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The effects of different carbon sources on biosynthesis of pectinolytic enzymes by *Aspergillus Niger*

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The aim of this work was to investigate the effects of different carbon sources on the nourishing base on the production of pectinolytic enzymes by *Aspergillus niger* with the aim of optimizing the medium for maximal enzyme production.

Growth and enzymes production by *Aspergillus niger* were evaluated on glucose, fructose, galactose, xylose, lactose, apple pectin and the dry apple pulp. Results of different carbon sources on base showed maximal endo-pectinolytic activity, endo-PG/328 U L^{-1} with the pressed apple pulp, compared with endo-PG/140 U L^{-1} with apple pectin, endo-PG/62 U L^{-1} with galactose, endo-PG/28 U L^{-1} with lactose, endo-PG/0.0 U L^{-1} with glucose and fructose and endo-PG/5.0 U L^{-1} without carbon source (control).

The growth of the microorganism (dry biomass) on different carbon sources showed maximum dry biomass, 4.5 g L^{-1} with glucose, compared with dry biomass, 4.3 g L^{-1} with fructose, 4.0 g L^{-1} with the pressed apple pulp, 3.5 g L^{-1} with galactose, 3.0 g L^{-1} with lactose, 2.2 g L^{-1} with apple pectin and 0.8 g L^{-1} without carbon source (control). Maximal endo-PG production, 328 U L^{-1} and dry biomass, 4.0 g L^{-1} by fungus *Aspergillus niger* was observed in a medium at pH initial, 4.0. The results presented here will be of commercial importance for using apple pulp as a carbon source for production of pectinolytic enzymes in submerged fermentation.

Keywords: Submerged fermentation, carbon sources, pH, pressed apple pulp, pectinolytic enzymes, *Aspergillus niger.*

Introduction

Pectin or other pectic substances are heterogeneous group of high molecular weight, complex acidic structural polysaccharides with a backbone of galacturonic acid residues linked by α(1-4) linkages (Kashyap et al., 2001). They constitute major components of the middle lamella, a thin layer of adhesive extracellular material found between the primary cell walls of adjacent young plant cells (Hoondal et al., 2002).

Pectic substances are classified into four main types based on the type of modifications of the backbone chain which are: protopectin, pectic acid, pectinic acid and pectin (Kashyap et al., 2001).

Protopectin is the water insoluble parent pectin substance found in the middle lamella of plant tissues. It yields soluble pectic substances such as pectin or pectinic acid upon restricted hydrolysis.

Pectic acid is a group designation applied to pectic substances mostly composed of galacturonans containing negligible amounts of methoxyl groups. The salts of pectic acid are called pectates.

Pectinic acid are the galacturonans containing various amounts of methoxyl groups. The salts of pectinic acids are either normal or acid pectinates. Under suitable conditions, pectinic acids are capable of forming gels with sugars and acids or, if suitably low in methoxyl content, with certain metallic ions.

Pectins are the soluble polymeric materials containing pectinic acids as the major component. They can form insoluble protopectins with other structural polysaccharides and proteins located in the cell wall (Kashyap et al., 2001).

There are basically three types of pectic enzymes: de-esterifying enzymes (pectinesterases), depolymerizing enzymes (hydrolases and lyases), and protopectinases. They can be further classified as: endo-, liquefying or depolymerizing enzymes or exo-, saccharifying enzymes (Alkorta et al., 1998; Kashyap et al., 2001).

Pectic enzymes are a group of enzymes that contribute to the degradation of pectin by various mechanisms. Elimination of pectic substances is an essential step in many food processing industries and wine industries. The principal objective is to reduce the viscosity of the solution so that it can be handled and processed easily. These enzymes are mainly synthesized by plants and microorganisms (Naidu and Panda, 1998). *Aspergillus niger* is used for the industrial production of pectinolytic enzymes. This fungus synthesizes polygalacuronases, polymethyl galacturonases, pectin lyases and pectin esterases. The advantage of *Aspergillus niger* is the possession of GRAS (Generally Regarded As Safe) status which permits the use of its metabolites in the food industry (Canal-Llauberes, 1993) and also the distribution of these enzymes is in appreciable amount. They have a significant role in maintaining ecological balance by causing decomposition and recycling of plant materials. Acidic pectic enzymes are widely used in the production and clarification of fruit juices and wines. They are also very important in maceration and solubilization of fruit pulps (Naidu and Panda, 1998; Pilnik and Voragen, 1993). Alkaline pectic enzymes have been used in several areas, including retting and degumming of fiber crops, textile processing, coffee and tea fermentations, paper and pulp industry, and oil extraction (Hoondal et al., 2002; Fogarty and Kell, 1983).

Pectinolytic enzymes are usually produced on solid or submerged fermentation (Friedrich et al., 1989; Bailey and Pessa, 1990; Schmidth et al., 1995; Kaur and Satyanarayana, 2004; Silva et al., 2005; Aguilar et al., 2008; Tsereteli et al., 2009). Submerged fermentations generally produce smaller quantities of secretory enzymes and solid fermentations are not susceptible to automation. For the industrial production of pectinolytic enzymes it is important to improve the fermentation conditions, for better production of extracellular enzymes on inexpensive carbon sources such as apple pomace, citric peels, pectin or other agricultural wastes which contain appreciable quantities of pectin (Alkorta et al., 1998; Maldonado and Navarro, 1986; Leuchtenberger et al., 1989; Aguilar and Huitron, 1986; Larious et al., 1989; Akinyele et al., 2010). The most authors describe the use of an optimized medium composition to increase the enzyme content (Pericin et al., 1992; Berovic and Ostroversnik, 1997; Shevchik et al., 1992; Tsereteli et al., 2009). The use of complex substrates like apple pulp, citric peels, pectin or other agricultural wastes is common. Since these materials are natural substrates for pectinolytic enzymes, they and their degradation products are inducers for the enzyme synthesis, e.g. galacturonic acid and pectic acid as well as polygalacturonic acid and pectin itself (Maldonado and Navarro, 1986). The use of saccharose or glucose as the substrate also influences enzyme synthesis, catabolite repression taking place if the concentration of the substrate is higher than 5-10 g L^{-1}. However, since pectin is a high molecular weight polysaccharide, the question arises as to how induction takes place or how the cells can sense the substrate in the outer environment. It has been suggested that some microorganisms can produce low levels of basal constitutive activities that degrade the polymeric substrate, and that the low molecular products of the reaction serve as inducers or energy sources to promote cell growth and to induce the other pectinases.

In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues such as cassava, sugar beet pulp, wheat bran and apple pomace. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new avenues have opened for their utilization. However, the huge amounts of residual plant biomass considered as waste can potentially be

converted into various different value added products including biofuels, chemicals and cheap energy sources for fermentation, improved animal feeds and human nutrients (Howard et al., 2003).

The use of low cost substrates for the production of industrial enzymes is one of the ways to reduce significantly production costs. This can be achieved using solid agricultural waste materials as substrates. The amount of enzymes produced by each substrate differs depending on the amount carbon source utilized by the organisms.

The aim of this work was to investigate the effects of different carbon sources on the nourishing base on the production of pectinolytic enzymes by *Aspergillus niger* with the aim of optimizing the medium for maximal enzyme production.

Growth and enzymes production by *Aspergillus niger* were evaluated on glucose, fructose, galactose, lactose, apple pectin and the pressed apple pulp.

Materials and methods

Micro-organism

The microorganism used in this work was the fungus *Aspergillus niger MK-15*, which was isolated from soil as a highly active producer of pectinolytic enzymes and was maintained on slant agar according to Czapek with 2% pectin. Spores from 3 days old agar slants were collected by adding sterile distilled water to each slant. The spores suspension was adjusted to a final concentration, $2 \cdot 10^6$ spores mL^{-1}. In the culture medium was added 3mL, or $6 \cdot 10^6$ spores.

Media and fermentation procedure

The fermentation medium contained the following constituents (g L^{-1}): $(NH_4)_2HPO_4$, 7.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; corn flour, 5.0. The sugars (g L^{-1}) were used as carbon sources as follows: glucose, 10.0; fructose, 10.0; lactose, 10.0; galactose, 10.0; apple pectin, 10.0; the pressed apple pulp, 10.0.

The base was previously sterilized by autoclaving at 121 ^{0}C for 30 min. The pressed apple pulp first is dryed and after is milled to the ground apple pulp particles with the diameter under 0.315 mm. The refuse apple pulp had the following content: moisture, 10÷12 %, ashes, 3÷5 %, proteins, 6÷6.2 %, and pectin, 9÷10 %.

The initial pH-value of medium was 4.0 without further adjustment. The initial pH value was adjusted at the range of 2-8 in experiments connected with investigations of pH effect on biosynthesis of pectinolytic enzymes.

All other process parameters such as inoculation, mixing, aeration, temperature, fermentation time were same. The growth of the microorganism and synthesis of pectinolytic enzymes were performed in 500 mL flasks, 100 mL base with rotational shaking 200 min^{-1} on a rotational laboratory shaker, at 30 ^{0}C within 120 h. Concentration of inoculum: suspension of $2 \cdot 10^6$ spores mL^{-1} up to 3 days old, added 3 mL, or $6 \cdot 10^6$ spores.

Enzyme assay

Endo-pectinolytic activity, based on change in the viscosity of the reaction mixture (0.35% pectin solution, buffered at pH /4.5 in 0.1 mol L^{-1} citrate) at 30 ^{0}C, was determined using Ostwald viscometer. The degree of degraded pectin under known amount of filtrate (enzyme) was calculated with the formula 100 (t1-t)/(t1-t2), where: (t1) is the flow time of the substrate control; (t) is the flow time of the test and (t2) is the flow time of water. 1U

is defined as the amount of enzyme which catalyzes hydrolysis of 1g pectin per 1 h at 40 ^{0}C. A specific activity was estimated as units of activity per 1 mg of a dry biomass.

Biomass production measurements

Biomass production was measured as dry weight. After filtering, the retained cell mass was dried at 100 ^{0}C to constant weight.

Results and discussion

The capacity of microorganisms to produce extracellular enzymes is influenced by environmental conditions such as temperature, pH, aeration, inoculums age and the presence of inducer or repressor substrates (El-Refai et al., 1984). Initially, the influence of the different carbon sources and initial pH of the cultivation medium of *Aspergillus niger* was investigated. All other process parameters such as inoculation, mixing, aeration, temperature, fermentation time were same.

TABLE 1. Endo-PG BIOSYNTHESIS AND GROWTH BY *ASPERGILLUS NIGER* IN RELATION TO THE CARBON SOURCES

Carbon sources	Experimentally found [a] biomass, g L^{-1}	Experimentally found [a] endo-PG/U L^{-1}	Calculated endo-PG/U g^{-1} d.b.[b]
Glucose	4.5 ± 0.08	0.0 ±0.0	0.0
Fructose	4.3 ± 0.08	0.0 ±0.0	0.0
Galactose	3.5±0.16	62.0 ±1.63	17.7
Lactose	3.0±0.24	28.0 ±2.16	9.3
The apple pectin	2.2 ± 0.08	140.0 ±4.08	63.6
The apple pulp	4.0±0.16	328.0 ± 8.64	82.0
Control	0.8 ± 0.08	5.0 ±3.74	6.2

Note: [a] Values are the average from 3 replicates ± SD. SD - standard deviation. [b]d.b.- dry biomass

TABLE 2. THE INFLUENCE OF INITIAL pH- VALUES OF THE MEDIUM ON endo-PG BIOSYNTHESIS AND GROWTH BY *ASPERGILLUS NIGER*

pH	Experimentally found [a] biomass, g L^{-1}	Experimentally found [a] endo-PG/U L^{-1}	Calculated endo-PG/U g^{-1} d.b.[b]
2.0	2.5 ± 0.16	260.0 ±6.16	104.0
3.0	3.5 ±0.08	300.0 ±4.89	85.7
4.0	4.0 ±0.16	328.0 ±5.88	82.0
5.0	3.8 ±0.16	280.0 ±10.80	73.7
6.0	3.5 ±0.08	205.0 ±3.55	58.6
7.0	3.0 ±0.08	100.0 ±6.97	33.3
8.0	2.1 ± 0.16	50.0 ±2.16	23.8

Note: [a] Values are the average from 3 replicates ± SD. SD - standard deviation. [b] d.b.- dry biomass

Results of different carbon sources on base in Table 1 showed maximal endo-PG/328 U L^{-1} with the pressed apple pulp, compared with endo-PG/140 U $L^{-1}$with apple pectin, endo-PG/62 U L^{-1} with galactose, endo-PG/28 U L^{-1} with lactose, endo-PG/ 0.0 U L^{-1} with glucose and fructose and endo-PG/5.0 U L^{-1} without carbon source (control).

The growth of the microorganism (dry biomass) on different carbon sources in Table1 showed maximum dry biomass, 4.5 g L^{-1} with glucose, compared with dry biomass, 4.3 g

L^{-1} with fructose, 4.0 g L^{-1} with the pressed apple pulp, 3.5 g L^{-1} with galactose, 3.0 g L^{-1} with lactose, 2.2 g L^{-1} with apple pectin and 0.8 g L^{-1} without carbon source (control).

An effect of pH of the medium on endo-PG biosynthesis and growth of the culture *Aspergillus niger* was studied at seven pH-values in a range of 2.0-8.0 (Table 2). The maximal endo-PG activity was observed in the medium with the acidic initial pH-values within a range of 3.0-4.0.

Maximal endo-PG production, 328 U L^{-1} and dry biomass, 4.0 g L^{-1} by fungus *Aspergillus niger* was observed in a medium at the initial pH/ 4.0 (Table 2).

From results in Table 1 can be see biosynthesis of endo-PG and growth of the culture *Aspergillus niger* in relation to the carbon sources. Biosynthesis of endo-PG is induced by pectic substances and inhibited in the presence of easy metabolized monosaccharides (glucose, fructose etc.) and some other compounds. Maximal endo-PG/328 U L^{-1} with the pressed apple pulp, compared with endo-PG/0.0 U L^{-1} with glucose and fructose.

The growth of the microorganism on different carbon sources (Table 1) showed maximum dry biomass, 4.5 g L^{-1} with glucose, compared with 2.2 g L^{-1} with apple pectin and 0.8 g L^{-1} without carbon source (control).

Similar results were obtained of many authors who described the use on different inexpensive carbon sources for better production of pectinolytic enzymes (Aguilar and Huitron, 1986; Maldonado and Navarro, 1986; Hours et al., 1988; Larious et al., 1989; Leuchtenberger et al., 1989; Pericin et al., 1992; Shevchik et al., 1992; Hang and Woodams, 1994; Berovic and Ostroversnik, 1997; Alkorta et al., 1998; Zheng et al., 2000; Kaur and Satyanarayana, 2004; Joshi et al., 2006; Zhong-Tao et al., 2009; Tsereteli et al., 2009).

Conclusion

Microbial enzymes are routinely used in many environmentally friendly and economic industrial sectors. Pectinolytic enzymes play an important role in food processing industries and alcoholic beverage industries. The production of food enzymes related to the degradation of different substrates. These enzymes degrade pectin and reduce the viscosity of the solution so that it can be handled easily. Optimization of physical parameters such as pH, temperature, aeration and agitation in fermenters should be done.

The different carbon sources on base as apple pectin and the pressed apple pulp stimulated the production of pectinolytic enzymes and the growth of the microorganism (dry biomass). The different carbon sources showed maximum dry biomass (d.b.) with glucose and fructose. The best carbon source on base for better production of pectinolytic enzymes was the pressed apple pulp with optimal concentration of 10.0 g L^{-1}. This residue, being renewable and in an abundant supply, represents a potential low cost material for microbial enzyme production. The significance of this agro-industrial residue as material for pectinolytic enzyme production is highlighted in this article. The results presented here will be of commercial importance for using pressed apple pulp as a carbon source for production of pectinolytic enzymes in submerged fermentation.

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