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Extraction Conditions Evaluation of Pectin Methylesterase Produced by Solid State Culture of *Aspergillus niger*

*Valeria BORSZCZ[1,2], Taísa Renata PIOTROSKI BOSCATO[1], Karine CENCE[1], Jamile ZENI[1], Rogério Luis CANSIAN[1], Geciane TONIAZZO BACKES[1] and Eunice VALDUGA[1]**

[1]Department of Food Engineering, URI Erechim, Rio Grande do Sul, Brazil; [2]Federal Institute of Education, Science and Technology of Rio Grande do Sul (IFRS), Erechim-RS, Brazil

**Corresponding author: veunice@uricer.edu.br*

Abstract

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Different solvents and extraction solutions (distilled water, NaCl, Tween 80, citrate buffer, and acetate buffer) were evaluated for enzymes recovery. The independent variables evaluated were agitation (12–348 rpm), time (4.8–55.2 min), and temperature (13.2–46.8°C) and 10 extraction cycles, using an experimental design (Central composite rotatable design 2^3). Pectin methyl esterase maximum recovery by solid state culture of *Aspergillus niger* was 11 U/g_{wm} (31 U/g_{dm}) using NaCl (0.1 mol/l) as solvent at a 5 : 1 (v/w) ratio, 30°C for 55.2 min, at 180 rpm, with one extraction cycle.

Keywords: pectinases; recovery; solid state culture

Pectin methylesterase (pectinesterase, PME, EC 3.1.1.11) catalyzes pectin de-esterification, yielding methanol and pectin with a lower esterification degree called pectate (Kohli *et al.* 2015), which acts as a substrate for polygalacturonase (PG, EC 3.2.1.15), a polysaccharide chain depolymerizing enzyme (Kohli *et al.* 2015).

Biotechnical processes, especially solid state cultivations are important since they are characterized by agroindustrial waste usage – i.e. orange peel – to produce extracellular enzymes, such as pectinases, thus reducing the environmental impacts caused by this byproduct (Santos *et al.* 2016). Other residues, such as wheat bran (Pitol *et al.* 2016), sugarcane bagasse (Biz *et al.* 2016; Pitol *et al.* 2016) and apple husk (Santos *et al.* 2016) have been used as supplements.

Different conditions for pectinase production and recovery by microorganisms have been studied for solid-state cultivation processes maximization, aiming at minimizing both time and cost and optimizing the products' yield, productivity, and quality. Therefore, the recovery of produced metabolites is quite relevant as the initial enzymes recovery stage is fermented material leaching, comprised of the preferential dissolution of one or more solutes of interest by contact of a solid with a liquid solvent, so-called solid-liquid extraction (Baiano 2014).

Different variables as well as on choosing the appropriate equipment influence the efficient leaching process development. Among the variables, solids preparation, solvent/mass ratio, process temperature, solvent's pH, liquid's superficial tension, system agitation level, and the presence or not of a chemi-

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cal reaction on the extraction could be highlighted (Rodríguez-Fernández *et al.* 2012).

Therefore, the aim of this work was to optimize the process parameters to pectin methylesterase recovery produced by solid state culture of *Aspergillus niger* ATCC 9642 using agro-industrial residues (orange peel, corn steep liquor, and parboiled rice water) and enzyme partial characterization.

MATERIAL AND METHODS

Extraction parameters evaluation. The solid-state cultivation (SSC) process was done using a polypropylene beaker (500 ml) containing a 12 g ground orange peel (*Citrus* genre, Valencia variety), 0.81 g wheat bran (Olfar Ind. & Com. Óleos Vegetais Ltd., Brazil), and 3 g corn steep liquor (Ingredion) mixture, 65% moisture content and $pH_{initial}$ of 4.3. The substrate mixture was sterilized in a vertical downward autoclave (AV75; Phoenix, Brazil) for 15 min at 1 atm. A 5.10^6 spores/g (wet matter) *A. niger* ATCC 9642 spores concentration was inoculated on the mixture and the latter incubated in a germination chamber (TE401; Tecnal, Brazil), under controlled relative humidity at 30°C, for 96 hours.

After cultivation period, 10 g of fermented medium diluted in solvents and/or solutions were used on the enzyme recovery process. The mixture was homogenized under controlled temperature, time period, and agitation speed. The mixture was then filtered and centrifuged (351R; MPW Med. Instruments, Poland) at 2756 *g* at 4°C for 15 min for spores and others insoluble materials removal. The supernatant (crude extract – enzymatic extract) was collected and stored for further analyses.

Initially, solvents and/or extractor solutions with distinguished ionic forces (distilled water, NaCl 0.1 mol/l, citrate buffer 0.005 mol/l (pH 5.4), acetate buffer 0.1 mol/l (pH 5.0), and Tween 80/0.1%, (v/v), at a 5 : 1 (v/w) ratio, homogenized at 175 rpm, at 20°C for 30 min were tested (Díaz *et al.* 2007) in order to recover the PME enzyme. The runs were performed in triplicate.

Afterwards, a comprehensive central composite rotatable design 2^3 (CCRD) was performed, aiming at optimizing and/or maximizing the pectinases recovery. Extraction temperature (13.2–46.8°C, X_1), time of contact (4.8–55.2 min, X_2), and agitation speed (12–348 rpm, X_3) were the studied independent variables, and NaCl 0.1 mol/l was used as the extraction solvent at a 5 : 1 (v/w) ratio. The dependent variable (response) was the enzymatic activity measurement ($U/g_{wet\ matter}$) for PME.

Successive extraction cycles (10 in total) were studied under the maximized conditions, with and without homogenization (20 min, 30°C, and 175 rpm) after the second cycle. For this purpose, 25 ml of NaCl (0.1 mol/l) was added to the solid fraction during the solid-liquid separation on each extraction cycle.

Analytical determinations. The bioproduction medium was filtered to separate the biomass for all PME activity determinations. The filtrate was determined by crude enzymatic extract: the pectin methylesterase (PME) activity was determined following the methodology described by Hultin *et al.* (1966). One PME unit was defined as the amount of enzyme capable to catalyse the pectin demethylation corresponding to 1 µmol consumption of NaOH min/ml, under assay conditions. The PME activity was expressed in units (*U*) per gram (g) of the substrate (wet matter – wm and dry matter – dm).

Statistical treatment. The other results were treated by analysis of variance (ANOVA), followed by the Tukey test. All analyses were performed using software Statistica version 5.0 (Statsoft Inc., USA).

RESULTS AND DISCUSSION

Table 1. presents PME activity values recovered from the fermented medium using different solvents (distilled water and NaCl) and extracting solutions (citrate buffer, acetate buffer, and Tween). The NaCl solvent (0.1 mol/l) was efficient on the PME extraction, obtaining 11 U/g_{wm} (31.4 U/g_{dm}) activities, without any significant difference ($P > 0.05$) from those obtained with acetate-buffer solution. In this

Table 1. PME enzyme activity using different types of solvents and solutions in the pectic enzymes extraction process

Solvents and solutions	PME activity (U/g_{wm})
Distilled water	5.64 ± 0.62^b
NaCl	11 ± 0.72^a
Citrate buffer	7.30 ± 1.26^b
Acetate buffer	12.42 ± 0.58^a
Tween	6.51 ± 0.63^b

Same letter between lines indicate the statistically significant difference at the level of 5% between lines (Tukey test).

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Table 2. Matrix of the central composite rotatable design (CCRD) 2^3 (real and coded values) and response of PME recovery (U/g_{wm})

Runs	Independent variables			PMe activity (U/g_{wm})
	X_1	X_2	X_3	
1	–1 (20)	–1 (15)	–1 (80)	7.78
2	1 (40)	–1 (15)	–1 (80)	10.46
3	–1 (20)	1 (45)	–1 (80)	8.32
4	1 (40)	1 (45)	–1 (80)	10.46
5	–1 (20)	–1 (15)	1 (280)	8.32
6	1 (40)	–1 (15)	1 (280)	7.78
7	–1 (20)	1 (45)	1 (280)	8.05
8	1 (40)	1 (45)	1 (280)	10.19
9	–1.68(13.2)	0 (30)	0 (180)	8.32
10	1.68 (46.8)	0 (30)	0 (180)	9.66
11	0 (30)	–1.68 (4.8)	0 (180)	8.98
12	0 (30)	1.68 (55.2)	0 (180)	10.99
13	0 (30)	0 (30)	–1.68 (12)	9.39
14	0 (30)	0 (30)	1.68 (348)	10.74
15	0 (30)	0 (30)	0 (180)	9.93
16	0 (30)	0 (30)	0 (180)	10.46
17	0 (30)	0 (30)	0 (180)	10.19

X_1 – temperature (°C); X_2 – time (min); X_3 – agitation (rpm); independent variable fixed

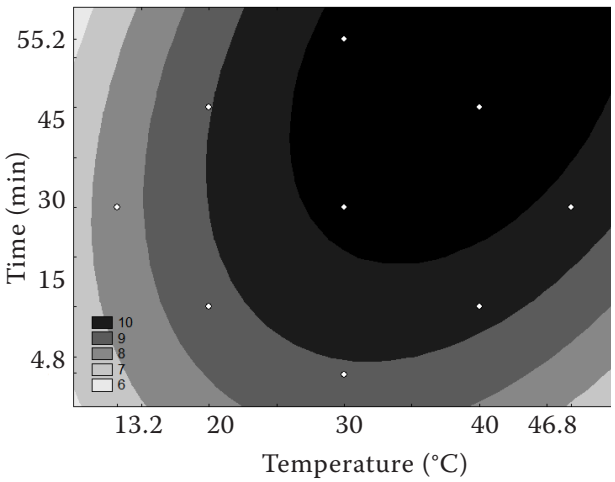

Figure 1. Contour curve for PME recovery (U/g_{wm}) as a time and temperature function.

way, the sodium chloride (0.1 mol/l) was established as the extracting solvent for the next stages.

Table 2 presents a CCRD 2^3 (real and coded values) on the enzymes recovery and on the PME activity response. It was noted that the maximum PME recovery was 11 U/g_{wm} (31.4 U/g_{dm}) using NaCl 0.1 mol/l, at 30°C and 180 rpm, for 55.2 min (run 12).

The results were statistically treated and Equation 1 present the second order coded models describing PME activity. Non-substantial factors were added to the lack of adjustment for the analysis of variance – ANOVA, the following coefficients were obtained correlation coefficient 0.75 and also calculated *F* of 1.65 greater than the *F* tabulated. Thus, validating the models and allowing contour curve construction for PME activity (Figure 1).

$$PME = 10.24 + 0.635\ (X_1) - 0.589\ (X_1)^2 + 0.444\ (X_2) \quad (1)$$

where: PME – pectin methylesteraseactivity (U/g_{wm}); X_1 – temperature (°C); X_2 – time (min)

According to Figure 1, a PME recovery optimization region of approximately 11 U/g_{wm} occured close to 30°C, at 180 rpm and 30 at 55.2 minutes. On the first extraction cycle, recoveries of approximately 9.7 U/g_{wm} (27.71 U/g_{dm}) occurred, from a total of 10 extraction cycles (data not shown). The yield reduced significantly ($P < 0.05$) on the second cycle for pectinases and subsequently kept practically constant ($P > 0.05$).

Some studies in the literature evaluated different parameters on pectinolytic enzymes recovery (Castilho *et al.* 1999, 2000; Santos *et al.* 2008; Rodríguez-Fernández *et al.* 2012; Sousa *et al.* 2012). It is worth mentioning that the temperature range obtained on the enzyme recovery optimization process (25–35°C) was considered noteworthy on industrial activities, as it corresponds virtually to room temperature, not implying additional energy costs during the extraction stage. On the other hand, enzymes are susceptible to deactivation, which would increase with increases in temperature and contact time. It suggests that studying and manipulating the extractive step operating conditions could be a useful tool for extracting preferably the desired enzyme from fermented solids containing a large number of different enzymes.

CONCLUSIONS

The extraction step was optimized by studying the combined influence of operating temperature, agitation, contact time, and solvent type. The maximum

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pectin methylesterase recovery by solid state culture of *Aspergillus niger* was 11 U/g_{wm} (31 U/g_{dm}) using NaCl (0.1 mol/l) as solvent at a 5 : 1 (v/w) ratio, 30°C for 55.2 min and 180 rpm, in one extraction cycle.

The present results demonstrated the simplicity of obtaining more concentrated enzyme extracts byoptimizing the extraction conditions, as it could be useful on extracting the desired biomolecule from fermented solids more selectively.

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