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Original scientific paper

The production of cellulase from the waste tobacco residues remaining after polyphenols and nicotine extraction and bacterial pre-treatment

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Abstract: Agricultural by-products are generated in large amounts in various industries, creating a serious disposal problem. Valorisation of tobacco waste for the extraction of value-added compounds and the production of enzymes could reduce both the problems of its disposal and the costs of cellulase production. Hitherto, there have been no reported studies concerning the utilization of tobacco residues (after extraction of polyphenols and nicotine, and fermentation pre-treatment by *Streptomyces fulvissimus* CKS7) for the production of cellulase (CMCase and avicelase) by *Paenibacillus chitinolyticus* CKS1. The optimal conditions for the polyphenols and nicotine extraction process were obtained using the response surface methodology: 60 s extraction time in water and 30 mL g^{-1} liquid/solid ratio. After the applied bacterial fermentation as a pre-treatment of tobacco residues, using *Paenibacillus* species, the extraction of polyphenols decreased by up to 10 %, while the extraction of nicotine increased by up to 35 %. Afterward, the maximum of cellulase activities (CMCase of 0.878 U g^{-1} and avicelase of 1.417 U g^{-1}) were achieved using of the strain CKS1.

Keywords: lignocellulosic waste; microwave-assisted extraction; solid-state fermentation; CMCase and avicelase activity.

INTRODUCTION

Solid waste from tobacco is classified as agroindustrial waste generated at various stages of tobacco processing after harvest and during the production of tobacco products. The disposal of this waste material is a serious problem, due to the presence of high carbon and nicotine contents.[1] It is classified as toxic and hazardous if the nicotine content exceeds 500 mg kg^{-1} dry weight. According to this, the disposal has to be controlled in order to avoid harmful effects to the

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environment. Cigarette companies have to pay for its disposal and the majority of the waste is destroyed by burning.[1,2] The utilization of tobacco residues has the potential of significant environmental and economic benefits, as a source of bioactive compounds instead of generation of the problems that may be caused by its disposal.

Tobacco waste has potential applications for soil amendment and the production of tailored organic fertilizer and desulfurization adsorbents.[3,4] In addition, it could be utilized for the extraction of valuable compounds. such as polyphenols and nicotine, and for the production of various enzymes during a fermentation process.[1,5] The concentration of chlorogenic acid and rutin is the highest among the polyphenol compounds in tobacco leaves.[6] The extraction of solanesol from tobacco leaves also produces large quantities of residues that still contain polyphenol compounds.[7]

Various efficient and advanced extraction techniques have been developed for the extraction of phenolic compounds, such as pressurized liquid extraction, microwave-assisted extraction (MAE), ultrasound-assisted extraction, soxhlet extraction and heat reflux extraction, as well as supercritical fluid extraction.[8] Among these treatments, MAE is a relatively new and promising green extraction method. This treatment is considered as efficient because of the reduction of both the extraction time and solvent consumption by rapid heating of the solvent and suspension. The absorption of energy in the sample, especially by polar molecules such as water, leads to cell disruption that facilitates the recovery of the compounds of interest. In addition, it is necessary to optimize the parameters of the extraction process to maintain the maximum amount of bioactive compounds in the obtained extracts.[8–10]

In addition, various lignocellulose residues, including tobacco waste, could be used in enzymes production as sources of carbon for microorganisms. Solid--state fermentation (SSF) as a cost-effective technology is increasingly being used in the production of enzymes and bioconversion of lignocelluloses waste biomass using cellulolytic microorganisms. The successful strategy to produce cellulolytic enzymes includes both microorganism selection and improved conditions of the fermentation process.[11,12]

The overall objective of this study was the reuse of tobacco waste for the extraction of compounds with added values (polyphenols and nicotine) and the production of cellulose before its disposal. The optimal range of extraction conditions of polyphenolic compounds and nicotine were determined by the use of response surface methodology (RSM). In addition, the influence of bacterial fermentation of the sample material by *Streptomyces fulvissimus* CKS7, as a pretreatment, on the extraction of compounds of interest was investigated. Subsequently, the exhausted material was utilized for the production of cellulose (avicelase and CMCase) by soil bacterium *Paenibacillus chitinolyticus* CKS1.

EXPERIMENTAL

Materials

Tobacco waste material. Tobacco residues from specially designed heated tobacco units (manufactured by the company Philip Morris International, Switzerland) were collected. The tobacco units were heated by IQOS electronics, where the tobacco was heated just enough to release a nicotine-containing vapour, but without burning the tobacco.[13] This residue was dried for 24 h in an oven at 37 °C and milled (IKA® A11 basic analytical mill) to a particle size range 0.063–0.1 mm (passed through a sieve of this diameter) and further used as a low-cost material for nicotine and polyphenol extraction and bacterial solid-state fermentation.

Chemicals. The total polyphenol content was measured using Folin–Ciocalteu reagent (purchased from MOL Belgrade, Serbia) and then calculated using gallic acid (GA, ≥98.5 % purity, purchased from Sigma–Aldrich, Denmark) as a standard. The nicotine content was measured using potassium permanganate (>99 % purity, purchased from Centrohem, Stara Pazova, Serbia) and sodium hydroxide (≥99 % purity) (purchased from Lachema, Czech Republic), and then calculated using (±)-nicotine (≥99 % purity, purchased from Sigma–Aldrich, Denmark) as a standard. The cellulase activity was determined using working solutions of Avicel (high purity, purchased from Merck, Germany) that were prepared fresh in pH 4.8 tri-sodium citrate buffer (≥99 % purity, purchased from Sigma–Aldrich, Denmark), DNS reagent (prepared from 3,5-dinitrosalicylic acid (>97 % purity, purchased from Alfa Aesar by Thermo Fisher (Kandel) GmbH, Germany) and potassium sodium tartrate tetrahydrate (>98 % purity, purchased from Lach-Ner, Czech Republic) and then calculated using glucose (≥99 % purity, Betahem, Belgrade, Serbia) as a standard.

Polyphenols and nicotine extraction

Equipment and procedure. The extraction of polyphenols and nicotine was performed using a domestic microwave oven (LG MC7849HS), with distilled water as the solvent. Batch experiments were realized in 100 mL Erlenmeyer flasks using tobacco waste at different liquid/solid ratios for a predetermined extraction time. The microwave oven was set at 180 W. After extraction, the mixture was filtered and the percentage of dry matter was measured on moisture analyzer (MA 9507, Iskra, Ljubljana, Slovenia). Each sample was diluted with distilled water to a concentration of 10 mg dry matter mL^{-1} for polyphenol analysis and 0.5 mg dry matter mL^{-1} for nicotine analysis.

Experimental design. Central composite design (CCD) within RSM was applied in order to determine the best combination of selected factors for the given responses: total polyphenols content (*TPC*, Y_1 / mg gallic acid equivalents (GAE) g^{-1} of extract dry matter) and nicotine content (Y_2 / μg mL^{-1}). The range values of the process variables of the extraction time and the liquid/solid ratio are shown in Table I.

TABLE I. Independent variables and their levels employed in the central composite design for the optimization of extraction of polyphenols and nicotine from tobacco waste

Independent variable	Values				
	$-\alpha$	–1	0	1	$+\alpha$
Extraction time, s	17.6	30	60	90	102.4
Liquid/solid ratio, mL g^{-1}	15.9	20	30	40	44.1

Pre-treatment by bacterial fermentation. Three points from design (runs 1, 2 and 11, Table II) were randomly selected for bacterial SSF experiments and evaluation of their effect

on the extraction of polyphenols and nicotine. After autoclaving samples of tobacco residues (which had been moistened with distilled water in a ratio of 1:4), 10 % inoculums of *Streptomyces fulvissimus* CKS7 were added. The samples were incubated for three days at 30 °C. The extraction of polyphenols and nicotine was performed as already described above (in the Experimental – Polyphenols and nicotine extraction section) and the results were compared with those of non-fermented samples.

Cellulase production

After extraction of polyphenols and nicotine, the solid residues of the tobacco samples (fermented and unfermented) were collected, dried and used as substrates for the production of cellulase by *Paenibacillus chitinolyticus* CKS1. The enzyme production was performed by the addition of 10 % inoculums of CKS1 and incubating at 30 °C for three days. Enzyme extraction was performed with 10 mL of 0.1 M tri-sodium citrate buffer (pH 4.8), and after filtration and centrifugation of the samples, the activity of cellulose in the supernatant was analyzed. All measurements were performed in triplicate.

Analytical methods

Determination of the total polyphenols content. The total polyphenols content (*TPC*) was determined by the Folin–Ciocalteu method with a slight modification.[14] An extract (0.1 mL with a dry matter concentration of 10 mg mL^{-1}) was mixed in a test tube with 0.5 mL of Folin–Ciocalteu reagent and 6 mL of distilled water. Then, 2 mL of 15 % Na_2CO_3 solution and 1.4 mL of distilled water were added. The absorbance was measured after 2 h, at 750 nm, against a blank, which was simultaneously prepared but distilled water was used instead of an extract sample. The results are expressed as gallic acid equivalents (GAE) through a calibration curve of gallic acid (1–1500 μg mL^{-1}).

Determination of nicotine content. The nicotine content was determined spectrophotometrically according to the Al-Tamrah method.[15] In a volumetric flask, 0.5 mL of potassium permanganate (0.0125 M) was swirled gently with 1 mL of sodium hydroxide (6.25 M). After the addition of 0.1 mL of an extract sample (with a dry matter concentration of 0.5 mg mL^{-1}) and 8.4 mL of distilled water, the mixture was heated in a water bath (100 °C) for 7.5 min. The samples were cooled to room temperature and measured at 610 nm against a reagent blank (using distilled water instead of an extract sample). The results are expressed according to a nicotine calibration curve in the concentration range 0.1–7.5 μg mL^{-1}.

Enzyme assay. The cellulase activity (CMCase and avicelase activity) was determined according to the DNS method[16] using of 1 % CMC or Avicel solution in tri-sodium citrate buffer (0.1 M, pH 4.8). Mixtures of enzyme sample and CMC or Avicel solution in volume ratio 1:1 were incubated for 30 min at 50 °C (CMCase) or 80 °C (avicelase).[17] The reaction was stopped by the addition of 1 mL of DNS reagent. After heating to 90 °C, cooling and dilution with 5 mL of distilled water, the samples were analyzed spectrophotometrically at 540 nm against the control (without enzyme incubation). One unit of CMCase or avicelase activity is defined as the amount of enzyme that released 1 μmol of glucose equivalents per min.

RESULTS AND DISCUSSION

Fitting the process parameters

In order to obtain the maximum content of extracted polyphenols and nicotine, according to the experimental design matrix derived from the CCD, the optimal combination of the two independent parameters was determined (Table II).

TABLE II. The values of the variables and the responses in the Central Composite design; Y_1: total polyphenols content, Y_2: nicotine content

Run	Variables		Responses	
	Extraction time, s	Liquid/solid ratio, ml g^{-1}	Y_1 / mg GAE g^{-1}	Y_2 / μg mL^{-1}
1	30.00	20.00	61.47	6.12
2	90.00	20.00	70.47	4.58
3	30.00	40.00	70.55	5.25
4	90.00	40.00	69.55	4.51
5	17.60	30.00	66.72	6.10
6	102.4	30.00	68.63	3.50
7	60.00	15.90	70.97	5.32
8	60.00	44.10	76.88	4.56
9	60.00	30.00	79.97	6.17
10	60.00	30.00	80.63	6.27
11	60.00	30.00	82.80	7.14
12	60.00	30.00	78.97	7.05
13	60.00	30.00	82.72	7.01

The relationships between the responses and the two tested factors were designed as second order responses by application of multiple regression analysis. They are presented by the following two equations:

$$Y_1 = 81.02 + 1.34A + 2.07B - 2.50AB - 7.37A^2 - 4.24\,B^2 \quad (1)$$

$$Y_2 = 6.73 - 0.75A - 0.25B + 0.20AB - 0.90A^2 - 0.83B^2 \quad (2)$$

where the Y_1 (total polyphenols content, mg GAE g^{-1} of dry extract) and Y_2 (nicotine content, μg mL^{-1}) are the responses, and the A (extraction time, s) and B (liquid/solid ratio, ml g^{-1}) are the independent variables based on the coded values.

It was found that a quadratic model is the most suitable model for both responses. The results of the analysis of variance ANOVA are presented in Table III. Significant model terms for the *TPC* response are B, AB, A^2 and B^2, while for the response of nicotine content, they are A, A^2 and B^2. These factors are significant according to the values of model terms $Prob > F$ <0.050. According to the obtained model, F-values (24.124 (Y_1) and 16.277 (Y_2)) and the lack of fit values (not significant ($p > 0.05$)), the models are significant and the quadratic models are adequate for predicting the extraction of both polyphenols and nicotine (Table III). Regression coefficient R-squared (R^2) of 0.945 (Y_1) and 0.921 (Y_2) indicate a good correlation between the actual (experimental) and predicted values of the responses.

Effect of extraction time and liquid/solid ratio on the extraction of polyphenols and nicotine

The effects of process parameters on the extraction of polyphenols and nicotine are shown in Figs. 1 and 2, respectively. The maximum *TPC* of 82.80 mg

GAE g^{-1} and nicotine content of 7.14 μg mL^{-1} of extract were obtained under the same conditions: 60 s of extraction time and 30 mL g^{-1} of liquid/solid ratio (Table II).

TABLE III. ANOVA for RSM parameters (*p*-values) fitted to a polynomial equation; *A* – extraction time; *B* – liquid/solid ratio; Y_1: total polyphenols content; Y_2: nicotine content; ns – not significant – *Prob* > *F* > than 0.050; s – significant – *Prob* > *F* < than 0.050

Source	Response	
	Y_1 / mg GAE g^{-1} of dry extract	Y_2 / μg mL^{-1}
Model	0.0003[s]	0.0010[s]
A	0.1131	0.0015[s]
B	0.0267[s]	0.1357
AB	0.0481[s]	0.3717
A^2	0.0001[s]	0.0008[s]
B^2	0.0011[s]	0.0012[s]
Lack of fit	0.2295[ns]	0.6722[ns]

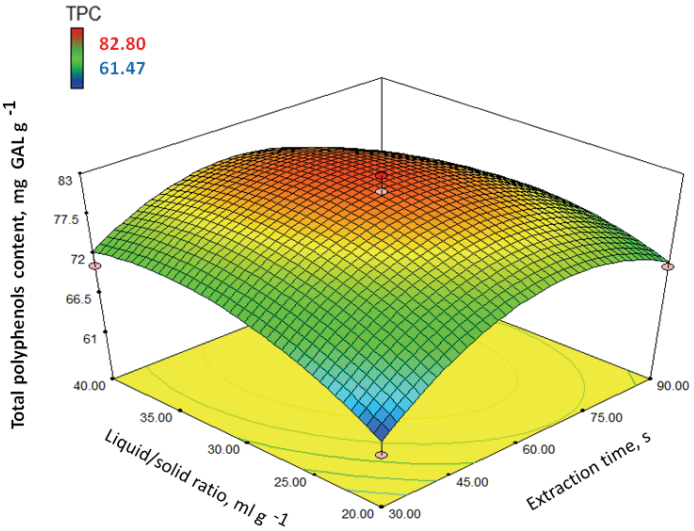

Fig 1. Contour plot of the combined effects of extraction time and liquid/solid ratio on the total content of polyphenols.

The total polyphenols and nicotine contents increased with increasing extraction time from 30 s to 60 s, and decreased when the values of this parameter ranged from 60 s to 90 s. Such a behaviour related to the duration of the extraction time, *i.e.*, decreasing TPC and nicotine contents when the sample was extracted longer than 60 s, may be explained by the effect of microwaves on bioactive compounds. More precisely, the relatively high wattage and high temperature during the longer exposure of the sample could disrupt the structure of polyphenols and nicotine and thus reduce the extraction efficiency.[14,18]

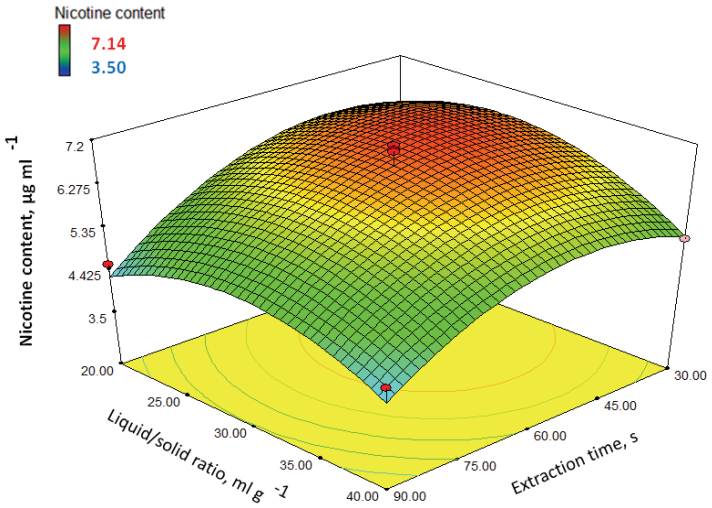

Fig 2. Contour plot of the combined effects of extraction time and liquid/solid ratio on the total content of nicotine.

When the parameter liquid/solid ratio was considered, both responses showed similar behaviour. The total polyphenols and nicotine content increased with increasing value of this parameter in the range of about 25–35 mL g^{-1} (Figs. 1 and 2). This means that the sample was present in the optimal amount for the volume of the solvent, thereby allowing the extraction of the maximal quantities of the bioactive compounds. Lower values of the liquid/solid ratio probably mean that insufficient solvent volume was added to the reaction system and thus was disabled to exploitation of the whole solid sample and extract more compounds of interest. On the other hand, higher values of liquid/solid ratio (greater than 35 ml g^{-1}) may result in solid particles aggregation, disabling solvent access to these parts of the sample and hindrance of the extraction process.

The extraction of the bioactive compounds of interest was performed distilled water because the applied analytical method for the determination of the nicotine content is sensitive to solvents such as ethanol, methanol and acetone. Previously in the literature, the bioactive compounds were extracted using various solvents, *i.e.*, water, ethanol, methanol, chloroform, dichloromethanol, diethyl ether and acetone. The efficiency of the extraction method mainly depends on the choice of the solvent.[19]

According to literature, different lignocellulosic materials were used in the extraction process of polyphenols. Singh and co-workers[20] extracted polyphenols from soybean crop (fermented and unfermented by *Trichoderma harzianum* NBRI-1055) using both water and methanol as the solvent. A maximum *TPC* of 181.32 mg GAL g^{-1} was obtained for the fermented sample employing water as solvent. In unfermented samples, using water and methanol, the maximum *TPC*

was 61.16 and 67.14 mg GAL g^{-1} of extract, respectively.[20] Upadhyay and co--workers[18] investigated the MAE for the isolation of polyphenols from coffee. They achieved optimal conditions with an extraction time of 5 min for the maximum response, which is 5-fold longer than determined in the present study. They presented a range of 12–24 mg GAL g^{-1} in aqueous extracts under the estimated extraction conditions, while the *TPC* in alcoholic extracts was even lower and ranged of 10–17 mg GAL g^{-1}. The applied power of microwave was also higher.[18]

In the study of Ruiz–Rodriguez,[21] the extraction of nicotine from tobacco leaves was performed by supercritical fluid extraction, and the content ranged from 0.01–0.05 mg mg^{-1} of extract.[21] In the present study, the nicotine content was lower and ranged from 0.007–0.014 mg mg^{-1} of extract, but it should be noted that the MAE was realized using tobacco waste and not tobacco leaves. Optimization of nicotine extraction from tobacco leaves using diethyl ether and petroleum ether as solvent was performed by Mulyadi and co-workers.[22] They also used RSM for the optimization of the process and obtained nicotine yield of about 5.43 %, which is higher than in the present aquatic extract (0.014 mg mg^{-1} of extract or 1.42 %). The lower value of the nicotine content of extracts in this study was justified because the extraction of nicotine was made using tobacco residues from which some amounts of nicotine had previously been extracted by consuming tobacco units.

Effect of bacterial fermentation on the extraction the polyphenols and nicotine

After the optimization of the extraction process, the effect of fermentation by *Streptomyces fulvissimus* CKS7 on the extraction of polyphenols and nicotine was examined. The results for the TPC and nicotine content after bacterial fermentation of tobacco waste are presented in Table IV.

TABLE IV. *TPC* and nicotine content in extracts after tobacco waste fermentation by *Streptomyces fulvissimus* CKS7; Y_1: total polyphenols content, mg GAE g^{-1}, Y_2: nicotine content, μg ml^{-1}, Y_{1F}: total polyphenols content after fermentation, Y_{2F}: nicotine content after fermentation

Run	Variable		Response			
			Before fermentation		After fermentation	
	Extraction time, s	Liquid/solid ratio, ml g^{-1}	Y_1 mg GAE g^{-1}	Y_2 μg ml^{-1}	Y_{1F} mg GAE g^{-1}	Y_{2F} μg ml^{-1}
1	30	20	59.66	6.07	60.47	7.21
2	90	20	71.27	4.49	67.14	6.96
11	60	30	82.43	7.14	74.80	7.26

In all tested samples, after the fermentation of tobacco residues by strain CKS7, the TPC was slightly lower and decreased from 1.65 to 10.70 %, while the content of nicotine was higher from 1.61 to 34.22 %. During the fermentation,

the compositional characteristic of the substrate has been altered by the action of microorganisms and it could be the reason of decreasing of *TPC* in extracts.

It could be presumed that the microorganism used polyphenols from the substrate for its own growth or caused the breakdown of the molecules by some of its metabolic activities. In the literature, there are contrary cases where the *TPC* increase or decrease after certain fermentation.[23,24] On the other hand, the nicotine content was increased after the fermentation, probably due to the action of the microorganism releasing some nicotine forms bound to other molecules.

CMCase and avicelase production during solid-state fermentation

The production of CMCase and avicelase by the bacterial strain *Paenibacillus chitinolyticus* CKS1 during solid-state fermentation using fermented and unfermented tobacco residues as a substrate was analyzed. This strain was selected because of their already proven cellulolytic activity.[25] The results are presented in Table V.

TABLE V. Cellulase production by *Paenibacillus chitinolyticus* CKS1 using tobacco waste as a substrate

Substrate sample	Enzyme activity, U g^{-1}	
	CMCase	Avicelase
Tobacco waste	0.548±0.046	0.817±0.032
Tobacco residues[a] – unfermented	0.864±0.038	1.050±0.036
Tobacco residues[a] – fermented by CKS7	0.878±0.027	1.417±0.053

[a]Tobacco residues after extraction of polyphenols and nicotine

The application of tobacco residues without any prior treatment (extraction or fermentation) gave the lowest values of cellulase activity. Fermentation of tobacco residues resulted in the production of both enzymes, CMCase and avicelase (Table V). The strain CKS1 demonstrated the dominance of exoglucanase activity that reached a value of 1.417 U g^{-1}, while the maximum of CMCase activity was 0.878 U g^{-1}. The structure of tobacco residues during SSF was changed by the action of the bacterial strain CKS7. This probably made the substrate more accessible for CKS1, due to the activity of various enzymes from CKS7 and enabled the increased production of cellulase.[26] In addition, the extraction of value-added compounds from tobacco improved the production of cellulase, in comparison with untreated residues.[27] The elimination of polyphenolic compounds probably reduced their negative impact on the manifestation of cellulase activity.[28,29]

In the literature, various species from genus *Paenibacillus* have been employed for the production of cellulases using both commercial and agricultural waste material.[30,31] The strain CKS1 also produced CMCase and Avicelase using commercial and agricultural waste material, such as medicinal herbs waste, sawdust and barley bran. The maximum obtained cellulase activity using barley

bran was 0.405 U mL^{-1} for CMCase and 0.433 U mL^{-1} for avicelase activity,[32] while the use of herbs waste gave 1.94 U mL^{-1} of avicelase activity.[16]

CONCLUSIONS

Valorisations of inexpensive raw materials, especially of agricultural origin, for the production of valuable biotechnological products are recent trends. The compounds extracted from tobacco waste with added value, polyphenols and nicotine, could be further processed in some commercial pharmaceutical products, while the less toxic tobacco residues could be utilized in the production of enzymes. The application of fermentation as a pre-treatment of tobacco waste with *Streptomices fulvissimus* CKS7 improved the extraction of nicotine and the production of cellulase. *Paenibacillus chitinolyticus* CKS1 expressed great potential for tobacco waste utilization, by using it for its own growth and metabolic activity and thereby producing a considerable amount of the cellulase.

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ИЗВОД

ПРОИЗВОДЊА ЦЕЛУЛАЗА ИЗ ОТПАДНОГ ДУВАНА ЗАОСТАЛОГ НАКОН ЕКСТРАКЦИЈЕ ПОЛИФЕНОЛА И НИКОТИНА И БАКТЕРИЈСКОГ ПРЕДТРЕТМАНА

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У различитим индустријама, агоиндустијски нуспроизводи настају у великим количинама и представљају проблем по питању њиховог одлагања. Искоришћење отпадног дувана за екстракцију компонената са додатном вредношћу и производњу ензима, може утицати на смањење количина насталог отпада и цену производње целулаза. Колико је познато, до сада у литератури нису забележени покушаји искоришћења остатака отпадног дувана (након екстракције полифенола и никотина и предтретмана ферментацијом помоћу *Streptomyces fulvissimus* CKS7) за производњу целулаза (ЦМЦаза и авицелаза) помоћу *Paenibacillus chitinolyticus* CKS1. Оптимални услови за екстракцију полифенола и никотина добијени су помоћу методологије одзивне површине: време екстракције од 60 s и однос течно/чврсто од 30 ml g^{-1}. Након примене бактеријске ферментације као предтретмана отпадног дувана, екстракција полифенола се смањила за 10 %, а екстракција никотина се повећала за 35 %. Након тога постигнуте су максималне активности целулаза (ендоглуканаза од 0,878 U g^{-1} и егзоглуканаза од 1,417 U g^{-1}), коришћењем соја CKS1.

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