APTEFF, 38, 1-190 (2007) UDC: 663.18:635.62:665.3
DOI:10.2298/APT0738127P BIBLID: 1450-7188 (2007) 38, 127-137
Original scientific paper

EXO-POLYGALACTURONASE PRODUCTION BY *Penicillium roqueforti* ON PUMPKIN OIL CAKE IN SOLID STATE FERMENTATION

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The feasibility of using pumpkin oil cake (PuOC), individual and in combination with wheat bran (WB), as substrate for the production of Exo-polygalacturonase (Exo-p) by starter culture Penicillium roqueforti in solid state fermentation (SSF) has been evaluated. The kinetics of enzyme production was investigated using PuOC alone in the range from 13 to 168 h, with moisture contents varying from 44% the ability to grow and produce Exo-p activity on this substrate, reaching a maximum value of 1451.75 U/g.d.w PuOC by the 5th day of fermentation. Fermentation experiments indicated that the water activity (a_w) influenced the enzyme production. A medium with a_w 0.932 and the fermentation time of 5 days were selected, as these conditions resulted in the highest pectolytic activity and were used for further investigation. A next step in this research was to examine the effect of the substrate combination, PuOC with wheat bran (WB), in different ratios. The addition of WB as carbon sources was found to have a significant influence on the enzymes yields. Exo-p activities were the highest with initial water activity of a $_w$ 0.932 and PuOC supplementation with WB (1:0.67).

KEYWORDS: Solid state fermentation, Pumpkin oil cake, *Penicillium roqueforti*, Exo-polygalacturonase

INTRODUCTION

Global oilseed production is the most widely cultivated crop in the world. From the world's total oil seed production of 395 million tonnes, about 36 % is used for making 145 million tonnes of oil and 101 million tonnes of oil meal and cake (1). Oil cake, or meal, is the residue left after oil extraction by pressing, and it is formed from the hull and non-oil components of oil seeds. Oil cake can be used as animal food, especially in dry seasons when pastures are scarce.

However, the use of oil cake for animal feed is limited because some of them have very low nutritional value and the presence of anti-nutritional factors such as phenolic components, which inhibit the ruminal symbiotes (2).

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In the production of fertilizers, relatively high levels of phenolic compounds in some oil cakes are a problem because they inhibit the germination properties (3). Therefore, most of this by-product is generally disposed of in open areas, leading to potentially serious environmental problems. Given this situation, it is necessary to look for the processes that would allow the controlled elimination of this residue or, even better, its industrial reutilization.

In recent years there has been an increasing trend in the efficient utilization and value-addition of oil cake, either alone or in combination with other industrial by-product such as wheat bran, coffee pulp, sugar beet, apple pomace and others. Biotechnological processes, especially the solid state fermentation (SSF), have contributed enormously to such reutilization. SSF is defined as the fermentation involving solids in the absence (or near absence) of free water. The substrate, however, must contain sufficient moisture to support the growth and metabolism of the microorganisms (4). Productivity of SSF, according to Nigam and Singh (5), is significantly affected by the medium moisture level. Gonzalez et al. (6) showed that in SSF, among all culture conditions the initial moisture level is one of the most critical. Ramana Murthy et al. (7) explained that initial water content determines two different but interrelated factors that affect microbial growth and product formation.

The application of oil cake in SSF bioprocesses not only provides an alternative substrate but also helps to solve some of the pollution problems caused by their accumulation. Several bioprocesses have been developed for its utilization as a raw material for the production of bulk chemicals and value-added fine products by SSF. As examples, the production of cephamycin C (8), lactic acid (9), mushrom and enzymes (10-22) can be cited. In a related area, there is a significant interest in using SSF technique to produce a variety of enzymes, mainly from molds, as indicated by the growing number of research papers published (23), and the marketing and development undertaken by a number of fermentation industries (24). Several advantages are often cited for SSF processes and these include that enzyme titers are higher in SSF than in submerged fermentation (SmF) (25).

The SSF processes offer a series of advantages over SmF (26). The culture conditions are more similar to the natural habitat of filamentous fungi, so that these are able to grow and excrete large quantities of enzymes. Product concentrations after the extraction are usually larger thanin the case of SmF and the quantity of liquid waste generated is lower. Additionally, these processes are of special economic interest for countries with abundance of biomass and agroindustrial residues, as these can be used as cheap raw materials.

Pectinases form the group of enzymes that degrade pectic substances, which are the structural polysaccharides present in vegetable cells, responsible for maintaining the integrity of plant tissues. Pectic substances are characterized by long chains of galacturonic acid residues. On these residues are carboxyl groups, which are sometimes modified by the addition of methyl groups, forming methoxyl groups. Pectic enzymes act by breaking glycosidic bonds of the long carbon chains (polygalacturonase, pectin lyase and pectate lyase) and by splitting off methoxyl groups (pectin esterase). Pectinase are produced by both SmF and SSF. The commercial preparations of pectinase are produced mainly from fungi, especially from *Aspergillus niger* (27). The microbial pectinase accounts approximately for 25% of the total worldwide enyzme sale (28). Among microbes, the fungi *Aspergillus, Penicillium and Rhizopus* have many advantages as enzyme producers, since they are normally GRAS (generally regarded as safe) strains and yield extracellular products, which makes easy the recuperation fermentation medium. In this respect we stu-

died the reutilization of pumpkin oil cake (PuOC) by starter culture *P. roqueforti* in exo-polygalacturonase (Exo-p). The fungal Exo-p (EC 3.2.1.67) are useful in the industrial applications such as fruit juice extraction and clarification, wine processing, oil extraction, maceration of fruits and vegetables, and in the production of oligogalacturonides as functional food components, *etc*. (29).

Previous studies carried out in the University of Novi Sad have suggested that PuOC can be used as an alternative substrate for Exo–p production in SmF using starter culture *P. roqueforti* (30). In the present work we studied the kinetics of Exo-p formation in SSF using media PuOC either alone or in combined with wheat bran (WB) and effect of different initial moisture levels on the enzyme production by *P. roqueforti*.

MATERIAL AND METHODS

Microorganism

P. roqueforti, starter culture, was propagated and preserved on 5% whole wheat flour and 2% agar slants at 4 °C. Inoculum for the experiments was prepared from fresh flour slants as described earlier (31). The inoculum concentrations were adjusted to 5×10^8 spores/g solid PuOC.

Solid state fermentation

A mass of 5 g of dry substrate was placed in a 300-ml Erlenmeyer flask and supplemented with the defined amount of mineral medium containing 0.2% KH_2PO_4, 0.1% $MgSO_4$ and 0.1% NaCl. The contents were sterilized by autoclaving at 121°C for 15 min. After cooling, the flakes were inoculated with 1 ml of spore suspension and incubated at 25°C for desired period.

Substrate for fermentation

PuOC used as the solid substrate for the production of Exo-p was from the variety Olinka. Typical batches of PuOC were collected in plastic bags from a local Oil Industry ”Pan Union” just after pressing and then stored at 4 °C until required. For every series of experiments, sub-samples (500 g) were taken, milled and sieved before using.

Effect of initial moisture and water activity

In order to elucidate the effect of water availability, substrate swelling and the oxygen diffusion, 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% KH_2PO_4, 0.1% $MgSO_4$ and 0.1% NaCl was used as the moistening agent.

Effect of supplementation with wheat bran

The effect of substrate supplementation with WB on the production of the Exo-p was also assessed. For the first set of experiments, the whole PuOC medium was supple-

mented with WB and five combination were prepared PuOC:WB (1:4; 1:1.5; 1:0.67; 1:0), at constant moisture content and water activity ($a_{w,}$ 0.932).

Sample preparation

During the fermentation, samples with and without inoculum were collected from the shaker at regular interval of 24 h (every day from the beginning of the process) for further analysis. Samples were extracted in 50 ml of Tween 80, for 30 min on a rotary shaker at 200 rpm and 30 °C, filtered and the filtrate was centrifuged at 10,000 rpm for 10 min. The supernatant was used for measuring the pH, reducing sugars (RSs), proteins and Exo-p activity.

In the second part of the experiment, samples were collected after five days from the beginning of the fermentation and they were treated in the same way as in the previous case.

All results shown below represent the average of three sets of experiments and error bars indicate the 95% confidence limit.

Analytical methods

The Exo-p activities were estimated by determining RSs released during hydrolysis of pectin. The RSs produced were quantified by a modification of the dinitrosalicylic acid (DNS) method (32). The enzymatic activity units (U) was defined as the amount of enzyme required to produce 1 μmol of product per 1h per gram of dry substrate PuOC.

Protein content was determined by the method of Lowry (33), with BSA as standard. The RSs in the medium were determined by the DNS method. The results were expressed as glucose using a calibration curve.

The moisture contents of the solid substrates were determined from the loss in weight after heating at 90 °C for 24 h and the water activity (a_w) was measured by „Testo" 650. 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.

RESULTS AND DISCUSSION

The basic aim of this work was to examine the feasibility and efficiency of *P. roqueforti* to grow and produce Exo-p activity using PuOC as substrate, by SSF technique.

Figs. 1 and 2 present the time courses of soluble proteins and pH, respectively during the SSF. Namely, it is known that the growth of microorganisms is connected with the amount of proteins and pH in medium. The increasing amount of soluble protein and pH indicate the growth of *P. roqueforti*, using PuOC as the sole nutrient source in the culture medium. Fig. 1 illustrates the time course of protein production during the fungi cultivation, in fermentation medium and in the medium without inoculums (WI). PuOC, itself, had a protein content of about 60%, so it was necessary to follow the changes in protein concentration in both inoculated and uninoculated substrates. The obtained results show an increase of the protein amount in the inoculated medium, which is in correlation with the fungi growth and enzyme production.

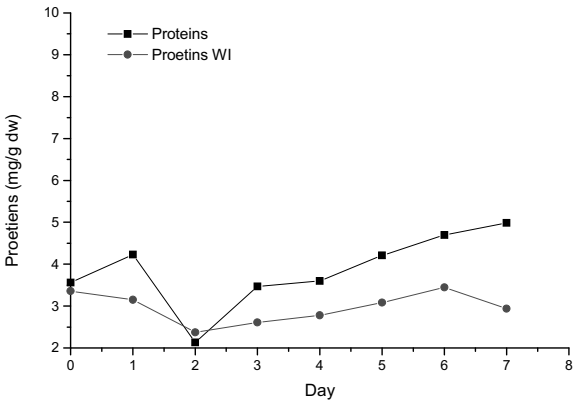

Fig. 1. Time course of soluble protein production in the SSF by *P. roqueforti*

During the fermentation, the pH increased from 6.25 to 8.14, which was obtained on 6th day of cultivation. The increase of the medium pH is in contradiction with the trend presented in (34, 35), in which a decrease in the pH of the medium during the fermentation of apple pomace and citrus pectin was observed. However, it corroborated other data from SSF (36-38). 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 (39, 40). 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 aminoacids were major carbon and energy sources in these fermentations, it is likely that protein hydrolisate is involved in this alkalization.

The pH decreased during the first day of fermentation (Fig. 2), after that it increased and achieved maximum level (pH 8) on the 6th day. When the concentration of soluble protein was very high (5 mg/g), the pH increased, probably due to microbial assimilation of organic acids.

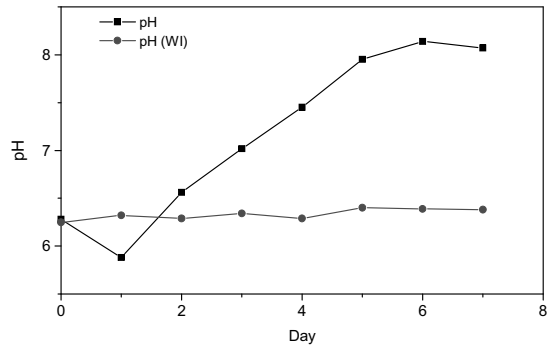

Fig. 2. Time course of pH during SSF by *P. roqueforti*

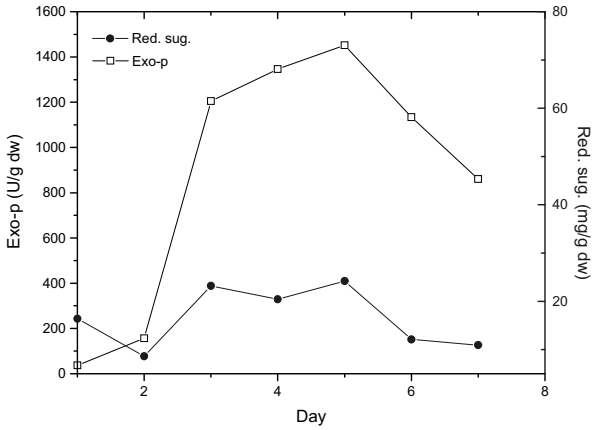

Fig. 3. Time course of Exo-p activities and RSs during SSF of PuOC by *P. roqueforti*

Fig. 3 shows the ability of *P. roqueforti* to synthesize and secret Exo-p and time course of RSs, during SSF, under the experimental condition employed. As can be seen, Exo-p activity increased from the beginning of fermentation and achieved the maximum value of 1452 U/g d.w. PuOC 5 days after inoculation. In the evolution of RSs, two trends have been clearly observed. During the first two days, a slight decline in RSs was observed. After the second day, the amount of RSs increased. This is in correlation with the increase of Exo-p activity. When the Exo-p activity reached a maximum, the amount of RSs was also the highest, 24.2 mg/g d.w. PuOC. After 5 days of fermentation, polysaccharides from the substrate were exhausted and Exo-p activity decreased, which was accompanied by the lowering of the amount of RSs.

Effect of moisture content of the solid substrate

Next step in this research was to examine the effect of initial moisture on the production of Exo-p activity by *P. roqueforti*. The effect of initial moisture content on Exo-p production by *P. roqueforti* is shown in Table 1.

Table 1. The effect of initial moisture on the production of Exo-p activity by *P. roqueforti*

w (%)	a_w	Exo-p (U/g dw)
44.44	0.932	288.2
50.00	0.962	271.1
54.50	0.974	104.1

The lowest moisture level tested in this work (44.44% w/w) resulted in very high Exo-p formation. The low enzyme activity at high substrate moisture levels could be at-

tributed to the decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer or increased formation of aerial hyphae. The results obtained at 54.50% moisture content shows that the enzyme formation by *P. roqueforti* also decreased at high moisture levels. On the contrary, in the media with very low water-availability (25%) fungi suffer modifications in their cell membrane, leading to transport limitations and affecting microbial metabolism. According to Raimbant and Alazard (41), who studied the influence of the different initial humidity levels on the growth of *A. niger*, the best results were obtained in media with moisture contents ranging from 50% to 55%. Based on the present results, moisture contents between 40% and 50% appeared to result in a compromise among water availability, substrate swelling and oxygen diffusion effects, favoring the pectinase formation by *P. roquefoerti*.

For further experiments a medium of 44.44 % moisture content with a fermentation time of 5 days was chosen. These conditions provided a high Exo-p activity and enhanced productivity.

Effect of supplementation with carbon sources

Further experiments were done to investigate the effect of combined medium (PuOC and WB), on the production of Exo-p, using the medium with 44% moisture content and fermentation time of 5 days. These conditions provided high pectinase activity and enhanced polygalacturonase productivity.

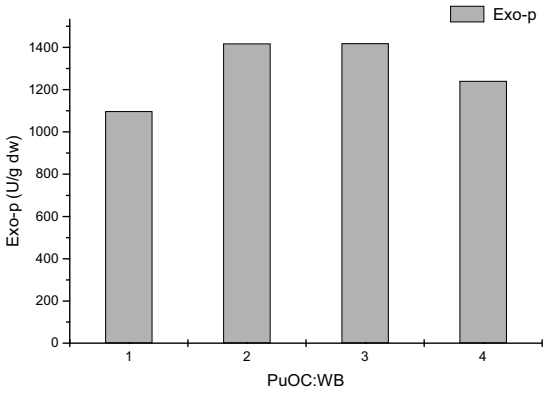

Fig 4. The effect of combined medium on Exo-p activity
*PuOC:WB (1:4; 1:1.5; 1:0.67; 1:0)

The best Exo-p activity, 1420.0 U/g dw PuOC (Fig. 4) was obtained in the combined medium containing PuOC and WB in a ratio 1:0.67.

CONCLUSION

From the results presented above, it may be concluded that *P. roqueforti* can be successfully cultured in PuOC to produce Exo-p. Maximum activity was reached 5th day from the beginning of the fermentation, which was followed with maximum values of the soluble protein, reduced sugars and pH.

The control of moisture content is an important parameter in solid fermentation. The production of polygalacturonases was influenced by the initial moisture of substrate, and it was highest at 44.44% moisture.

The addition of WB as carbon source was found to have a significant influence on the enzymes yields. Exo-p activities were the highest with initial water activity of 0.932 and PuOC supplementation with WB (1:1).

ACKNOWLEDGMENT

This work was supported by Project No. 371007 from the Ministry of Science and Environmental Protection of the Republic of Serbia.

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ПРОДУКЦИЈА ЕГЗО-ПОЛИГАЛАКТУРОНАЗА ГЉИВОМ *Penicillium roqueforti* НА ПОГАЧИ ОД СЕМЕНА УЉАНЕ ТИКВЕ ФЕРМЕНТАЦИЈОМ НА ЧВРСТОЈ ПОДЛОЗИ

Драгиња М. Перичин, Сенка З. Мађарев, Љиљана М. Радуловић и Марија М. Шкрињар

У овом раду испитана је могућност употребе тиквине уљане погаче (PuOC-pumpkin oil cake), посебно и у комбинацији са пшеничним мекињама (WB-wheat bran), као супстрата за продукцију егзо-полигалактуроназе (Егзо-п), стартер културом *P. roqueforti*, ферментацијом на чврстој подлози. Кинетика синтезе ензима на самој погачи праћена је у временском интервалу од 13 до 168 h, а влажност подлоге је варирана у интервалу од 44% до 54%, док је активност воде (a_w) била од 0,932 до 0,974. Добијени резултати показују да *P. roquefoerti* има способност раста

и продукције ензима на испитиваном супстрату, достижући максималну вредност активности од 1452 U/ml с.м. PuOC, петог дана ферментације. Изведени ферментациони експерименти указују да активност воде утиче на продукцију ензима. Медијум са a_w 0.932 пружа најбоље услове за продукцију Егзо-п. Медијум са a_w 0.932 и време ферментације од 5 дана изабрани су као оптимални услови за следеће истраживање. У следећој фази истраживања праћен је ефекат комбиноване подлоге која је садржала PuOC и WB у различитим односима. Додатак WB значајно је утицао на принос ензима, па је тако највећа активност Егзо-п забележена на подлози састављеној од PuOC и WB, у односу 1:0,67.

Received 11 June 2007
Accepted 5 September 2007