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Editors
Radoje V. Pantovic
Zoran S. Marković

EcoIst '15

Hotel "PUTNIK", Kopaonik, SERBIA
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TECHNICAL FACULTY BOR

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SUGAR BEET PULP AND MOLASSES AS A SOLID STATE FERMENTATION MEDIA FOR CELLULASE PRODUCTION BY *Paenibacillus chitinolyticus* CKS1

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ABSTRACT

Sugar beet pulp (SBP) and molasses, by-products from sugar industry were used as a substrate for cellulase production in this study. Solid state fermentation (SSF) was performed by a natural isolate *Paenibacillus chitinolyticus* CKS1. Data showed that cellulose in SBP could be used as a substrate to produce both cellulases, CMCase and Avicelase. An optimum solid: moisture ratio for maximum cellulase production was investigated. Maximum CMCase 3.159 U/g and Avicelase activity 4.840 U/g was obtained at the fourth day of incubation with 10% of inoculum. The cellulase production during SSF on SBP indicates that this process is economically very justified.

Key words: Cellulases, Sugar beet pulp, Molasses, *Paenibacillus chitinolyticus* CKS1.

INTRODUCTION

Sugar beet pulp (SBP) and molasses, the two main by-products of the sugar industry, are produced in large amounts annually which creates disposal problems [1, 2]. SBP is a lignocellulosic by-product with low economic value, mostly used as animal feed [3, 4] is mainly composed of polysaccharides consisting of approximately (dry basis) 22–24 wt.% cellulose, 30 wt.% hemicelluloses and 15–25 wt.% pectin [5]. Molasses from the sugar beet processing contain up to 54% sugars [1]. Sugarcane molasses are used as nutrient medium for the cellulase production [6] while literature data about using sugar beet molasses are very limited. SBP is used as a substrate for microbial protein production [1] and in a recent years for ethanol production [7].

The cellulase production on SBP and molasses by *Paenibacillus* sp. is a new approach. Cellulases are industrially important enzymes with a potential to convert cellulose into fermentable sugars which can then be converted into value-added products or bioenergy [8]. Cellulases are enzymes that hydrolyze cellulose while the mechanism of enzymatic activity differs between the different enzyme classes: endoglucanases

(carboxymethyl cellulases; CMCases), exoglucanases (Avicelases) and β-glucosidases [9-11]. Endoglucanases cut the amorphous cellulose polysaccharide chain at random internal sites and generate oligosaccharides of various lengths [12]. Exoglucanases are active on the reducing or non-reducing ends of the cellulose polysaccharide chains and liberate either glucose or cellobiose as major products [12].

Solid state fermentation (SSF) is used for cellulase production because of its several benefits like high enzyme titer, low labor cost, lower capital input, etc.[13].

In this research we used SBP and molasses, for the cellulase production by a novel strain *Paenibacillus chitinolyticus* CKS1. SSF was applied for cellulose production including both CMCase and Avicelase. Due to the application of SBP and molasses as a cheap and abundant substrates for the enzyme production, it may be considered that the economic aspect of this method is very promising.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Paenibacillus chitinolyticus CKS1 was natural isolate from soil sample (sequence accession number KP715850). The inoculum was prepared by growing the microorganism in 300 ml Erlenmeyer flask with 50 ml of ISP1 broth containing 3 g/l yeast extract and 5 g/l casein hydrolisate. The medium was inoculated at 30 °C for 24 hours in a rotary shaker at 150 rpm and was used for the fermentation process.

Fermentation substrates

SBP (Fibrex 620, Nordic Sugar, Denmark) and molasses (ethanol Factory Alpis, Kovin, Serbia) were used as fermentation substrates. SBP was grounded in a mortar and pestle to a particle size of 800μm -2mm. Molasses was diluted in distilled water to a concentration of 2%.

Solid state fermentation - SSF

Solid state fermentation was carried out according to Moftah et al. [14] with some modifications. 5 g of SBP and molasses as a moistening agent was placed into the 300 ml Erlenmayer flask Solid (SBP):moisture ratio was adjusted as follows - 1:1 (4.5 ml molasses), 1:2 (9.5 ml molasses) and 1:3 (14.5 ml molasses). The media in flasks were autoclaved at 121 °C for 20 min. In each flask 10% of inoculum (v/w) was added. Fermentation was carried out at 30 °C for 4 days. To extract the enzyme, a known quantity of the fermented media was mixed with 0.1 M acetate buffer pH 4.80 (1:5, w/v) by shaking on a rotary shaker (190 rpm, 30 min, 25 °C); then, the whole contents were centrifuged at 6.000 rpm for 10 min (4 °C), and the supernatant was used as a crude enzyme extract and was further analysed for cellulase activity.

For solid:moisture ratio 1:1, which shown the maximum cellulase production, the effect of different concentrations of inoculum (5, 10 and 15 % (v/w)) and different time of incubation (2, 3, 4, 5 and 6 days) on cellulase production was studied.

Enzyme assay for cellulase

Cellulase activity was measured by reduction of 3,5-dinitrosalicylic acid in the presence of glucose released by enzymatic hydrolysis of cellulose [15].

CMCase (endogluconase) activity was determined as follows: 500 µl of enzyme solution (crude supernatant) was mixed with 500 µl 1 % CMC in 0.1 M acetate buffer pH 4.80 and incubated at 50°C for15 min in a rotary shaker at 150 rpm. After incubation, 1 ml of DNS reagent was added. The reaction mixture was then boiled for 15 min. After cooling at room temperature 5 ml of distilled water is added to each tube and absorbance was measured at 540 nm against the blank. One unit of CMCase activity was defined as the amount of enzymes that released 1 µmol of glucose per minute. It is expressed as U/g where *g* is the gram of solid substrate (SBP) used.

Avicelase (exoogluconase) activity was determined at 80 °C using the same procedure as for CMCase with the exception that 1% Avicel was used as substrate instead of CMC.

RESULTS AND DISCUSSION

Investigation of cellulase production during growth of *P. chitinolyticus* CKS1 on SBP and molasses showed that microorganism produced cellulases, with different efficiency, at all tested solid:moisture ratio (Figure 1) .

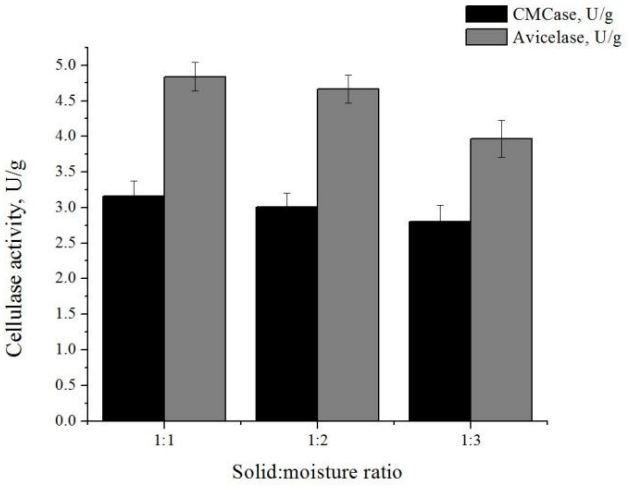

Figure 1. Cellulase production by P. *chitinolyticus* CKS1 on SBP an molasses with different solid:moisture ratio

The literature data shows that the production of cellulases is inducible and affected by the nature of the carbohydrate used during fermentation [16]. SBP contains cellulose that can be an inducer for the cellulase production. SBP is used as a substrate for cellulase production by fungi *Trichoderma* [17, 18] but until now there has been no literature data about cellulase production using SBP by *Paenibacillus* sp. *P.*

chitinolyticus CKS1 produced CMCase and Avicelase after four days of incubation at different solid:moisture ratio. For the strain CKS1, the optimum solid: moisture ratio, during SSF, appeared to be 1:1 for maximum CMCase 3.159 ± 0.213 U/g and Avicelase activity 4.840 ± 0.201 U/g. The moisture content in the substrate can be considered as an important factor in SSF and microbial growth [19]. Low moisture content may lead to poor accessibility of nutrients and a lower degree of substrate swelling, resulting in poor microbial growth and decreased enzyme production. On the other hand, higher moisture contents appeared to cause decreased porosity, loss of particle structure and development of stickiness, which, in turn, prevented oxygen penetration [14]. For *Paenibacillus curdlanolyticus* DSMZ 10248 an optimum solid (palm kernel cake): moisture ratio was 1:1 and for *Paenibacillus polymyxa* ATCC 842 was 1:0.8 [19].

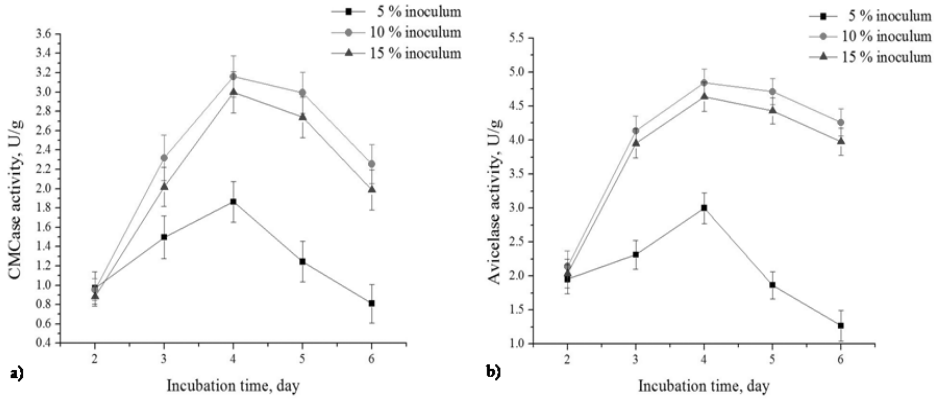

Figure 2. Cellulase production by *P. chitinolyticus* CKS1 using SBP during six days of incubation: a) CMCase activity and b) Avicelase activity.

The pattern of cellulase production, for both CMCase and Avicelase, indicates that the cellulase activity increased during the first four days, reached the maximum on day 4 and then decreased at the end of cultivation (Figure 2 a-b). The decrease of cellulolytic activity could be a consequence of changed conditions in the medium (pH change, production of inhibiting by- products), or due to the depletion of nutrients in the fermentation medium as seen for other bacterial strains [20]. The fourth day of incubation during SSF was found to be optimal for maximum CMCase production for other *Paenibacillus* sp. [19]. The importance of inoculum size with regard to microbial fermentation processes is generally accepted. There is a significant increase in cellulase production with an increase in inoculum concentration from 5 to 10 % and found to be maximum at 10% (Figure 2 a-b). Maximum CMCase activity 3.159 ± 0.213 U/g and Avicelase activity 4.840 ± 0.201 U/g was reached after four days of incubation with 10% of inoculum. Higher inoculum size 15 % resulted in a decrease in enzyme production. This demonstrates that inoculum density does not exert an unlimited effect on fermentation processes. High inoculum density can reduce enzyme production due to competition for available nutrients while low density can result in a reduce of enzyme secretion, owing to a drop in cell numbers [21].

CONLCUSIONS

SBP and molasses, as by-products from sugar industry could be a good substrate for cellulase production by P. *chitinolyticus* CKS1. During SSF, the optimum solid: moisture ratio appeared to be 1:1 for maximum cellulase activity. The maximum CMCase and Avicelase activity was reached at the fourth day of incubation with 10 % of inoculum. In addition, the use of SBP and molasses for the enzyme production is economically very suitable. The conversion of cellulose in SBS into fermentable sugars, could make these substrates suitable for the application in bioethanol production.

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REFERENCES

1. Nigam, P. and M. Vogel, Bioconversion of sugar industry by-products—molasses and sugar beet pulp for single cell protein production by yeasts. Biomass and Bioenergy, p. 339-345, 1991.
2. Nigam, P., Process selection for protein-enrichment: fermentation of the sugar industry by-products molasses and sugar beet pulp. Process Biochemistry, p. 337-342, 1994.
3. Olmos, J.C. and M.Z. Hansen, Enzymatic depolymerization of sugar beet pulp: Production and characterization of pectin and pectic-oligosaccharides as a potential source for functional carbohydrates. Chemical Engineering Journal, p. 29-36, 2012.
4. Kracher, D., et al., Fungal secretomes enhance sugar beet pulp hydrolysis. Biotechnology journal, p. 483-492, 2014.
5. Sun, R. and S. Hughes, Fractional extraction and physico-chemical characterization of hemicelluloses and cellulose from sugar beet pulp. Carbohydrate polymers, p. 293-299, 1998.
6. Goldbeck, R., et al., Cellulase production from a new strain Acremonium strictum isolated from the Brazilian Biome using different substrates. Bioresource technology, p. 797-803, 2013.
7. Rezi, T.I., I. Markovi, and R. Ludwig, Integrated hydrolyzation and fermentation of sugar beet pulp to bioethanol. Journal of microbiology and biotechnology, p. 1244-1252, 2013.
8. Rubin, E.M., Genomics of cellulosic biofuels. Nature, p. 841-845, 2008.
9. Kuhad, R.C., R. Gupta, and A. Singh, Microbial cellulases and their industrial applications. Enzyme research, p. 2011.
10. Bhat, M., Cellulases and related enzymes in biotechnology. Biotechnology advances, p. 355-383, 2000.

11. Kim, B.-K., et al., Purification and characterization of carboxymethylcellulase isolated from a marine bacterium,< i> Bacillus subtilis</i> subsp.< i> subtilis</i> A-53. Enzyme and Microbial Technology, p. 411-416, 2009.
12. Sadhu, S. and T.K. Maiti, Cellulase production by Bacteria: A Review. British Microbiology Research Journal, p. 235-258, 2013.
13. Singhania, R.R., et al., An integrative process for bio-ethanol production employing SSF produced cellulase without extraction. Biochemical Engineering Journal, p. 2015.
14. Moftah, O.A.S., et al., Adding value to the oil cake as a waste from oil processing industry: production of lipase and protease by Candida utilis in solid state fermentation. Applied biochemistry and biotechnology, p. 348-364, 2012.
15. Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, p. 426-428, 1959.
16. Kumar, R., S. Singh, and O.V. Singh, Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. Journal of industrial microbiology & biotechnology, p. 377-391, 2008.
17. Moosavi-Nasab, M. and M. Majdi-Nasab, Cellulase Production by Trichoderma reesei using Sugar Beet Pulp. Iran Agricultural Research, p. 107-116, 2008.
18. Olsson, L., et al., Influence of the carbon source on production of cellulases, hemicellulases and pectinases by Trichoderma reesei Rut C-30. Enzyme and Microbial Technology, p. 612-619, 2003.
19. Alshelmani, M.I., et al., Characterization of cellulolytic bacterial cultures grown in different substrates. The Scientific World Journal, p. 2013.
20. Karim, A., et al., Hyper production of cellulose degrading endo (1, 4) β-d-glucanase from Bacillus licheniformis KIBGE-IB2. Journal of Radiation Research and Applied Sciences, p. 2014.
21. Reddy, L., et al., Optimization of alkaline protease production by batch culture of< i> Bacillus</i> sp. RKY3 through Plackett–Burman and response surface methodological approaches. Bioresource technology, p. 2242-2249, 2008.

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