Evaluate of pumpkin oil cake as substrate for the cellulase production by *Penicillium roqueforti* in solid state fermentation

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Abstract

Evaluate of pumpkin oil cake as substrate for the carboxyl methyl cellulase (CMCase) production by Penicillium roqueforti in solid state fermentation was the main aim of this study. Comparisons were made for CMCase production using wheat bran (WB) and different oil cakes as substrates. WB served as the best carbon source for CMCase synthesis by P. roqueforti as it gave the highest enzymes activity (53.06 U gds^{-1}) (units per gram dried solid). Reasonably good quantities of enzyme were produced on mixed substrate (PuOC+WB) and on PuOC (pumpkin oil cake) as sole nutrient source, which were 48.49 U gds^{-1} and 37.07 U gds^{-1}, respectively. The results could be considered interesting as they indicated the suitability of PuOC as substrate in compare to WB. Knowing that the enzyme synthesis was related to the availability of moisture, variation in initial moisture content of substrate was investigated. Substrate with initial moisture of 44.44 %, showed the highest CMCase yield (36.0 U gds^{-1}) and was used for the further investigation. The influence of incubation time on the enzyme production during SSF was also evaluated. CMCase production increased progressively with incubation time and maximal activity was obtained after fifth days (59.9 U gds^{-1}).

Keywords: carboxyl methyl cellulase - *Penicillium roqueforti* - pumpkin oil cake - solid state fermentation.

Abbreviations

C	
carboxyl methyl cellulase	CMCase
P	
pumpkin oil cake	PuOC
S	
solid state fermentation	SSF
soybean cake	SBC
sunflower oil cake	SuOC
W	
water activity	a_w
wheat bran	WB

Introduction

Cellulases are a group of hydrolytic enzymes capable of degrading cellulose to the smaller glucose units. Cellulase is a complex enzyme having chiefly endoglucanase (EC 3.2.1. 4), exoglucanase (EC 3.2.1. 91) and β-glucosidase (EC 3.2.1. 21) [1]. Cellulase action is generally initiated by the random acting endoglucanase (carboxyl methyl cellulase – CMCase) at the amorphous region within cellulose chain to produce cellooligosaccharides [2]. Cellulasses account for approximately 20% of the world enzyme market [3]. The use of this enzyme has increased, continuously, especially in food (production of fruit and vegetable juices, oligosaccharides as functional food ingredients and low-calorie food substituents), brewery and wine industries [4, 5]. Indeed, the demand for this enzyme is growing more rapidly than ever before, and this demand has become the driving force for research on cellulases. Several studies are focused for cellulase use in the bioconversion of agro-industrial waste [6]. Some of them, such as rice husk, tea waste, wheat straw, bagasses, grapevine trimmings dust, wheat bran etc. are successfully used as substrates for the cultivation of microorganisms to produce the cellulase. However, wheat bran (WB) holds the key, and has most commonly been used in this enzyme production [7].

Oil cakes/oil meals present by-products in oil industry, obtained after oil extraction from the seeds. In recent years there has been an increasing the biotechnological interest on the utilization of these residues as substrates in biotechnological processes [8, 9]. They have been shown as suitable both carbon and nitrogen sources, and reported to be good substrate for enzyme production using fungal species in solid state fermentation (SSF). In search of oil cake application for cellulase production, Prasertsan et al., [10] find that palm oil cake is convenient substrate.

Pumpkin oil cake (PuOC) is a by-product obtained after oil extraction from dried pumpkin seed. Its only use is to feed animals and no other application has been found, yet. A large amount of this by-product is generally disposed of in open areas, leading to potentially serious environmental problems. Due to their composition (soluble sugars 1.10 %, crude proteins 63.52 %, crude fibers 4.50 %, lipids 8.66 % and trace amounts of minerals), pumpkin oil cake (PuOC) could also served as a good substrate for enzyme production. As it is known that soluble cellooligosacharides enter the cell and convert into an inducers that triggers cellulase induction [11] and that PuOC has a quite amount of soluble sugars it is to be expected that cellulase synthesis will be induced using this cake as substrate for enzyme production.

The objective of this study was to evaluate the potential of PuOC as a substrate for the production CMCase, using a GRAS strain of *P. roqueforti* in SSF. In this work compare of PuOC and some other oil cakes with, WB were examined. The effects of initial moisture of substrate and incubation time were, also, investigated.

Material and methods

Solid-state fermentation

SSF was carried out at WB, PuOC and soybean cake (SBC) as sole substrate and also at the mixed substrate, made of a oil cake (PuOC, SBC and sunflower oil cake (SuOC)) and

WB, in combination (1.5:1), for enzyme production. A mass of 5g of the dry substrate was introduced in 300 ml Erlenmeyer flask, moistened with required amount of salt solution containing 0.2 % (w/v) KH_2PO_4, 0.1 % (w/v) $MgSO_4$ and 0.1 % (w/v) NaCl. The contents were sterilized by autoclaving at 121 ˚C for 20 min. After cooling, the flakes were inoculated with 1 ml of spore suspension of *Penicillium roqueforti* and incubated at 28 ˚C and samples were collected from the shaker fourth days of cultivation for analysis.

For further experiments sole PuOC was used. Samples were prepared at the same way as earlier and were collected from the shaker at regular interval of 24 h (every day from the beginning of the process) for analysis.

To extract the enzyme form fermented substrate, a known quantity of medium was mixed with 0.1 % (v/v) Tween 80 by shaking on a rotary shaker (180 rpm, 1h); then the whole contents were centrifuged at 10 000g for 15 min (4 ˚C) and the supernatant was used as crude enzyme extract.

Effect of initial moisture and water activity

In order to elucidate the effect of water availability and substrate swelling, different moisture levels were tested (44–50 %, on dry weight basis). In all cases PuOC was used as the solid substrate. The mineral medium containing 0.2 % (w/v) KH_2PO_4, 0.1 % (w/v) $MgSO_4$ and 0.1 % (w/v) NaCl was used as the moistening agent.

Water activity (a_w) was measured by "Testo 650" (Testo, Germany). In order to measure a_w value, substrate fills half of the container. The time of adjustment takes approx. 30 min at constant temperature, depending on substrate need to be measured.

Enzyme assay

Carboxyl methyl cellulase activity was assayed according to the procedure reported by Rodionova et al. [12] using cellulose as a substrate. One unit of cellulase activity was defined as the amount of enzyme that produced 1 micromol of glucose per s at pH 5.0 and 50°C.

Analytical methods

Protein content was determined by the method of Lowry (13), with BSA as standard. Concentration of reducing sugars was examined by DNS method by Miller (14). The results were expressed as glucose using a calibration curve.

Results and discussions

First step in this work was to evaluate PuOC as possible substrate for CMCase production, comparing its possibility with some other oil cakes and with WB. The results obtained are shown in Fig. 1. Evidently WB served as the best carbon source for CMCase synthesis by *P. roqueforti* as it gave the highest enzymes activity (53.06 U gds^{-1}). Reasonably good quantities of enzyme were produced on mixed substrate (PuOC+WB) and on PuOC as sole nutrient source, which were 48.49 U gds^{-1} and 37.07 U gds^{-1}, respectively. Although other substrate also resulted in enzyme production, the quantities were less then with WB and PuOC. The results could be considered interesting as they indicated the suitability of PuOC as substrate in compare to WB, which has generally been considered an ideal substrate for enzyme production in SSF (7).

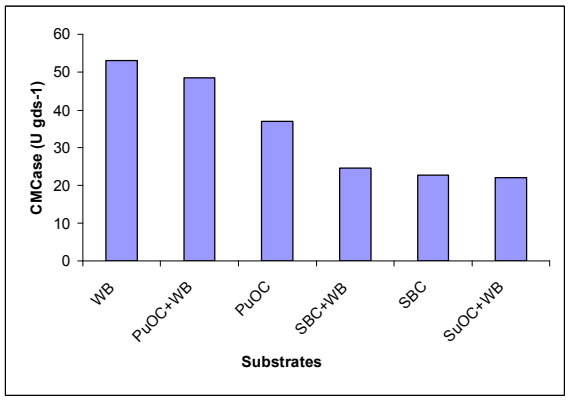

Figure 1. CMCase production on different substrates.

WB – wheat bran, PuOC– pumpkin oil cake, SBC – soybean cake, SuOC – sunflower oil cake

Next step was to investigate the effect of initial moisture of PuOC and the effect of incubation time on CMCase production in SSF. Substrate moisture is a crucial factor in SSF and its importance for enzyme production has been well established. Variation in initial moisture content of substrate showed that the enzyme synthesis was related to the availability of moisture. Substrate with initial moisture of 44.44 %, had the highest CMCase yield (36.0 U gds^{-1}) and was used for the further investigation.

Table 1. The effect of initial moisture on the production of CMCase by *P. roqueforti* on PuOC

w (%)	a_w	CMC (U gds^{-1})
44.44	0.932	36.0
50	0.962	25.7
54.5	0.974	23.8

During the time course study, CMCase activity was detected. Fig. 2. shows that CMCase production, increased progressively with incubation time and maximal activity was obtained after fifth days (59.9 U gds^{-1}). When the CMCase activity reached maximum, amount of reducing sugars was the highest (24.2 mg gds^{-1}), too. In the beginning of fermentation, a slight decline in reducing sugars was observed. After the second day, the reducing sugars increased due to degradation of cellulose from the medium by synthesized CMCase.

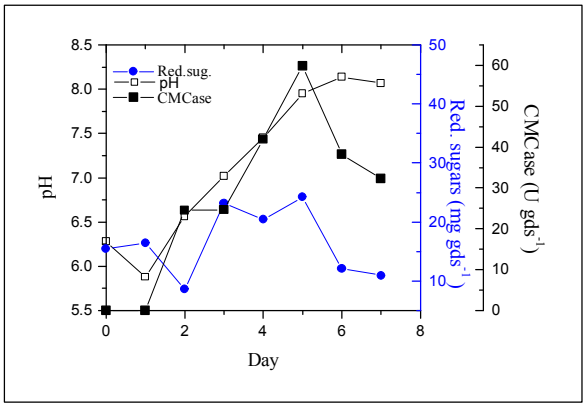

Figure 2. Time courses of CMCase production, reducing sugars and pH during SSF of PuOC by *P. roqueforti*

During the fermentation, the pH increased from 6.25 to 8.14, which was obtained on 6th day of cultivation. The increase of medium pH corroborated other data for enzyme production from *Penicillium* spp. [15, 16, 17) Alkalinization of the medium in the fermentative processes has generally been associated with ammonia release, resulting from protein metabolism breakdown, and the main mechanism is likely to be the oxidation of amino acids during their utilization as energy sources [18, 19]. Other possible alkali-generating metabolic reactions include the uptake and oxidation of the anions of organic acids. Considering that proteolytic activity in the media was detectable (result not shown) and that protein and amino acids were major carbon and energy sources in these fermentations, it is likely that protein hydrolisate is involved in this alkalization.

Conclusion

From the results presented in this work we can conclude that PuOC could be used as substrate for CMCase production by *P. roqueforti* in SSF. Like some other oil cakes (Ramachandran et al., 2006), bioprocess utilization of PuOC is attractive due to relatively cheaper availability and the presented results could be of commercial importance for CMCase production, also decreasing environmental problems caused by PuOC disposing of in open areas.

Acknowledgement

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