**Extraction of polyphenols and nicotine and the production of cellulase using tobacco waste**

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*Abstract*:

The agricultural by-products are generated in large amounts in various industries, creating a serious disposal problem. Valorization of tobacco waste for the extraction of value-added compounds and the production of enzyme can reduce both, the problems of its disposal and the costs of cellulase production.

Up to now, there were no reported papers about the utilization of tobacco residues (after extraction of polyphenols and nicotine, and fermentation pretreatment by *Streptomyces fulvissimus* CKS7) for the production of cellulase (CMCase and Avicelase) by *Paenibacillus chitinolyticus* CKS1. The optimal conditions of the polyphenols and nicotine extraction process were obtained using Response surface methodology: 60s of extraction time in water and 30 ml g-1 of liquid/solid ratio. After the applied bacterial fermentation as a pretreatment of tobacco residues, using the *Paenibacillus* species, the extraction of polyphenols decreased up to 10%, while the extraction of nicotine increased up to 35%. Afterward, the maximum of cellulase activities (CMCase of 0.878 U g-1and Avicelase of 1.417 U g-1) were achieved by using of the strain CKS1.

*Keywords:*

lignocellulosic waste; microwave-assisted extraction; solid-state fermentation; CMCase and Avicelase activity.

RUNNING TITLE: VALUE-ADDED PRODUCTS FROM TOBACCO WASTE

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 the high carbon (C) and nicotine content1. 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 environment. Cigarette companies have to pay for its disposal and the majority of the waste is destroyed by burning1,2. The utilization of tobacco residues has the potential of significant environmental and economic benefits, as source of bioactive compounds instead of generation of the problems which may be caused by its disposal.

Tobacco waste has potential applications for soil amendment and the production of tailored organic fertilizer and desulfurization adsorbents3,4. In addition, it can be utilized for the extraction of valuable compounds such as polyphenols and nicotine, as well as for the production of various enzymes during fermentation process1,5. The concentration of chlorogenic acid and rutin is the highest among the polyphenol compounds in tobacco leaves6. The extraction of solanesol from tobacco leaves also produces large quantities of residues that still contain the polyphenol compounds7.

Various efficient and advanced extraction techniques have been developed for extracting of phenolic compounds, such as pressurized liquid extractor, microwave-assisted extraction (MAE), ultrasound-assisted extraction, soxhlet extraction and heat reflux extraction, as well as supercritical fluid extraction8. Among these treatments, MAE is a relatively new and promising green extraction method. This treatment is considered as an efficient because of reducing of both of the extraction time and the solvent consumption by rapid heating of 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 compounds of interest. In addition, it is necessary to optimize the extraction process parameters to maintain the maximum amount of bioactive compounds in the extracts obtained8-10.

In addition, various lignocellulose residues, including tobacco waste, can 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 fermentation process conditions11-12.

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 using 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 has been investigated. Afterward, the exhausted material has been utilized for the production of cellulose (Avicelase and CMCase) by soil bacterium *Paenibacillus chitinolyticus* CKS1.

EXPERIMENTAL

***Materials***

*Tobacco material*

Tobacco waste material - tobacco residues from specially designed heated tobacco units (manufactured from the company Philip Morris International, Switzerland), was collected. The tobacco units were heated by IQOS electronics, where the tobacco was just enough heated to release a nicotine-containing vapor, but without burning the tobacco13. This residue was dried for 24 h using an oven at 37°C and milled (IKA® A11 basic Analytical mill) to a particle size ranged from 0.063 to 0.1 mm (passed through a sieve with this diameter) and further was used as a low-cost material for nicotine and polyphenol extraction and bacterial solid-state fermentation.

*Chemicals*

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. 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. Cellulase activity was done 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 by 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*

Polyphenols and nicotine extraction was performed using household microwave oven (LG MC7849HS), with distilled water as a solvent. Batch experiments were done in 100 ml Erlenmeyer flasks, with tobacco waste and different liquid/solid ratio and with a predetermined time of extraction. The microwave oven was set at 180 W. After extraction, the mixture was filtrated 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 1](https://ezproxy.nb.rs:2055/science/article/pii/S1383586613004589%22%20%5Cl%20%22t0010).

Table 1. Independent variables and their levels employed in a Central Composite Design for the optimization of extraction of polyphenols and nicotine from tobacco waste.

|  |  |
| --- | --- |
| **Independent variable** | **Values** |
| **-α** | **-1** | **0** | **1** | **+α** |
| Extraction time, s | 17.6 | 30 | 60 | 90 | 102.4 |
| Liquid/solid ratio, ml g-1 | 15.9 | 20 | 30 | 40 | 44.1 |

***Pretreatment by bacterial fermentation***

Three points from design (run 1, run 2 and run 11, Table 2) were randomly selectedfor bacterial SSF experiments and evaluation of their effect on the extraction of polyphenols and nicotine. After autoclaving the samples of tobacco residues (which were moistened by distillate water in a ratio of 1:4 (w/v)), the 10 % inoculums of *Streptomyces fulvissimus* CKS7 was 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 *Polyphenols and nicotine extraction* section) and compared with nonfermented samples.

***Cellulase production***

After extraction of polyphenols and nicotine, solid residues of tobacco sample material (fermented and unfermented) was collected, dried and used as a substrate for the production of cellulase by *Paenibacillus chitinolyticus* CKS1. The enzyme production was carried out by adding 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 done in triplicate.

***Analytical methods***

*Determination of total polyphenols content*

The total polyphenols content (TPC) was determined by Folin–Ciocalteu method with a slight modification14. In the test tube, the 0.1 ml of the extract (with a dry matter concentration of 10 mg ml-1) was mixed with 0.5 ml of Folin–Ciocalteu reagent and 6 ml of distilled water. Then, 2 ml of 15% Na2CO3 solution and 1.4 ml of distilled water were added. The absorbance was measured after 2 h, at 750 nm, with the blank, that was simultaneously prepared and distilled water was used instead of the extract sample. The results were expressed as gallic acid equivalents (GAE) through the calibration curve of gallic acid (1–1500 μg ml-1).

*Determination of nicotine content*

The nicotine content was determined spectrophotometrically according to Al-Tamrah method15. In the volumetric flask, 0.5 ml of potassium permanganate (0.0125 M) was swirled gently with 1 ml of sodium hydroxide (6.25 M). After adding of 0.1 ml of the 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 distillate water instead of extract sample). The results were expressed according to the calibration curve of nicotine concentration of 0.1 to 7.5 μg ml-1.

*Enzyme assay*

Cellulase activity (CMCase and Avicelase activity) was determined according to DNS method16 using of 1% (w/v) CMC or Avicel solution in tri-sodium citrate buffer (0.1 M, pH 4.8). The mixtures of enzyme sample and CMC or Avicel solution in ration 1:1 (v:v) were incubated 30 min at 50°C (CMCase) and 80°C (Avicelase)17. Reaction was stopped by adding of 1 ml of DNS reagent. By cooking, 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 was defined as the amount of enzyme that released 1 μmol of glucose equivalents per minute.

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 two independent parameters was conducted (Table 2).

Table 2. The values of the variables and the responses in the Central Composite design

|  |  |  |
| --- | --- | --- |
| **Run** | **Variables** | **Responses** |
|  | Extraction time, s | Liquid/solid ratio, ml g-1 | ***Y1* /**mg GAE g-1 | ***Y2* /** 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 |
| ***Y1***: total polyphenols content, ***Y2***: nicotine content. |

The relationship between the responses and the two tested factors was designed as a second order response using applying a multiple regression analysis and presented by the two following equations (Eqs. [(1)](https://ezproxy.nb.rs:2055/science/article/pii/S001623611830543X%22%20%5Cl%20%22e0020), [(2)](https://ezproxy.nb.rs:2055/science/article/pii/S001623611830543X%22%20%5Cl%20%22e0025)):

*Y1*=81.02+1.34 *A*+2.07 *B*-2.50 *AB*-7.37 *A2*-4.24 *B2*(1)

*Y2*=6.73-0.75 *A*-0.25 *B*+0.20 *AB*-0.90 *A2*-0.83 *B2* (2)

where the *Y1* (total polyphenols content, mg GAE g-1 of extract dry matter) and *Y2* (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 coded values.

It was found that the quadratic model is the most suitable model for both of the responses. The results of the analysis of variance ANOVA are presented in [Table 3.](https://ezproxy.nb.rs:2055/science/article/pii/S1876107013003295#tbl0010) Significant model terms for the TPC response are *B*, *AB*, *A*2 and *B*2, while for the response of nicotine content are *A*, *A2* 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 (*Y1*) and 16.277 (*Y2*)) 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 3). [Regression coefficient](https://ezproxy.nb.rs:2055/topics/earth-and-planetary-sciences/regression-coefficients) R-squared (R2) of 0.945 (Y1) and 0.921 (Y2) indicates a good correlation between the actual (experimental) and predicted values of responses.

Table 3. ANOVA for RSM parameters fitted to polynomial equation; *A-* Extraction time; *B*- Liquid/solid ratio; ***Y1***: Total polyphenols content; ***Y2***: nicotine content

|  |  |
| --- | --- |
| **Sourse** | **Response** |
| ***Y1* /** mg GAE g-1 of extract | ***Y2* /** μg ml-1 |
| p-value Prob > F | p-value Prob > F |
| 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 |
| *A2* | 0.0001**s** | 0.0008s |
| *B2* | 0.0011**s** | 0.0012**s** |
| Lack of fit | 0.2295**ns** | 0.6722**ns** |
| **ns**-not significant - "Prob > F" > than 0.050**s**-significant- "Prob > F" < than 0.050  |
|  |

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

The effects of process parameters on the extraction of polyphenols and nicotine are shown in Fig. 1 and 2. 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 2).



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



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

Total polyphenols and nicotine content were increasing with an increase of the extraction time from 30 s to 60 s, and decrease when the values of this parameter ranged from 60 s to 90 s. Such a behavior related to the extraction time duration, meaning to the decrease of TPC and nicotine when the sample was extracted longer than 60 s, may be explained by the microwave effect 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 efficiency14,18.

When the parameter liquid/solid ratio was observing, both responses were also shown similar behavior. Total polyphenols and nicotine content were increasing with increase of this parameter in the range of about 25-35 ml g-1 (Fig.1 and 2). That means that the sample is in the optimal dose with the volume of the solvent, allowing the extraction of maximal quantities of bioactive compounds. Lower values of liquid/solid ratio probably mean that insufficiently solvent volume was added in the reaction system, thus was disabled to exploit the whole solid sample and extract more compounds of interest. On the other side, higher values of liquid/solid ratio (greater than 35 ml g-1) may be referred to solid particles aggregation, disabling the solvent access to these parts of the sample and hindering the extraction process.

The extraction of bioactive compounds of interest was done in distillate 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: water, ethanol, methanol, chloroform, dichloromethanol, ether and acetone. The efficiency of the extraction method mainly depends on the choice of the solvents19.

According to literature, different lignocellulosic materials were used in the extraction process of polyphenols. Singh and co-workers20 extracted polyphenols from soybean crop (fermented and unfermented by *Trichoderma harzianum* NBRI-1055) using both water and methanol as solvent. Maximum of TPC of 181.32 mg GAL g-1 was obtained for the fermented sample and with employing of water as solvent. In unfermented samples, using water and methanol, maximum of TPC was 61.16 mg GAL g-1 and 67.14 mg GAL g-1 of extract20. Upadhyay and co-workers18 investigated the MAE for the isolation of polyphenols from coffee. They achieved optimal conditions during the extraction time of 5 min for the maximum response, which is 5 fold longer than shown in this study. They presented the range of 12–24 mg GAL g-1 in aqueous extracts under the estimated extraction conditions, whereas the TPC in alcoholic extracts was even lower and ranged of 10–17 mg GAL g-1. The applied power of microwave was also higher18.

In the study of Ruiz-Rodriguez21 the extraction of nicotine by supercritical fluid extraction from tobacco leaves was performed, and the content was ranged from 0.01 -0.05 mg mg-1 of extract21. In this study, the nicotine content was lower and ranged from 0.007 - 0.014 mg mg-1 of extract, but it should be noted that MAE was done using tobacco waste, instead of tobacco leaves. Optimization of nicotine extraction from tobacco leaves using ether and petroleum ether as solvent was performed by Arie Febrianto Mulyadi and co-workers22. They also used RSM for the optimization of the process and obtained nicotine yield of about 5.43%, which is higher than in our 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 were previously extracted by consuming tobacco units.

*Effect of bacteria fermentation on polyphenols and nicotine extraction*

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 of TPC and nicotine content after bacterial fermentation of tobacco waste are shown in Table 4.

**Table 4.** TPC and nicotine content in extracts after tobacco waste fermentation by *Streptomyces fulvissimus* CKS7

|  |  |  |
| --- | --- | --- |
| **Run** | **Variables** | **Responses** |
|  |  |  | **Before fermentation** | **After fermentation** |
|  | Extraction time, s | Liquid/solid ratio, ml g-1 | ***Y1* /** mg GAE g-1 | ***Y2* /** μg ml-1 | ***Y1F* /** mg GAE g-1 | ***Y2F* /** 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 |
| ***Y1***: total polyphenols content, mg GAE g-1,***Y2***: nicotine content, μg ml-1,***Y1F***: total polyphenols content after fermentation, ***Y2F***: nicotine content after fermentation. |

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. There is a presumption 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 the opposite cases where TPC increase or decrease after certain fermentation23,24. On the other hand, nicotine content was increased after the fermentation, probably due to the microorganism action of releasing of some bonded nicotine forms with other molecules.

*CMCase and Avicelase production during solid-state fermentation*

The production of CMCase and Avicelaseby 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 activity25, and the results are presented in Table 5.

**Table 5.** 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\*-unfermented | 0.864±0.038 | 1.050±0.036 |
| Tobacco residues\*fermented by CKS7 | 0.878±0.027 | 1.417±0.053 |
| \*Tobacco residues after extractionof polyphenols and nicotine |

The application of tobacco residues without any prior treatment (extraction or fermentation) gave the lowest values of cellulase activity. Fermented tobacco residues favored the production of both enzymes, CMCase and Avicelase (**Table 5**). Strain CKS1 demonstrated the dominance of exoglucanase activity that reached the activity 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 has probably made the substrate more accessible for CKS1, due to the activity of various enzymes from CKS7 and enabled the increased production of cellulase26. Also, the extraction of value-added compounds from tobacco improved the production of cellulase, in comparison with untreated residues27. 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* were employed for the production of cellulases using both, commercial and agricultural waste material30,31. Also, strain CKS1 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 activity32, while the use of herbs waste gave 1.94 U ml-1 of Avicelase activity16.

CONCLUSION

Valorizations of inexpensive raw materials for production of valuable biotechnological products, especially from agricultural origin, are recent trends. Extracted compounds with added value, polyphenols and nicotine can be further processed in some commercial pharmaceutical products, while less toxic tobacco residues can be utilized in the production of enzymes. The application of fermentation as pretreatmentof tobacco waste with *Streptomices fulvissimus* CKS7 improved the extraction of nicotine and the production of cellulase. The *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 enzyme.

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И З В О Д

ЕКСТРАКЦИЈА ПОЛИФЕНОЛА И НИКОТИНА И ПРОИЗВОДЊА ЦЕЛУЛАЗА ИСКОРИШЋЕЊЕМ ОТПАДНОГ ДУВАНА

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У различитим индустријама, агоиндустијски нус-производи 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 секунди 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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