higher and lower than those in the present study. Also, these activities were reached after 2 days of culture incubation, as in the present study; and after that period, the activity was decreased.

It is interesting to note that the strain 224 showed higher Avicelase than CMCase activity while growing on waste tobacco. Considering that there are no available literature data about the cellulolytic potential of the *Sinorhizobium* sp., it can be stated that the strain 224 is the first reported *Sinorhizobium* sp. with the predominant activity of Avicelase. For the efficient hydrolysis of cellulose, exoglucanases and endoglucanases should act in synergy. Therefore, the cellulases secreted by *S. meliloti* could be categorized as predominantly exoglucanases with complementary and lower endoglucanases activity.

On the other hand, the utilization of various agro-waste materials during SmF, such as wheat bran, cotton seed, rice bran, rice straw and pomegranate, to express the CMCase activity of *Aspergillus flavus*, was reported by Gomathi et al. (2012). The highest CMCase production was showed using wheat bran after 3 days of incubation, with 4% inoculum concentration and waste substrate concentration of 3%. In fact, the application of smaller amounts of inoculum provides advantages in the production of enzymes, as production costs could be reduced in this way. The maximal CMCase activity was 3.3 U/mL (Gomathi et al. 2012). In addition, Mihajlovski et al. (2017) also employed RSM to optimize the production of CMCase from *P. chitinolyticus* CKS1 using barley

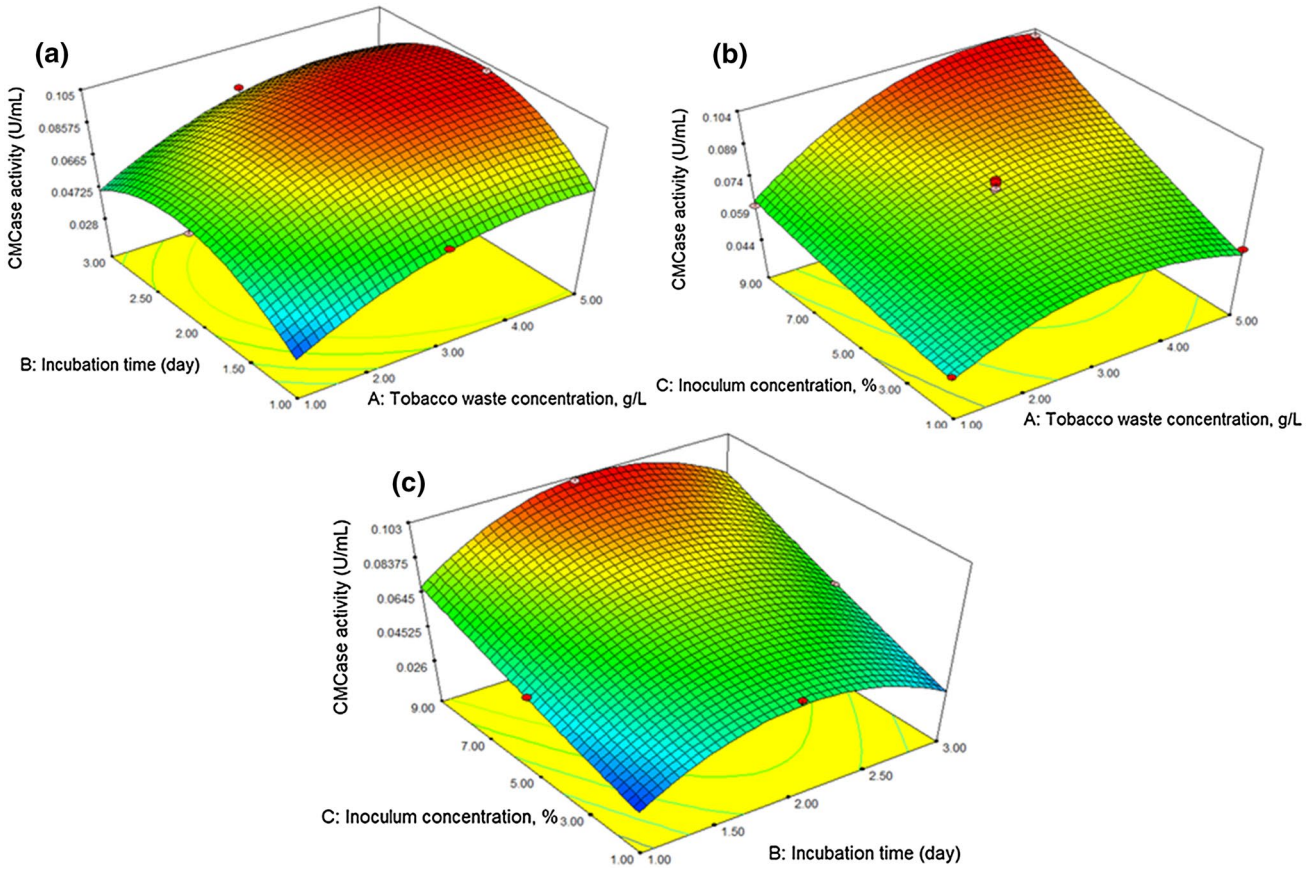

Fig. 3 Contour plot of combined effects of: *AB* (**a**), AC (**b**) and *BC* (**c**) on CMCase activity U/mL. The actual values of other factors are: *C* (**a**, 9%); *B* (**b**, 2 days) and *A* (**c**, 5 g/L)

Table 5 Cellulase production during SmF using modified substrates

Substrate	Cellulase activity (U/mL)	
	Avicelase	CMCase
H_2SO_4-modified tobacco	0.098	0.112
NaOH-modified tobacco	0.124	0.063
Unmodified tobacco	0.131	0.101

bran. The maximum CMCase activity (0.405 U/mL) was obtained with a similar inoculum concentration, lower substrate concentration of 4% and after a prolonged incubation time of 3 days (Mihajlovski et al. 2017).

Submersed fermentation using modified waste tobacco

Under optimal conditions obtained from RSM (substrate concentration 5 g/L, 10% inoculum concentration and 2 days of incubation time), the modified substrates were tested for cellulase production. The results are present in Table 5.

The modification of tobacco waste did not contribute to the improvement in the production of both enzymes during SmF, except for a slight increase in CMCase activity with applying H_2SO_4 pretreatment. Avicelase activities between untreated and NaOH pretreatment of substrate were similar. Pretreatment of wheat bran with H_2SO_4 and NaOH also did not contribute to the increase in cellulases production, as reported by Bansal et al. 2012, where the highest CMCase activity was obtained by utilizing untreated substrate.

Solid-state fermentation using tobacco waste

The production of enzymes Avicelase and CMCase from soil bacterial strain *S. meliloti* 224 by solid fermentation using unmodified and modified tobacco waste as a substrate was analyzed, and the results are presented in Fig. 4.

The use of the same amount of bacteria inoculums, which were growing in YMB (YMB culture) and CMC (CMC culture) medium before fermentation, yielded to different results. The use of YMB culture favored higher production of Avicelase, while the use of CMC culture led to higher CMCase production. Application of the acclimated culture

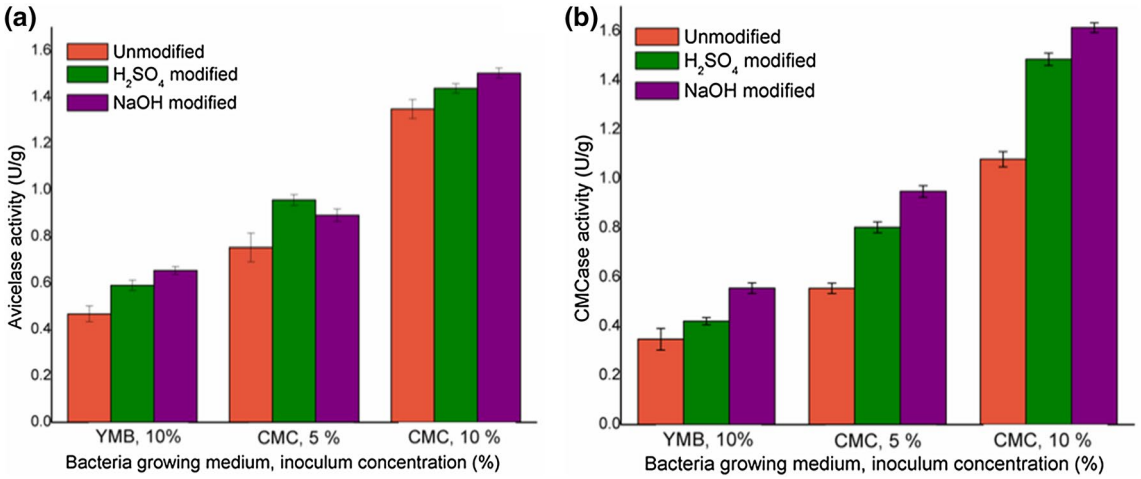

Fig. 4 Production of: Avicelase (**a**) and CMCase (**b**) using tobacco waste by strain 224 with and without prior acclimatization of bacterial culture

achieved an increase in Avicelase activity by 2.9-fold and in CMCase activity by about 3.1-fold. This can be explained by the effect of a bacterial passaging on the production of cellulases, where the microorganism is forced to use cellulosic material as a source of carbon (Mihajlovski et al. 2016). In addition, the increased percentage of acclimatized inoculum increased the activity of both enzymes, Avicelase and CMCase.

The applied chemical pretreatment of the tobacco substrate improved the production of CMCase and Avicelase. The modification with NaOH was proved as a better solution for the pretreatment of substrate in the production of cellulase during SSF. Maximum Avicelase and CMCase activities reached 1.503 U/g and 1.615 U/g using the NaOH-modified tobacco waste and 10% inoculum of acclimatized bacterial culture, respectively. Application of NaOH pretreatment to tobacco waste causes swelling of the material. It further leads to an increase in the internal surface and a decrease in the degree of polymerization. The diluted NaOH treatment also causes a decrease in the crystallinity of the lignocellulosic material, which probably increases the activity of the CMCase (Karp et al. 2013). Application of the modified substrate (H_2SO_4 and NaOH modified) achieved an increase in Avicelase activity of 1.5- to 2.5-fold and in CMCase activity of about 1.3- and 2.1-fold, respectively.

In the literature, tobacco waste without any previous treatment was used to produce cellulase by fungi: *Aspergillus terreus*, *A. niger*, *Phanerochaete chrysosporium*, *Trametes versicolor* and *Trametes hirsute* (Narasimha et al. 2006; Oliveira et al. 2008; Su et al. 2016). Oliveira et al. (2008) produced CMCase utilizing black and Virginia tobacco dust waste as a substrate. During fermentation of black tobacco dust by *A. niger*, the obtained values for the assayed CMCase activity were very low, while fermentation with *A. terreus* gave the CMCase activity of 0.4163 U/mL (incubation time of 4 days, 31 °C). When Virginia tobacco dust was used as a substrate, the activities of CMCases from *A. niger* and *A. terreus* were 0.2128 U/mL and 0.3729 U/mL after 3 and 5 days of fermentation, respectively (Oliveira et al. 2008). Application of other fungi, *P. chrysosporium*, *T. versicolor* and *T. hirsute* in the production of cellulases during SSF of tobacco waste, gave the highest CMCase (0.51 U/mL) and Avicelase activity (0.51 U/mL) by using *T. versicolor* (inoculation of 10 days, 28 °C, relative humidity of 80%) (Su et al. 2016).

Conclusion

Soil bacterium *Sinorhizobium meliloti* strain 224 was found to be capable to produce both Avicelase and CMCase using tobacco waste as substrate with predominant Avicelase activity. Submerged fermentation favored Avicelase production and the usage of unmodified substrate. (Maximum Avicelase activity was 0.131 U/mL.) On the other hand, solid-state fermentation gave higher CMCase activities using the NaOH-modified substrate and expressed the maximum activity of 1.615 U/g. The presence of Avicelase and CMCase in strain 224 is a rare occurrence in bacteria and makes the despair significant in bioconversion of lignocelluloses waste and reduction in the amount of agro-industrial waste. The crude enzyme, produced by using the tobacco waste by strain 224, could be employed in eco-friendly processes of cellulose-based material bioconversion to useful products.

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Compliance with etical standard

Conflict of interes The authors declare that they have no conflict of interest.

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