Bioprocess Biosyst Eng (2015) 38:749–760
DOI 10.1007/s00449-014-1315-1

ORIGINAL PAPER

Utilization of orange peel, a food industrial waste, in the production of exo-polygalacturonase by pellet forming *Aspergillus sojae*

Ali Oguz Buyukkileci · Marcello Fernandez Lahore · Canan Tari

Received: 23 September 2014 / Accepted: 19 October 2014 / Published online: 29 October 2014

Abstract The production of exo-polygalacturonase (exo-PG) from orange peel (OP), a food industrial waste, using *Aspergillus sojae* was studied in submerged culture. A simple, low-cost, industrially significant medium formulation, composed of only OP and $(NH_4)_2SO_4$ (AS) was developed. At an inoculum size of 2.8×10^3 spores/mL, growth was in the form of pellets, which provided better mixing of the culture broth and higher exo-PG activity. These pellets were successfully used as an inoculum for bioreactors and 173.0 U/mL exo-PG was produced. Fed-batch cultivation further enhanced the exo-PG activity to 244.0 U/mL in 127.5 h. The final morphology in the form of pellets is significant to industrial fermentation easing the subsequent downstream processing. Furthermore, the low pH trend obtained during this fermentation serves an advantage to fungal fermentations prone to contamination problems. As a result, an economical exo-PG production process was defined utilizing a food industrial by-product and producing high amount of enzyme.

Keywords Exo-polygalacturonase · *Aspergillus sojae* · Food industry waste · Orange peel · Low-cost medium

Introduction

Citrus peel, which constitutes 50 % of fresh fruit weight, is the main by-product of citrus processing fruit juice industry. The world production of citrus production was reported to be 88 million tons per year [1], whereas only part of it was utilized as cattle feed, organic fertilizer or in the extraction of pectin [2]. The presence of high amount of soluble and insoluble carbohydrates in the citrus peel makes it an attractive substrate for bioprocesses. It can be utilized directly for the production of hydrolytic enzymes [3–8]. The insoluble polysaccharides of OP such as cellulose, hemicelluloses and pectin can be degraded to sugars by pretreatments and enzymatic hydrolysis, and used for the production of value-added products such as ethanol and various enzymes [9–12].

Pectin, a complex polysaccharide found mainly in the primary cell wall of plants, is hydrolyzed by a group of enzymes called pectinases. Pectinases have long been used in fruit juice industry to increase juice yield and clarity by degrading the pectic substances present in the fruit [13]. There are several other applications of pectinases in food, textile, and paper industries and in waste water treatment [14, 15]. Pectinases (pectinolytic enzymes) are classified mainly in three groups: protopectinases, de-esterifying pectinases (pectin esterases) and depolymerizing pectinases (hydrolases and lyases). Pectin and pectate lyases (transeliminases) cleave pectin and pectic acid, respectively, through β-elimination, generating an unsaturated C bond. Polymethyl galacturonase and polygalacturonases, which act on pectin and pectic acid, respectively, are the hydrolytic pectinases. Depolymerizing pectinases act either randomly (endo-) or on the terminal residue (exo-). Pectinases are widely distributed among fungi, bacteria and plants; however, commercial production depends mainly on fungal sources [16]. Pectinolytic enzymes can be produced by several species such as, *Aspergillus, Penicillium, Fusarium, Rhizopus, Trichoderma, Bacillus* and *Erwinia* [17, 18]. *Aspergillus niger* has been the most studied fungus and is

A. O. Buyukkileci · C. Tari (✉)
Department of Food Engineering, İzmir Institute of Technology, Gulbahce Campus, 35430 Urla, İzmir, Turkey
e-mail: canantari@iyte.edu.tr

A. O. Buyukkileci · M. F. Lahore
School of Engineering and Science, Chemical Sciences, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany

the most commonly used fungal species for industrial production of pectinases [16]. It has previously been shown that *A. sojae* can produce exo-polygalacturonase in submerged [8, 19, 20] and solid-state cultures [7, 21].

In submerged culture, fungi exhibit different morphological forms ranging from dispersed mycelial growth to pellets of various sizes. Desired morphology depends on the fungus and the product, e.g., penicillin production by *P. chrysogenum* requires free mycelia, while pellets are required for citric acid production by *A. niger* [22, 23]. The main factors affecting the morphology are: type of strain, pH, temperature, medium composition, aeration, and inoculum size besides the genetic factors. Generally, filamentous growth yields higher biomass with highly viscous broths of non-Newtonian behavior [24]. Due to the high viscosity, higher agitation rates, more power is required to attain desired mixing and oxygen transfer [25]. Pellet morphology has the advantage of exhibiting non-viscous broth with Newtonian behavior, so that better mixing and aeration can be provided and also separation of biomass from the broth is simpler [19]. However, the interior of the pellets based on it compactness degree can be sometimes nutrient and oxygen limited, where growth and product synthesis are impaired [24, 26].

A. sojae was previously shown in submerged cultures to utilize efficiently some agro-industrial products as substrates for exo-PG production [8]. In this study, utilization of OP, a food industrial waste product, was investigated in an attempt to propose an economical exo-PG production process with high yield and desired pellet morphology, which could ease the subsequent downstream processing and decrease the operational cost, encountered in most industrial fungal fermentations. Moreover, developing a very simple and cost-effective media formulation by utilizing the waste and returning in into a value-added product such as the exo-PG would not only reduce the raw material cost but also would serve as an example where similar fungal fermentation is used in the production of other products. Such a medium formulation due to its low pH formation trend would also serve as a solution to microbial contamination problems faced by many industrial fungal fermentations causing high economical losses. Therefore, taking these points into consideration, medium components were optimized and cultural conditions yielding fungal pellets were specified.

Materials and methods

Materials

OP (*Aurantii amari epicarpium et mesocarpium*) (Bombastus–Werke AG, Freital, Germany) was purchased from the local market in Bremen, Germany. All other chemicals were analytical grade and obtained from Applichem (Darmstadt, Germany), Sigma (St. Louis, MO, USA), or Fluka (Steinheim, Germany).

Microorganism

A. sojae mutant derived from *A. sojae* ATCC 20235 by random UV mutation was used in this study. The organism was maintained and sporulated on solid medium at 30 °C for 5 days. This medium was composed of (g/L) glycerol (45), sugar beet syrup (45), peptone (18), NaCl (5), $FeSO_4{\cdot}7H_2O$ (0.015), KH_2PO_4 (0.060), $MgSO_4{\cdot}7H_2O$ (0.050), $CuSO_4{\cdot}5H_2O$ (0.012), $MnSO_4{\cdot}H_2O$ (0.015), and agar (20). Spores were collected and suspended in 0.01 g/L Tween 80. Spore concentration in the suspension was determined by counting the spores on a hemocytometer. A volume of spore suspension required to obtain the desired initial spore concentration was added to broth to initiate fermentation.

Shake flask cultures

Flask cultures were carried out in 250 mL flasks with 50 mL working volume. After sterilization at 121 °C for 15 min, flasks were inoculated with 4.0×10^5 spores/mL, unless otherwise stated. Incubation was done at 30 °C and 250 rpm in shaking incubators (New Brunswick Scientific, NJ, USA).

Preliminary exo-PG production tests were carried out in the presence of OP, maltrin (60 g/L) and AS (8 g/L). Medium optimization studies were conducted accordingly, and selected medium was used in the subsequent fermentations. Effect of spore concentration was investigated both in the optimum medium and also in M2 medium, which was composed of OP (34 g/L), maltrin (140 g/L), AS (8 g/L), $NaH_2PO_4{\cdot}H_2O$ (3.3 g/L) and $Na_2HPO_4{\cdot}2H_2O$ (3.2 g/L). M2 was used as seed culture medium in bioreactor studies.

Bioreactor cultures

The optimum medium was tested in 5 L (Biostat B plus, Sartorius, Gottingen, Germany) and 30 L (Techfors; Infors AG, Bottmingen-Basel, Switzerland) bioreactors with 4 and 18 L working volume, respectively. Five-litre bioreactor was sterilized at 121 °C for 45 min in the autoclave, while 30 L was sterilized in situ at 121 °C for 15 min. The bioreactors were inoculated either directly with spores or with a seed culture (see section "Results and discussion"-"Use of pellets as inoculum" for seed culture conditions). In both bioreactors temperature, stirring speed and aeration were kept at 30 °C, 500 rpm and 0.5 vvm, respectively. In these cultures, pH was not maintained except the constant pH study in 5-L bioreactor, in which pH was kept constant at 4.0 by automatic addition of NaOH (4 N). Batch cultures

Table 1 FCCC experimental design and the results of the first (a) and second (b) optimization study (coded levels are given in parentheses)

Run no.	Factor A: OP (g/L)	Factor B: Maltrin (g/L)	Factor C: AS (g/L)	Response: activity (U/mL)
a				
1	22.5 (0)	120 (+1)	7 (0)	96.20
2	40.0 (+1)	120 (+1)	2 (−1)	97.17
3	22.5 (0)	60 (0)	12 (+1)	100.50
4	22.5 (0)	60 (0)	7 (0)	103.48
5	5.0 (−1)	0 (−1)	2 (−1)	1.37
6	5.0 (−1)	60 (0)	7 (0)	11.21
7	22.5 (0)	60 (0)	7 (0)	94.69
8	22.5 (0)	0 (−1)	7 (0)	56.65
9	40.0 (+1)	0 (−1)	2 (−1)	156.51
10	40.0 (+1)	0 (−1)	12 (+1)	165.94
11	22.5 (0)	60 (0)	2 (−1)	96.67
12	40.0 (+1)	60 (0)	7 (0)	179.45
13	22.5 (0)	60 (0)	7 (0)	120.00
14	22.5 (0)	60 (0)	7 (0)	100.03
15	22.5 (0)	60 (0)	7 (0)	96.84
16	5.0 (−1)	0 (−1)	12 (+1)	8.67
17	5.0 (−1)	120 (+1)	2 (−1)	27.47
18	22.5 (0)	60 (0)	7 (0)	83.00
19	5.0 (−1)	120 (+1)	12 (+1)	20.76
20	40.0 (+1)	120 (+1)	12 (+1)	186.43

Run no.	Factor A: Maltrin (g/L)	Factor B: AS (g/L)	Response: activity (U/mL)
b			
1	0 (−1)	0.5 (−1)	0.2
2	40 (0)	0.5 (−1)	8.8
3	80 (+1)	5 (−1)	100.8
4	40 (0)	2.75 (0)	152.7
5	40 (0)	2.75 (0)	144.0
6	0 (−1)	5 (+1)	140.9
7	80 (+1)	0.5 (−1)	8.8
8	40 (0)	2.75 (0)	130.0
9	40 (0)	5 (+1)	103.4
10	40 (0)	2.75 (0)	116.2
11	0 (−1)	2.75 (0)	152.1
12	80 (+1)	2.75 (0)	154.4
13	40 (0)	2.75 (0)	129.7

in the bioreactors were conducted in duplicates and results of single representative runs were reported.

Two fed-batch cultures with different feeding rates were conducted in the 5-L bioreactor under the conditions similar to that of batch cultures. Fermentations were started as batch with a working volume of 2.5 L inoculated with 5 % seed culture. Fermentation was run batch wise for 24 h, afterwards feeding started and continued until the culture volume reached 4.1–4.4 L. Sterile feed medium formulated according to the results of the medium optimization study was added to the bioreactor via a peristaltic pump.

Analytical methods

At intervals, samples were collected from the shake flasks and bioreactors, centrifuged and kept at 4 °C for enzymatic analysis or at −18 °C for sugar analysis. Total sugar concentration was measured by Phenol–Sulfuric acid method according to DuBois et al. [27]. Sucrose solutions with known concentrations were used as standards.

Exo-PG assay was based on measuring the amount of reducing sugar released upon incubation of the enzyme sample with its polymeric substrate. Culture filtrate

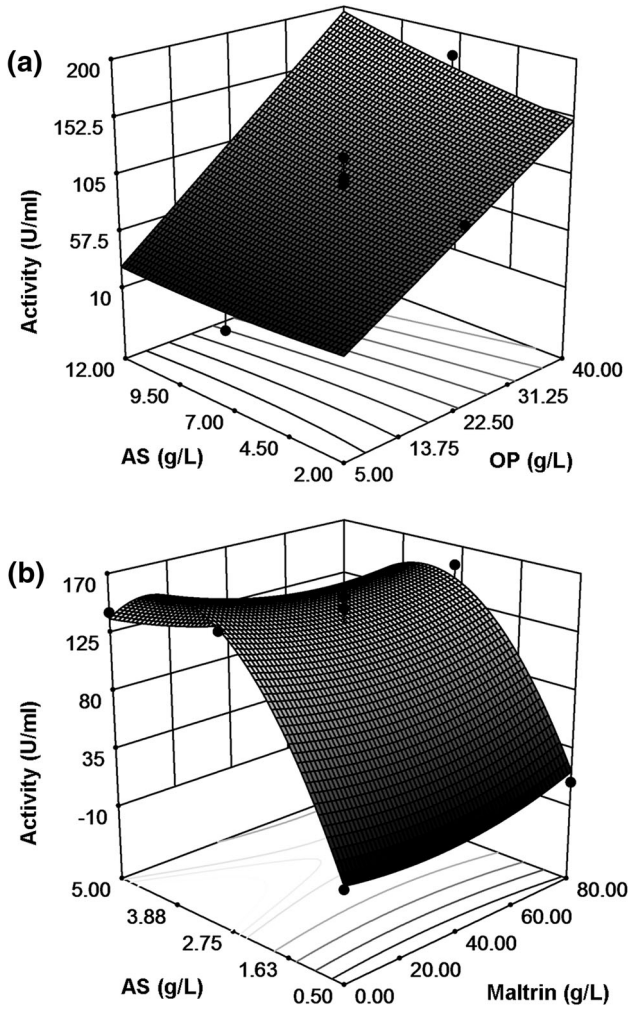

Fig. 1 3D model graph showing the interaction effects of **a** AS and OP in the presence of 60 g/L maltrin **b** of AS and maltrin on exo-PG activity in the first and second optimization studies, respectively

(86 µL) containing exo-PG was mixed with 100 mM acetate buffer (pH 4.8) containing 2.4 g/L of polygalacturonic acid (400 µL) [28]. After incubating at 40 °C in a water bath for 20 min, enzymatic reaction was stopped by immersing the reaction tubes in boiling water for 10 min. Reducing sugar released was quantified by Nelson–Somogyi method using galacturonic acid as the standard [29, 30]. One unit of exo-PG activity was defined as the enzyme that catalyzes the release of 1 µmol of product per unit volume of culture filtrate per unit time under assay conditions.

Experimental design

Medium composition was optimized using response surface methodology, face centered central composite (FCCC) design. Concentrations of the medium components were used as the factors, while exo-PG activity was taken as the response. A second-order polynomial of the following form was fitted,

$$Y = \beta_{\mathrm{o}} + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_j X_i X_j + \varepsilon \qquad (1)$$

where Y is the predicted response, k is the number of factor variables, β_0 is the model constant, β_{i} is the linear coefficient, X_i is the factor variable in its coded form, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and ε is the error factor. The variables were coded according to Eq. 2:

$$x = \frac{\text{actual} \; - \; (\text{low level} + \text{high level})/2}{(\text{high level} \; - \; \text{low level})/2} \qquad (2)$$

The critical significance level was selected as 5 % ($\alpha = 0.05$). The statistics software Design Expert (version 7.1.5 Trial, by STAT-EASE Inc., Minneapolis, USA) was used to design experiments and analyze the data.

Results and discussion

Optimization of culture medium

Initially, two OP concentrations (25 and 50 g/L) were tested in the presence of maltrin and AS. Maltrin, which was composed of maltodextrins and corn syrup solids, was used successfully as a carbon source for *A. sojae* in previous studies [20, 31, 32]. Exo-PG activities obtained with 25 and 50 g/L of OP were 85.0 and 109.7 U/mL, respectively. Although activity was higher, the fermentation broth was too viscous using 50 g/L of OP. Therefore, in the following optimization study, the upper limit of OP concentration was set to 40 g/L. FCCC design with the coded and actual levels of the factors and the activities after 120 h are shown in Table 1a. According to the ANOVA results, the significant ($p < 0.05$) terms besides the model were the OP and AS concentrations and their interactions and the quadratic term of maltrin concentration. The resulting model equation in terms of coded factors was as follows:

$$\begin{aligned} \text{Activity} \; = \; & 106.88 \; + \; 79.09\,A \; + \; 2.38\,B \; + \; 11.75\,C \\ & - \; 8.76\,A\,B \; + \; 12.39\,A\,C \; + \; 7.63\,B\,C \\ & - \; 0.045\,A^2 \; - \; 9.17\,B^2 \; + \; 1.61\,C^2 \end{aligned} \qquad (3)$$

According to the model, exo-PG activity increased linearly with OP concentration in the range of this design (5–40 g/L) (Fig. 1a). Although a similar trend was observed for the concentrations of AS, a careful inspection of the results showed that even the lowest amount of AS yielded considerable activity values. Interestingly, OP supplemented with sugar beet syrup was previously shown to have an adverse effect on exo-PG production by the

Table 2 Design and results of validation experiments in shake flask cultures (250 mL flasks with 50 mL working volume)

Run number	OP (g/L)	Maltrin (g/L)	AS (g/L)	Predicted activity (U/mL)	Measured activity (U/mL)
1	40	4.0	4.55	149.4	143.3
2	40	26.3	3.51	149.6	138.6
3	40	0.0	2.75	153.9	141.2
4	40	80.0	2.75	144.2	140.1

Activities are average of two runs

same strain when used at concentrations above 10 g/L [8]. Similarly, based on a study conducted by Nighojkar et al. [33], PG production by alginate immobilized *A. niger* in submerged culture decreased when OP concentration was above 15 g/L. Teixeira et al. [34] also observed decreased exo-PG production by *A. japonicus* when the pectin concentration in the liquid medium was increased from 5 to 10 g/L. On the other hand, Galiotou-Panayotou et al. [35] reported a consistent increase in PG activity with the increase of pectin concentrations up to 30 g/L.

According to the results of the first optimization study, another RSM design (FCCC) was conducted, in which OP concentration was kept constant at 40 g/L and the ranges of maltrin and AS concentrations were narrowed down to 0–80 and 0.5–5.0 g/L, respectively (Table 1b). Based on the results of this study, the model was significant and the lack of fit was insignificant at the 95 % confidence level. There was a good agreement between the predicted (0.887) and the adjusted R^2 (0.951) terms. The linear AS and the quadratic AS^2 terms were the factors affecting the activity significantly ($p < 0.05$). The model equation of the second design was as follows:

$$\text{Activity} = 136.21 - 4.85A + 54.53B - 12.17AB + 12.87A^2 - 84.29B^2 \quad (4)$$

Model plots showed that maximum activity lied in the AS concentration range of 2.75–5 g/mL (Fig. 1b). Maltrin did not have a significant influence on the activity in the ranges (0–80 g/L) studied (Fig. 1b). Validity of the model was tested by conducting experiments at four different conditions selected to obtain high activity based on the model graph (Table 2; Fig. 1b) and according to the recommendations of the software. The measured values were in good agreement with the predicted ones (Table 2). In the presence of 40 g/L OP and 2.75 g/L AS, 141.2 U/mL exo-PG could be produced without addition of maltrin. This showed that, a simple medium, OP supplemented with AS, was sufficient to yield significant amount of PG. No additional carbon source was needed to support the growth of the microorganism and PG production, which showed that the soluble sugar content of the OP was sufficient. Around 23–25 % of the dry weight of OP is the soluble (non-structural) carbohydrates composed mainly of glucose, fructose and sucrose [10, 11, 36–38]. OP also contains insoluble cellulose, hemicellulose as well as pectin at appreciable amounts [10, 38]. These may have been hydrolyzed during the cultivation by the probable hydrolytic enzymes of *A. sojae* such as cellulases or hemicellulases, to yield additional simple sugars. Apart from that, the complex nature of the OP eliminated the addition of mineral salts or high amount of nitrogen sources to the culture medium [2]. El-Sheekh et al. [5] also used OP powder efficiently as the sole carbon source for pectinase production by *A. carneus*, while no activity was detected when the OP was replaced by mono- and disaccharides. Same authors also demonstrated that pectinase activity was stimulated, whereas cellulase and hemicellulase activities were inhibited by AS [5]. In another study, the same amount of (15 g/L) OP and pectin yielded similar PG activity values; however, activity was less when they were supplemented by glucose, peptone and yeast extract [33].

Effect of spore concentration

In the above-mentioned shake flask cultures *A. sojae* grew in free dispersed mycelial form, which resulted in a culture broth with high apparent viscosity [24]. This phenomenon was discussed in the previous reports of our group and *A. sojae* was shown to form pellets in submerged culture under the optimized conditions [19, 20, 32]; whereas in another study, the same organism formed small mycelial clumps (small groups of entangled mycelia) in the presence of OP and sugar beet syrup as substrates [8]. It is well known that inoculum size is one of the main factors influencing the growth form and product formation in fungal cultures [22, 39, 40]. Therefore, the effect of inoculated spore concentration on the morphology and exo-PG activity was investigated in OP-AS and in M2 medium, in an effort to obtain a pellet forming, less viscous and high exo-PG producing condition. The latter medium was optimized previously for exo-PG production by the same strain [31]. These two media were inoculated with 4.0×10^5, 4.0×10^4 or 2.8×10^3 spores/mL of culture volume. Inoculum size influenced two cultures differently (Table 3; Fig. 2). In OP-AS medium, free mycelia or mycelial clumps were the dominant morphology at high inoculum sizes. On the other hand, fluffy pellet formation was observed at low inoculum size; however, there were free mycelia in the broth as well as the pellets. In M2 medium, pellets were formed almost at all inoculums levels; however, at the low level, small compact pellets were dominant with only small amount of free mycelia around the pellets (Fig. 2). Exo-PG activity in the OP-AS medium

Table 3 Effect of spore concentration on PG activity and morphology in shake flask cultures (250 mL flasks with 50 mL working volume)

Medium	Spore concentration (spores/mL)	Activity after 72 h (U/mL)	Activity after 120 h (U/mL)	Morphology
OP-AS	4.0×10^5	83.1	141.2	Free mycelia
OP-AS	4.0×10^4	79.7	137.1	Mycelial clumps
OP-AS	2.8×10^3	72.2	138.7	Pellets–Free mycelia
M2	4.0×10^5	11.8	50.2	Large pellets–clumps
M2	4.0×10^4	20.3	80.8	Clumps
M2	2.8×10^3	65.1	155.7	Pellets

Activities are average of two runs

was not influenced by the inoculum size; 141.2 and 138.7 U/mL PG activities were observed after 120 h at high and low inoculum sizes, respectively (Table 3). In the M2 medium, the effect was drastic; the exo-PG activity increased significantly as the spore concentration decreased (Table 3). At low inoculum level, 155.7 U/mL PG activity could be obtained, which was ca. 12 % higher compared to levels obtained in OP-AS medium. Comparison of the enzyme activity and the morphology in the shake flask cultures showed that formation of pellets in OP-AS medium did not improve the exo-PG activity, whereas in M2 medium more enzyme activity was observed when the pellets were smaller. Considering the high exo-PG production levels and pellet growth, the spore concentration of 2.8×10^3 spores/mL was concluded to be the best inoculum size for both media in the ranges investigated. The enzyme activity trend observed in M2, but not in OP-AS medium, was in accordance with the results of Friedrich et al. [41], who observed decreased PG activity with increasing inoculum size in *A. niger* cultures, while pectin lyase showed the opposite relation. Same authors added potassium hexacyanoferrate II hydrate to culture medium and morphology changed from loose pellets to smaller compact ones associated with a marked increase in PG enzyme activity [41].

The effect of spore concentration was also tested in the 5-L bioreactor with a working volume of 4 L. The OP-AS medium was inoculated with either 4.0×10^5 or 2.8×10^3 spores/mL, where exo-PG activity (Fig. 3a) and pH and dissolved O_2 (Fig. 3b) profiles were compared. At high inoculum size, activity was 80 U/mL after 120 h and increased slightly to 94 U/mL in the next 48 h. These values were approximately 40 % lower than the ones obtained in the shake flask cultures using the same medium and the same inoculum concentration. The initial pH of the

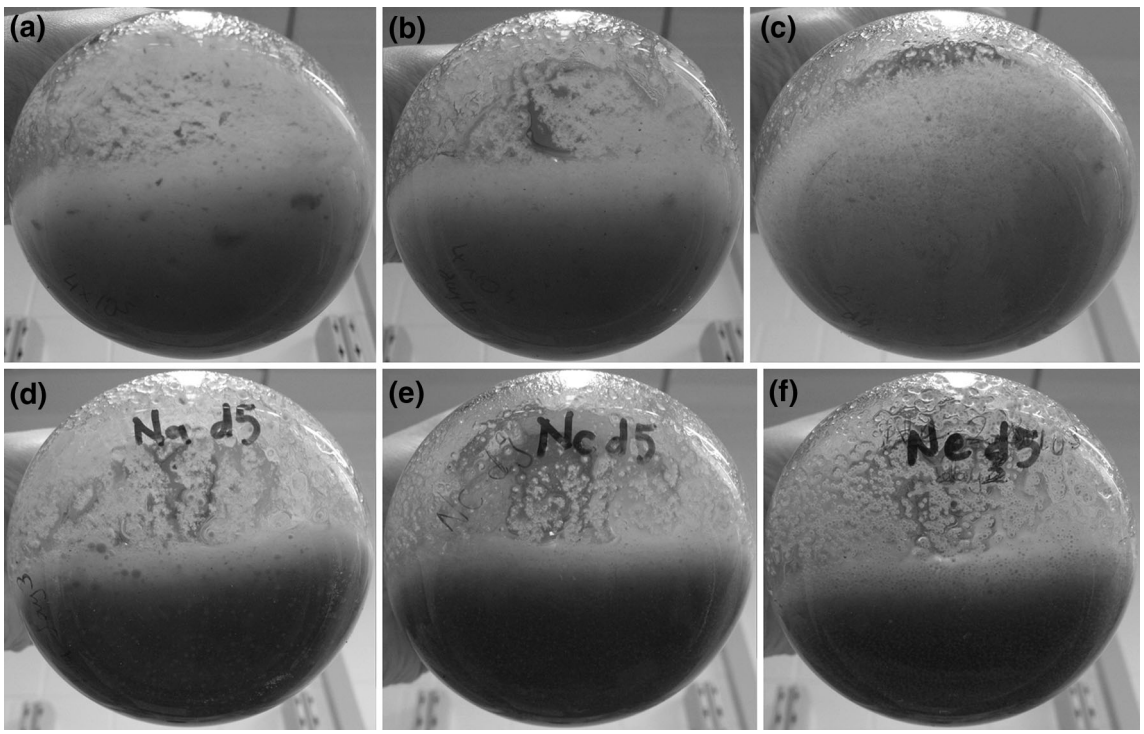

Fig. 2 Flask photographs showing the effect of spore concentration on morphology after 96 h. **a**, **b**, and **c** OP-AS medium; **d**, **e**, and **f** M2 medium; **a**, **d** 4×10^5 spores/mL; **b**, **e** 4×10^4 spores/mL; **c**, **f** 2.8×10^3 spores/mL

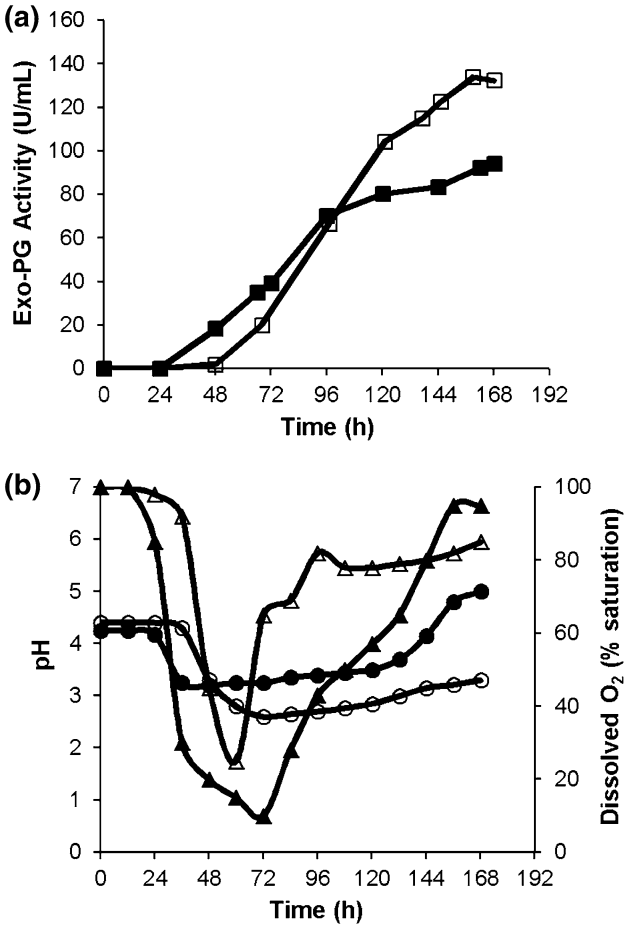

Fig. 3 Effect of inoculum size on PG production in OP-AS medium in the 5-L bioreactor. **a** Exo-PG activity, **b** pH (*circles*) and dissolved O_2 (*triangles*). *Closed marks* 4×10^5 spores/mL, *open marks* 2.8×10^3 spores/mL

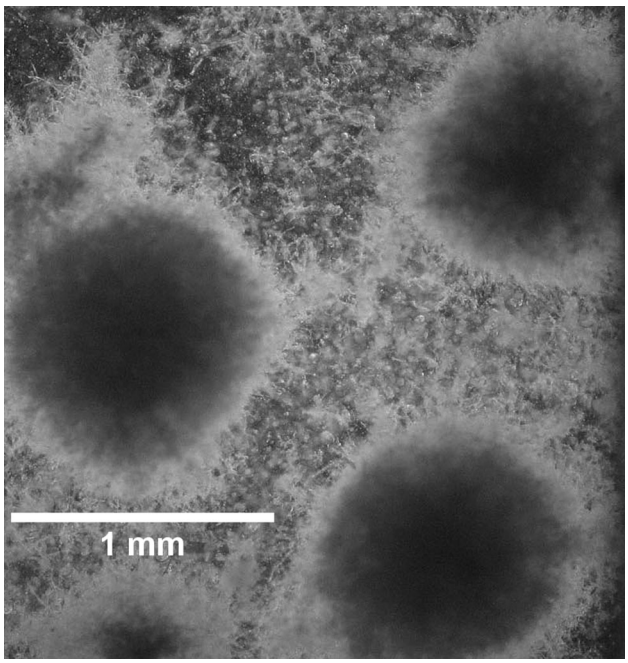

Fig. 4 Appearance of fungal pellets and free mycelia under microscope after 96 h in the 5-L bioreactor inoculated with 2.8×10^3 spores/mL

medium was around 4.3 and decreased sharply between 24 and 36 h to 3.2. Starting from 48 h, pH increased slowly to 5 in the next 120 h. Similarly, dissolved O_2 showed a sharp decrease between 24 and 72 h and increased afterwards. At low inoculum size, on the other hand, enzyme production started later, but the activity increased to 134.1 U/mL in 160 h. pH trend was similar to the high inoculum size case, but the values were slightly lower. Dissolved O_2 trend was also similar; however, the increase following the sharp decrease was rapid, so that level was generally higher than the former case. Decreasing the inoculum size resulted in approximately 45 % increase in the exo-PG activity in the bioreactor, whose effect was more pronounced after 96 h (Fig. 3a). The lag phase of enzyme production with low inoculum size was longer than the one with larger inoculum size shown in Fig. 3a; however, the maximum exo-PG productions of two fermentation process both reached their maximum level simultaneously. In the early phase of the low inoculum size culture, biomass concentration was probably low as a result of the low number of inoculated spores, thus no exo-PG was detected in the first 48 h (Fig. 3a). On the other hand, enzyme production rate was high in this culture compared to one in high inoculum case (Fig. 3a). Although enzyme production started earlier in the latter case, the low dissolved O_2 levels (Fig. 3b) may have inhibited the enzyme production, thus resulted in a lower production rate. As a result of these, the maximum exo-PG productions were obtained in similar time points. Exo-PG production rates between 48 and 120 h were calculated as 1.31 and 0.91 U/mL h for the low and high inoculum sizes, respectively. In the same medium, the tested inoculum sizes had an influence on the activity levels in the fermenter, unlike in the shake flasks. This could be attributed to different aeration and mixing regimes in two systems pronouncing the effect of inoculum sizes (Table 3; Fig. 3a).

At high inoculum size, free and clumped mycelia were observed, while at low inoculum size pellets were the dominant growth form. Pellets had compact cores and hairy regions in the outer parts and there were free mycelia surrounding the pellets (Fig. 4). Growth form in the former case created mixing problems in the bioreactor, which was apparent by the poorly mixed zones in the broth in high inoculum size culture. Inefficient mixing and probably higher amount of biomass in the high inoculum case resulted in poor aeration of the broth, as can be deduced by the lower dissolved O_2 profile [22]. Consequently, enzyme

production was impaired in this culture. In the latter case, the problem was not so severe, although there were poorly mixed regions in the culture broth as well, probably due to the free mycelia associated with the pellets (Fig. 4). However, it should not be overlooked that enhancement of PG production in pellet form can be partly a result of altered cellular metabolism and enzyme secretion due to limitations in oxygen and nutrient supply and in removal of metabolic products [42].

Tari et al. [20] optimized the culture conditions for *A. sojae* ATCC20235 and observed increased PG activity with increasing inoculum size. Maximum PG activity (13.5 U/mL) was achieved at an inoculum size of 4×10^5 spores/mL, where the growth was as small pellets. This contradictory result can be explained by the presence of a less viscous broth containing simple carbon sources (maltrin and glucose) in that study. In this study, however, the OP generated a viscous broth, so that pelleted growth, thus sufficient mixing and aeration could only be obtained at a lower inoculum size (2.8×10^3 spores/mL). In another study, where agro-based products were utilized for exo-PG production by *A. sojae* M5/6, the inoculum size of 4×10^5 spores/mL resulted in mycelial clumps and viscous culture broths in the presence of all substrates [8]. Hermersdörfer et al. [42] observed increased PG synthesis by *A. niger* in a synthetic medium when it grew as a compact mycelia, which was defined as a large ball with a diameter of 40 mm. The PG activity was considerably lower when the growth was in the form of either diffuse mycelia or pellets of 2–10 mm in diameter [42]. A similar trend was observed for *R. oryzae* where low inoculum size induced compact large mycelial mass and high lactate production, whereas the fungus formed dispersed mycelia and produced low lactate but high ethanol at high inoculum sizes [39]. Couri et al. [43], on the other hand, explained the higher PG synthesis by the loose pellet morphology of *A. niger* induced by the addition of some metal ions. Therefore, the relation between the morphology and product synthesis in fungal cultures is complex and depends on the strain and culture conditions, among other factors.

Use of pellets as inoculum

In the above study pH, dO_2 and exo-PG activity profiles showed that in the first 36–48 h of the low inoculum size run there was only a little microbial activity in the culture (Fig. 3a, b). In this part of the study, a seed culture was used to inoculate the bioreactor in an effort to decrease the length of the lag phase. When the effect of spore concentration was investigated, small pellets were obtained in M2 medium (Table 3; Fig. 2). These pellets had the potential to be used as an inoculum for exo-PG production in OP-AS medium. In fact, high levels of exo-PG could be obtained

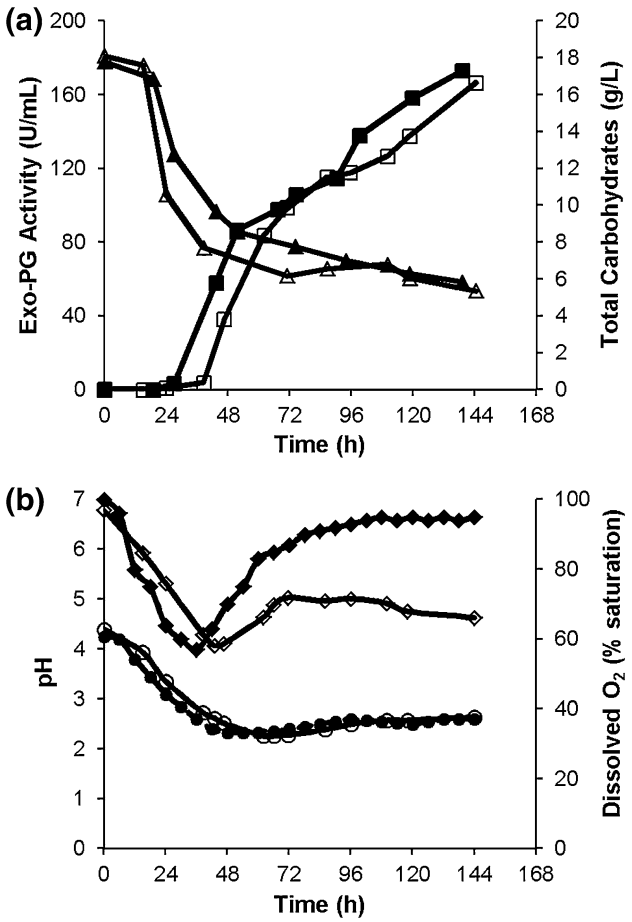

Fig. 5 **a** Activity (*squares*) and carbohydrate utilization (*triangles*), and **b** pH (*circles*) and dO_2 (*rhombs*) profiles of 4 L (*closed marks*) and 18 L (*empty marks*) batch cultures inoculated with seed culture

in M2 medium itself; however, OP-AS medium was a simpler and more economical medium.

A. sojae mutant was grown in the form of pellets in M2 medium inoculated initially with 2.8×10^3 spores/mL in shake flask for 48 h and 200 mL of this culture was added as a seed culture to the 5-L bioreactor containing 3.8 L OP-AS medium. Similarly, 30-L bioreactor with a working volume of 18 L was inoculated with 900 mL of the same seed culture. Throughout the cultivations in the bioreactors inoculated by pelleted fungus, pellets were the dominant morphology. There were a few free mycelia as well; however, there was no apparent mixing problem. Cultures at two scales followed similar activity, sugar utilization (Fig. 5a), and pH trends (Fig. 5b), while there was a difference in dO_2 levels (Fig. 5b) in the later stages of the cultivations. Exo-PG activity started earlier compared to spore-inoculated case and showed a steep increase at a rate of 3.3 U/mL h after 24–36 h of inoculation. After 96 h, enzyme levels at both scales were around 120 U/mL. The endpoint exo-PG activities were measured as 173.0 U/mL after 139 h and 166.5 U/mL after 144.5 h in 4 and 18 L

Table 4 Feeding profiles of fed-batch cultures

Time (h)	Feeding rate (mL/h)	
	Fed-batch 1 (FB1)	Fed-batch 2 (FB2)
0–24	0	0
24–48	21.6	10.8
48–72	27.0	13.5
72–96	32.4	16.2
96–120	–	18.9
120–128	–	21.6

cultures, respectively. Thus, using a seed culture, in which *A. sojae* formed pellets, enhanced the exo-PG level and the productivity.

The fast increase in enzyme activity was associated with the fast sugar utilization. Initial concentration of total sugars was 18 g/L which decreased sharply in 48 h to 5 g/L sugar remaining unutilized until the end of the fermentation (Fig. 5a). Similar declining trend was observed for both pH and dO_2 profiles after which pH stabilized around 2.5 until the end of the cultivation (Fig. 5b). The decrease in pH can be attributed to the organic acids produced by the organism as metabolic end products. dO_2 level decreased to 60 % of saturation in 2 days and increased afterwards. These indicated that microbial activity stopped or continued at a very low rate after 48–72 h. These can be explained by the low pH (2.5), which was below the physiological values. On the other hand, exo-PG production did not stop at low pH, but continued at a lower rate compared to that of the first phase. Overall productivity value was calculated as 1.2 U/mL h and the yield based on the final exo-PG level and the initial OP amount was around 4,200 U/g OP.

When the culture pH was kept constant at 4, the sugar utilization followed a similar trend to the uncontrolled pH run for the first 72 h; however, it continued in the next 72 h and most of the sugar was utilized unlike the uncontrolled pH run. dO_2 decreased sharply to zero in about 72 h and increased after 96 h. These indicated a prolonged activity of the organism compared to uncontrolled pH run. However, the maximum attainable activity was 26.6 U/mL and the secondary increase phase in activity, which was apparent at low pH after the sugar utilization terminated, was not observed at pH 4.

It was previously shown that exo-PG production by *A. sojae* was induced at low pH values obtained under uncontrolled pH run [8]. Fontana et al. [13] observed very similar kinetics for PG production by *A. oryzae* in a liquid medium containing wheat bran and citrus pectin. In the stirred tank reactor, pH and dO_2 decreased sharply in 24–36 h and increased afterwards. Cell concentration did not increase after around 30–40 h, while both exo- and endo-PG productions continued at least for 96 h [13]. Malvessi et al. [44] also obtained highest PG activity by *A. oryzae* at an initial pH of 4, where pH was slightly below 3 between 24 and 72 h.

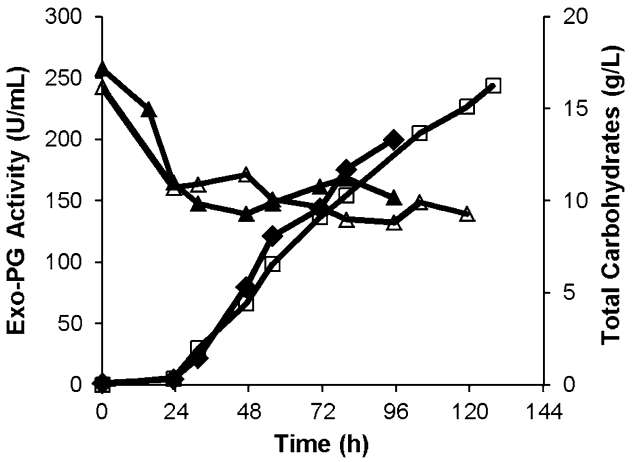

Fig. 6 Exo-PG activity (*squares*) and total carbohydrate (*triangles*) profiles of fed-batch cultures; *closed marks* FB1, *open marks* FB2

Fed-batch culture

As indicated above, exo-PG level increased with increasing OP concentration; however, high OP concentrations generated highly viscous broths. Therefore, its level was limited to 40 g/L throughout the study. In an effort to overcome this limitation, fed-batch cultures were conducted. The feed OP concentration was set to 80 g/L, above which concentration the medium in the feeding tank was too viscous that it could not be agitated by the magnetic stirrer and fed using the peristaltic pump. Two different feeding modes were used (Table 4); in the fast feeding run (FB1), cultivation was terminated 96 h after inoculation, while slow feeding (FB2) allowed a culture time of 127 h. Feedings were stopped when the culture volumes increased to 4.1–4.4 L in the 5-L bioreactor.

Two runs followed similar fermentation kinetics (Fig. 6). Total carbohydrate concentration decreased to 10 g/L in 24 h and remained at this value until the end of the fermentation. Thus, it was higher during the fed-batch culture compared to batch cultures (Fig. 5a). Exo-PG activity followed almost the same trend in two cultures; however, an extended production was achieved in FB2 due to prolonged fermentation time. The endpoint exo-PG activities were 200 U/mL after 96 h in FB1 and 244 U/mL after 127 h in FB2 (Fig. 6). Exo-PG productivities were calculated for the total cultivation time as 2.1 and 1.9 U/mL h for FB1 and FB2, respectively. These values were considerably higher than the ones obtained in batch

fermentations. On the other hand, product yield was higher in the batch culture. In FB1 and FB2, total of 295 and 267 g OP was supplied including the batch phase, which correspond to yield values of 3012 and 3738 U/g OP, respectively. pH and dO_2 trends were similar to batch cultures. pH decreased to 2.5 in 48 h, whereas dO_2 showed a minimum between 24 and 36 h (50 % saturation) and increased slowly to 80 % saturation in time.

Results showed that exo-PG activity was 1.4-fold higher in fed-batch culture than in batch culture (Figs. 5a, 6). Slow feeding rate used in FB2 was sufficient to obtain enhanced enzyme production. The lower enzyme titer in the batch culture can be explained by depletion of a nutrient, which was overcome by addition of the nutrients in the fed-batch culture. Depletion of nutrients in the batch culture, rather than low pH, can also be the reason for decreased production rates, since similar low pH values did not result in a rate decline in fed-batch culture. Although higher total carbohydrate levels were obtained in fed-batch cultures, AS or nutrients other than carbohydrates provided by the complex nature of OP could be the reason for enhanced exo-PG production. Enzyme yield, which is an industrially important parameter, was higher in batch cultures although end point enzyme level was low, because of the low utilized OP amount. However, considering the low cost of OP, low yield in fed-batch culture could be ignored, since the final exo-PG activity and the productivity values were considerable higher compared to batch culture.

The enzyme levels obtained in this study were higher compared to the ones reported in most of the other reports. Favela-Torres et al. [17] compared 24 studies in which exo-PG was produced from pectin, glucose or plant biomass using several different microorganisms in submerged fermentations. Among the activities reported, only three of them (162, 221 and 239 U/mL, respectively) were comparable to the ones obtained in this study, while in one study 500 U/mL exo-PG was reported [34, 45–47]. Most of the other reported values were one order of magnitude lower. In previous studies of our group, 145 U/mL exo-PG was produced in a more complex medium containing sugar beet syrup and OP [8] and 93.5 U/mL exo-PG was produced from OP, supplemented with maltrin and phosphate salts [31] using the same *A. sojae* strain. Corresponding productivity values in fungal cultures were generally below 2 U/mL h, even if high activity values were obtained [8, 31, 46, 47], except that *A. japonicus* produced exo-PG at a rate of 10.41 U/mL h [34] In a more recent study, 230 U/mL exo-PG activity could be obtained by *A. carbonarius* in 24 h, which also resulted in exceptionally high productivity values (9.6 U/mL h) [48]. Rangarajan et al. [49] utilized OP extract successfully as a substrate for exo-PG production in submerged culture using *A. niger* and reported a yield of 6,800 U/g of OP used. *A. giganteus* produced 48.5 U/mL PG on orange bagasse [50], while activity was below 1 U/mL with immobilized *A. niger* growing on OP [33]. It should be noted that, unlike most of the other studies, in this study, a single low cost substrate (OP), which was supplemented solely with a simple inorganic N source (AS), was utilized.

Conclusion

A very simple and cost-effective medium was defined for exo-PG production by *A. sojae*. The medium was composed of OP, which is a food industrial by-product, and an inorganic nitrogen source, AS and no other sugar or mineral salt source were necessary. The problem of mixing and aeration was overcome by inducing pellet formation obtained by decreasing the inoculated spore concentration. Further enhancement of reactor performance and exo-PG activity was achieved using a seed culture, in which *A. sojae* was pre-grown in the form of small pellets, to inoculate the bioreactors containing OP-AS medium. Fed-batch culture prevented depletion of nutrients and allowed higher exo-PG activity and productivity than the batch culture. Low operational pH was also advantageous, since it eliminated the pH maintenance and decreased the risk of bacterial contamination. Thus, the proposed process for exo-PG production has the potential to be applied to industrial scale easily.

Acknowledgments This work was supported by TÜBITAK (Turkey) and BMBF (Germany) through the Intensified Cooperation Project of IntenC 107O602 and EUROTRANSBIO, PGSYS/ETB-2008-44.

References

1. Marin FR, Soler-Rivas C, Benavente-Garcia O, Castillo J, Perez-Alvarez JA (2007) By-products from different citrus processes as a source of customized functional fibres. Food Chem 100:736–741
2. Lopez JAS, Li Q, Thompson IP (2010) Biorefinery of waste orange peel. Crit Rev Biotechnol 30:63–69
3. Mamma D, Kourtoglou E, Christakopoulos P (2008) Fungal multienzyme production on industrial by-products of the citrus-processing industry. Bioresour Technol 99:2373–2383
4. Giese EC, Dekker RFH, Barbosa AM (2008) Orange bagasse as substrate for the production of pectinase and laccase by *Botryosphaeria rhodina* MAMB-05 in submerged and solid state fermentation. Bioresources 3:335–345
5. El-Sheekh MM, Ismail AMS, El-Abd MA, Hegazy EM, El-Diwany AI (2009) Effective technological pectinases by *Aspergillus carneus* NRC1 utilizing the Egyptian orange juice industry scraps. Int Biodeterior Biodegrad 63:12–18
6. Tao NG, Shi WQ, Liu YJ, Huang SR (2011) Production of feed enzymes from citrus processing waste by solid-state fermentation with *Eupenicillium javanicum*. Int J Food Sci Technol 46:1073–1079

7. Heerd D, Yegin S, Tari C, Fernandez-Lahore M (2012) Pectinase enzyme-complex production by *Aspergillus* spp. in solid-state fermentation: a comparative study. Food Bioprod Process 90:102–110
8. Buyukkileci AO, Tari C, Fernandez-Lahore M (2011) Enhanced production of exo-polygalacturonase from agro-based products by *Aspergillus sojae*. Bioresources 6:3452–3468
9. Oberoi HS, Vadlani PV, Nanjundaswamy A, Bansal S, Singh S, Kaur S, Babbar N (2011) Enhanced ethanol production from Kinnow mandarin (*Citrus reticulata*) waste via a statistically optimized simultaneous saccharification and fermentation process. Bioresour Technol 102:1593–1601
10. Pourbafrani M, Forgacs G, Horvath IS, Niklasson C, Taherzadeh MJ (2010) Production of biofuels, limonene and pectin from citrus wastes. Bioresour Technol 101:4246–4250
11. Wilkins MR, Widmer WW, Grohmann K (2007) Simultaneous saccharification and fermentation of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol. Process Biochem 42:1614–1619
12. Wilkins MR, Widmer WW, Grohmann K, Cameron RG (2007) Hydrolysis of grapefruit peel waste with cellulase and pectinase enzymes. Bioresour Technol 98:1596–1601
13. Fontana RC, Polidoro TA, da Silveira MM (2009) Comparison of stirred tank and airlift bioreactors in the production of polygalacturonases by *Aspergillus oryzae*. Bioresour Technol 100:4493–4498
14. Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. Bioresour Technol 77:215–227
15. Ribeiro DS, Henrique SMB, Oliveira LS, Macedo GA, Fleuri LF (2010) Enzymes in juice processing: a review. Int J Food Sci Tech 45:635–641
16. Jayani RS, Saxena S, Gupta R (2005) Microbial pectinolytic enzymes: a review. Process Biochem 40:2931–2944
17. Favela-Torres E, Volke-Sepulveda T, Viniegra-Gonzalez G (2006) Production of hydrolytic depolymerising pectinases. Food Technol Biotechnol 44:221–227
18. Zeni J, Cence K, Grando CE, Tiggermann L, Colet R, Lerin LA, Cansian RL, Toniazzo G, de Oliveira D, Valduga E (2011) Screening of pectinase-producing microorganisms with polygalacturonase activity. Appl Biochem Biotechnol 163:383–392
19. Gogus N, Tari C, Oncu S, Unluturk S, Tokatli F (2006) Relationship between morphology, rheology and polygalacturonase production by *Aspergillus sojae* ATCC 20235 in submerged cultures. Biochem Eng J 32:171–178
20. Tari C, Gögus N, Tokatli F (2007) Optimization of biomass, pellet size and polygalacturonase production by *Aspergillus sojae* ATCC 20235 using response surface methodology. Enzyme Microb Technol 40:1108–1116
21. Ustok FI, Tari C, Gogus N (2007) Solid-state production of polygalacturonase by *Aspergillus sojae* ATCC 20235. J Biotechnol 127:322–334
22. Pazouki M, Panda T (2000) Understanding the morphology of fungi. Bioprocess Eng 22:127–143
23. Paul GC, Priede MA, Thomas CR (1999) Relationship between morphology and citric acid production in submerged *Aspergillus niger* fermentations. Biochem Eng J 3:121–129
24. Papagianni M (2004) Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol Adv 22:189–259
25. Amanullah A, Christensen LH, Hansen K, Nienow AW, Thomas CR (2002) Dependence of morphology on agitation intensity in fed-batch cultures of *Aspergillus oryzae* and its implications for recombinant protein production. Biotechnol Bioeng 77:815–826
26. Grimm LH, Kelly S, Krull R, Hempel DC (2005) Morphology and productivity of filamentous fungi. Appl Microbiol Biotechnol 69:375–384
27. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
28. Panda T, Naidu GSN, Sinha J (1999) Multiresponse analysis of microbiological parameters affecting the production of pectolytic enzymes by *Aspergillus niger*: a statistical view. Process Biochem 35:187–195
29. Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 153:375–380
30. Somogyi M (1952) Notes on sugar determination. J Biol Chem 195:19–23
31. Gogus N, Taze BH, Demir H, Tari C, Unluturk S, Lahore MF (2014) Evaluation of orange peel, an industrial waste, for the production of Aspergillus sojae polygalacturonase considering both morphology and rheology effects. Turk J Biol 38:537–548
32. Oncul S, Tari C, Unluturk S (2007) Effect of various process parameters on morphology, rheology, and polygalacturonase production by *Aspergillus sojae* in a batch bioreactor. Biotechnol Prog 23:836–845
33. Nighojkar S, Phanse Y, Sinha D, Nighojkar A, Kumar A (2006) Production of polygalacturonase by immobilized cells of *Aspergillus niger* using orange peel as inducer. Process Biochem 41:1136–1140
34. Teixeira MFS, Lima JL, Duran N (2000) Carbon sources effect on pectinase production from *Aspergillus japonicus* 586. Braz J Microbiol 31:286–290
35. Galiotou-Panayotou M, Rodis P, Kapantai M (1993) Enhanced polygalacturonase production by *Aspergillus niger* NRRL-364 grown on supplemented citrus pectin. Lett Appl Microbiol 17:145–148
36. Grohmann K, Cameron RG, Buslig BS (1995) Fractionation and pretreatment of orange peel by dilute acid hydrolysis. Bioresour Technol 54:129–141
37. Talebnia F, Pourbafrani M, Lundin M, Taherzadeh MJ (2008) Optimization study of citrus waste saccharification by dilute-acid hydrolysis. Bioresources 3:108–122
38. Widmer W, Zhou WY, Grohmann K (2010) Pretreatment effects on orange processing waste for making ethanol by simultaneous saccharification and fermentation. Bioresour Technol 101:5242–5249
39. Buyukkileci AO, Hamamci H, Yucel M (2006) Lactate and ethanol productions by *Rhizopus oryzae* ATCC 9363 and activities of related pyruvate branch point enzymes. J Biosci Bioeng 102:464–466
40. Papagianni M, Moo-Young M (2002) Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. Process Biochem 37:1271–1278
41. Friedrich J, Cimerman A, Steiner W (1990) Production of pectolytic enzymes by *Aspergillus niger*—effect of inoculum size and potassium hexacyanoferrate II-trihydrate. Appl Microbiol Biotechnol 33:377–381
42. Hermersdorfer H, Leuchtenberger A, Wardsack C, Ruttloff H (1987) Influence of culture conditions on mycelial structure and polygalacturonase synthesis of *Aspergillus niger*. J Basic Microbiol 27:309–315
43. Couri S, Pinto GAS, de Senna LF, Martelli HL (2003) Influence of metal ions on pellet morphology and polygalacturonase synthesis by *Aspergillus niger* 3T5B8. Braz J Microbiol 34:16–21
44. Malvessi E, da Silveira MM (2004) Influence of medium composition and pH on the production of polygalacturonases by *Aspergillus oryzae*. Braz Arch Biol Tecn 47:693–702

45. Beg QK, Bhushan B, Kapoor M, Hoondal GS (2000) Effect of amino acids on production of xylanase and pectinase from Streptomyces sp QG-11-3. World J Microbiol Biotechnol 16:211–213
46. Hadj-Taieb N, Ayadi M, Trigui S, Bouabdallah F, Gargouri A (2002) Hyperproduction of pectinase activities by a fully constitutive mutant (CT1) of *Penicillium occitanis*. Enzyme Microb Technol 30:662–666
47. Villas-Boas SG, Esposito E, de Mendonca MM (2002) Novel lignocellulolytic ability of *Candida utilis* during solid-substrate cultivation on apple pomace. World J Microbiol Biotechnol 18:541–545
48. Nakkeeran E, Gowthaman MK, Umesh-Kumar S, Subramanian R (2012) Techno-economic analysis of processes for *Aspergillus carbonarius* polygalacturonase production. J Biosci Bioeng 113:634–640
49. Rangarajan V, Rajasekharan M, Ravichandran R, Sriganesh K, Vaitheeswaran V (2010) Pectinase production from orange peel extract and dried orange peel solid as substrates using *Aspergillus niger*. Int J Biotechnol Biochem 6:445–453
50. Pedrolli D, Gomes E, Monti R, Carmona E (2008) Studies on productivity and characterization of polygalacturonase from *Aspergillus giganteus* submerged culture using citrus pectin and orange waste. Appl Biochem Biotechnol 144:191–200