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Short communication

Bioconversion of wheat bran for polygalacturonase production by *Aspergillus sojae* in tray type solid-state fermentation

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ABSTRACT

Wheat bran was tested as the solid substrate for the tray-type solid-state fermentation (SSF) production of polygalacturonase (PG) enzyme by *A. sojae* mutant strain – a high-PG activity producer. PG production of *A. sojae* was found to reduce as the thickness of the substrate increase from 8 mm to 14 mm at 90% relative humidity. An interaction between the thickness of the bed and relative humidity of the environment was determined with the help of experimental design and statistical analysis tools. As a result, the PG activity could be enhanced by 31% as the process conditions optimized. Additionally, 11 mm thickness and 70% relative humidity were selected as the PG production favoring conditions with the maximum PG activity of 298 U/g substrate in tray type of SSF without the addition of any nutritive or inducing supplements into wheat bran. The kinetic study conducted in the trays revealed the presence of reduction in the water activity on the 4th day of the SSF process under stated conditions. The productivity of the process conducted under optimized conditions was 3.41 U/g substrate^{-1} h^{-1} for the 4th day of the SSF.

1. Introduction

Among the food enzymes pectinases are the complex and diverse group of enzymes that degrade the pectic substances (Gummadi et al., 2007). Pectin and other pectic substances are complex polysaccharides playing an important role in the firmness of plant tissues. Pectinases (or pectinolytic enzymes) holding a share of 25% in the global sales of food enzymes are produced from microbiological sources. These enzymes have variety of applications in food industry such as in clarification of fruit juices, extraction of vegetable oils, curing of coffee and cocoa, refinement of vegetable fibers and in the manufacture of pectin-free starch (Singh et al., 1999; Jayani et al., 2005). Therefore it is of great importance to discover new pectinase-producing microbial strains and optimize their enzyme production conditions in order to meet this increasing demand in both submerged and solid state fermentations. It is stated that submerged fermentation (SmF) is generally used for the production of industrially demanded enzymes, employing mostly genetically modified strains (Pandey et al., 2000). However, it is necessary to reduce the high production cost of SmF and find alternative methods. At this point, solid-state fermentation (SSF) is a method to be considered as an attractive alternative.

Industrially important enzymes can be produced by solid-state fermentation (SSF) techniques especially using the fungal metabolism. Agro-industrial residues are the most popular substrates due to their low-cost and availability. Wheat bran can be accepted as a sustainable by-product with a high annual disposal rate and low cost for the production of industrially important enzymes using SSF technique (Demir and Tari, 2014).

Among many types of SSF bioreactors, tray-type bioreactor is the most preferred SSF system for the production of many industrial enzymes (Thomas et al., 2013). They are mainly composed of trays placed in a climatic controlled chamber. These trays can be with or without perforation. The climatic chamber is selected according to the stage of the scale. The scale-up is relatively easier, which can be done by increasing the number of trays. The position of the trays inside the bioreactor should also be considered by optimizing the gap between the trays. However, this system has handicaps such as difficulty in sterility maintenance, requirement of large space and labor, separate sterilization of the substrate bed (Krishna, 2005; Mitchell et al., 2006; Bhargav et al., 2008).

Recently, Bhavsar et al. (2011) performed a scale-up study in

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enamel-coated metallic trays having dimensions of 28 × 24 × 4 cm, 45 × 30 × 4 cm, and 80 × 40 × 4 cm for the production of phytase from *Aspergillus niger* NCIM 563. They have scaled-up the process from 10 g substrate to 1000 g in a Koji type analogous bioreactor. Some reproducible enzyme activity results were reported that was found to be encouraging for pilot-scale production. Ruiz et al. (2012) investigated the production of pectinase by *A. niger* Aa-20 and lemon peel pomace (LPP) as support and carbon source in a column-tray solid-state bioreactor in which they obtained high levels of fungal biomass and enzyme production.

The polygalacturonase production potential of *Aspergillus sojae* (wild type and mutant strains) by SSF was revealed by author's research group (Ustok et al., 2007; Demir et al., 2012). Recently, Demir and Tari (2014) optimized the PG production by an *A. sojae* mutant strain using only wheat bran and water at flask scale and achieved significantly high PG activity (535.4 U/g substrate) that constituted the motivation of the current study. The objective of this study was to determine the bioconversion potential of wheat bran for the production of polygalacturonase by an *A. sojae* mutant strain in the trays as a scale-up step to the pilot scale SSF process. In order to investigate the possible interaction between substrate bed thickness and relative humidity of the environment, an experimental design was set up and its results were analyzed with the statistical tools.

2. Materials and methods

2.1. Microorganism and propagation

A. sojae ATCC 20235 (wild type) was purchased in the lyophilized form from Procochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. This wild type culture was randomly mutated using ultraviolet light exposure according to the modified method of Nicholás-Santiago et al. (2006) by Jacobs University gGmbH, Bremen and used as the mutant in this study.

2.2. Preparation of inoculum

After the propagation step on YME plates, the spore suspensions used as inoculum were obtained on molasses agar slants given by Göğüş et al. (2006). The inoculum preparation procedure for SSF was according to Demir et al. (2012). Besides, spore/g substrate was estimated by dividing the total number of obtained spores to the amount of substrate used in the extraction step.

2.3. Solid substrate

Wheat bran (supplied from Hazal Flour and Feed Manufacturing Company, Turgutlu, Manisa, Turkey) was used as the solid-state fermentation medium. 150–250 μm particle size fraction of the wheat bran was obtained by sieving approximately 100 g of batches in a sieve shaker (Retsch AS 200 Basic, Germany) for 15 min. The physicochemical composition of wheat bran used in this study was characterized and summarized in Demir and Tari (2014).

2.4. Solid-state fermentation

The effect of solid substrate thickness experiments were conducted in the 1500 cc Borcam ® (borosilicate glass) casseroles with inner diameter of approximately 14 cm. These casseroles were; inert, tolerable to the temperatures up to 300 °C, easy to prepare and handle and could conduct the heat of the incubating environment to the solid substrate with a thermal conductivity of (20 °C) 1.14 W/m°C. Appropriate amount of wheat bran was placed in each casserole. The thickness of the wheat bran in the casserole was determined by measuring the depth of the flattened wheat bran layer from ten different points (5 points from the inner region and 5 points from the side region) by Torq 150 mm Digital Caliper before the addition of water. The average of these 10 measurements was calculated as the thickness of the solid substrate. The investigated solid substrate thickness values were 8 ± 0.3, 11 ± 0.2 and 14 ± 0.5 mm that were equivalent to 50, 75 and 100 g of dry solid substrate amount values, respectively.

Half of the appropriate amount of distilled water calculated to maintain 62% initial moisture content was added to the medium before sterilization and dispersed by a glass baguette until a homogenous mixture was maintained. The other half of the water was sterilized and used for inoculation including 10^7 spore/g substrate of inoculum concentration. The content of the container was mixed gently with a sterile glass baguette to maintain homogenous distribution of the inoculated fungal spores. The casserole was covered with 3 piles of sterile bones during the fermentation and also with aluminum foil at the autoclaving and handling steps. Fermentation was performed in the Memmert HCP-108 Humidity Chamber at the desired relative humidity (%) and 37 °C for 4 days.

2.5. Enzyme extraction

After a gentle dry homogenization, 10 g of the fermented sample was placed in a 250 ml Erlenmeyer flask and mixed with Tween 80 (0.02%) solution in the ratio of 1:10 (sample amount: Tween 80 solution) in two replicates and shaken at 150 rpm and 25 °C for 30 min. Afterwards, the pH of the mixture was recorded and filtered through the cheese cloth and centrifuged at 4 °C, 5000 rcf for 15 min. The supernatant was separated and used immediately for enzyme, protein and total sugar assays.

2.6. Enzyme activity, total sugar and protein determination

Polygalacturonase (PG) activity was assayed according to the modified procedure of Panda et al. (1999) using 2.4 g l^{-1} of polygalacturonic acid as substrate. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions (pH 4.8 and 40 °C). Galacturonic acid (Sigma, St. Louis, MO) was used as standard for the calibration curve of PG activity. Total sugar content of the crude enzyme was determined with the phenol – sulphuric acid method of Dubois et al. (1956). Protein content of the crude enzyme was analyzed with the Bradford protein content determination method (Bradford, 1976).

2.7. Experimental design and statistical analysis

The single and interactive effects of two factors on the enzymatic polygalalcturonase activity (U/g substrate) were investigated using a factorial D-optimal design. The tested factors were relative humidity of the fermentation environment (70, 80, 90%) and thickness of the bed (8, 11, 14 mm). Design Expert software (version 7.0.0, Stat-Ease Inc., Minneapolis, USA) was used to construct the design with 12 runs and the ANOVA ($p < 0.05$). The 2-D contour plot (Fig. 3) was generated with the help of the software.

3. Results and discussion

3.1. Effect of solid substrate thickness on PG activity

The thickness of the substrate placed in fermentation bed of tray type bioreactors has importance with respect to the formation of

oxygen and temperature gradients in the bed. As the microorganisms used in the SSF are almost in direct contact with the gaseous oxygen in the air and water content is quite low compared to the SmF, the overall reaction involves the transport of oxygen and water into the microbial biomass, the generation of metabolic heat due to respiration and the transport of heat and carbon dioxide from the interior of the substrate into the gas phase. However, due to the heat and mass transfer resistances in the biomass, concentration and temperature gradients will occur which should be minimized in the design of a proper SSF bioreactor (Raghava Rao et al. 1993). At this point, the thickness of the substrate in the bed becomes a critical factor and should be optimized in order to prevent overheating and guarantee the aerobic conditions in static type bioreactors. With this perspective, the substrate thickness in the fermentation bed was investigated in the present study. SSF was conducted in the round borosilicate glass casseroles that were placed in a humidity chamber adjusted to 90% relative humidity (RH). A regular incubator was not used in order to prevent the drying of the substrate due to the wider area exposed to circulated heated air (37 °C). This set of experiments also had the characteristic approach to an upper scale i.e. tray type bioreactor. Wheat bran was placed in the casseroles with the measured thicknesses of 8 ± 0.3, 11 ± 0.2 and 14 ± 0.5 mm and the accumulation density (Bhanja et al., 2007) values of 1.137, 1.705, 2.274 g substrate/cm^2 of fermentation bed, respectively. Accumulation density value is believed to be useful for the scale-up of this process, since it is the amount of substrate per unit bed area, values were calculated to express the amount of substrate that should occupy the area of 1 cm^2 in any other container with different dimensions. Additionally, no agitation was applied to the fermentation medium during the incubation period with the intent of preventing possible contamination. The PG activity, specific PG activity, final pH and consumption of total carbohydrate results were presented in Fig. 1.

As can be seen in Fig. 1a, the PG activities obtained for three of the investigated thicknesses were lower than the maximum PG activity (535.4 U/g substrate) obtained in the Erlenmeyer flask scale under the optimized conditions (Demir and Tari, 2014). This reduction in the PG activity addressed that the optimized conditions achieved at the down scale should be reviewed in the framework of the scale-up strategies before their use at the upper scales. The reason for this low PG activity might be due to the selection of an unsuitable relative humidity level. Sekar and Balaraman (1998) observed that the optimum relative humidity of the environment (95%) led to an increase of 48% (compared to 80% RH) in the yield (g/kg of bran) of the target metabolite Cyclosporin A. The views taken from each tray at the end of the fermentation period (Fig. 2) supports this idea. As can be seen from these (top and inner) views, the fungus and wheat bran complexes agglomerated and formed relatively large aggregates at all thicknesses probably because of the high relative humidity (90%) of the environment. These aggregates might have limited the transfer of oxygen to the interparticle spaces and resulted in poor growth of the fungus (Fig. 2).

When a comparison was made among the PG activities obtained for each thickness (Fig. 1a), the results indicated an inverse relation between the bed thickness and PG production. This result revealed the limitation of the oxygen and moisture as the depth of the bed increases. Sargin and Öngen (2003) similarly observed approximately 38% reduction in the xylanase activity of *Trichoderma longibrachiatum* as the substrate thickness increased from 0.5 cm to 1.0 cm at the non-aerated condition. In another study, Bhanja et al. (2007) observed that the yield of α-amylase (from *Aspergillus oryzae* IFO-30103) increased slightly with the increase of bed height from 0.5 to 1.0 cm. However, the yield of α-amylase declined with the further (2, 3 and 4 cm) increase in the bed height in the enamel

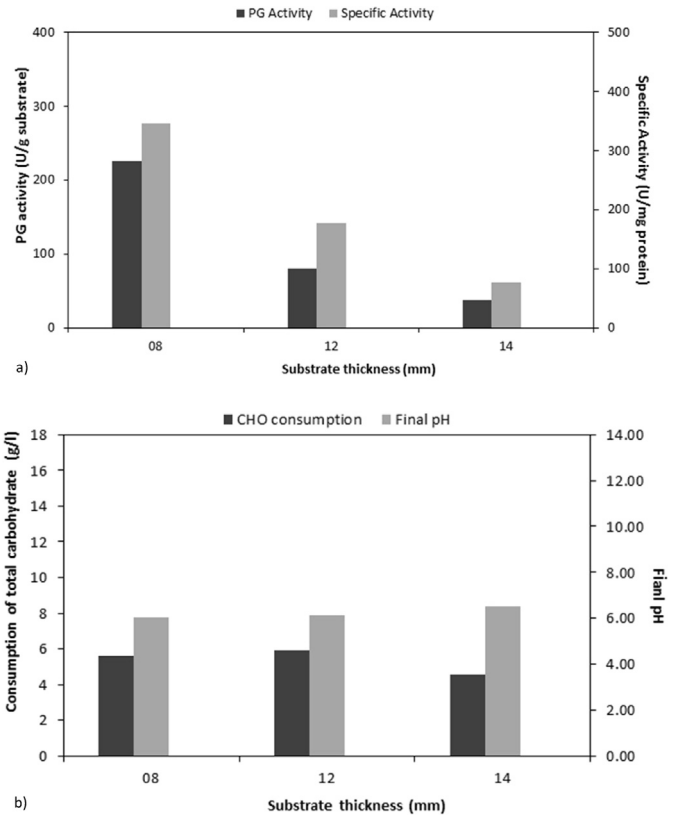

Fig. 1. (a) PG activity and specific activity, (b) consumption of total carbohydrate and final pH profiles of PG production by *Aspergillus sojae* mutant fermented in the glass casseroles (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content, substrate particle size: 100–250 μm, relative humidity: 90%).

coated metallic trays (30 × 25 × 4 cm). Another concern was the temperature gradient occurring throughout the bed. Chen et al. (2005) have placed probes in the trays every 3 cm bed height (0, 3, 6 and 9 cm) from the bottom to the top of the bed. With this method, they have observed nearly 2.5 °C medium temperature difference between 0 and 6 cm levels of the bed (with the air pulsation method).

The specific activity results for 8, 11 and 14 mm thicknesses plotted in the Fig. 1a were 76%, 88% and 95% lower than the maximum one (1467.2 U/mg protein) obtained and previously published by the authors in a study at the flask-scale under similar conditions (Demir and Tari, 2014). It is thought that the reason for these low specific activities was; high protein contents of the crude enzymes rather than their low PG activity values. This idea was supported with the relatively (compared to flask) higher number of protein bands obtained with the enzyme extract of tray (data not shown) in the SDS-PAGE profile. Even the SSF was conducted with the same substrate and same fungus strain, the tray type of fermentation is quite different from the flask type. The most important difference is the increase in the contact surface area of the fermentation medium with the air. This means the fungus is exposed to more oxygen and may show difference in the metabolite synthesis during the adaptation period. The total carbohydrate consumption given in the Fig. 1b showed that the fungus was able to consume the carbohydrate to a reasonable level but could not reduce the pH below 6.0 (Fig. 1b) which was achieved at the flask scale.

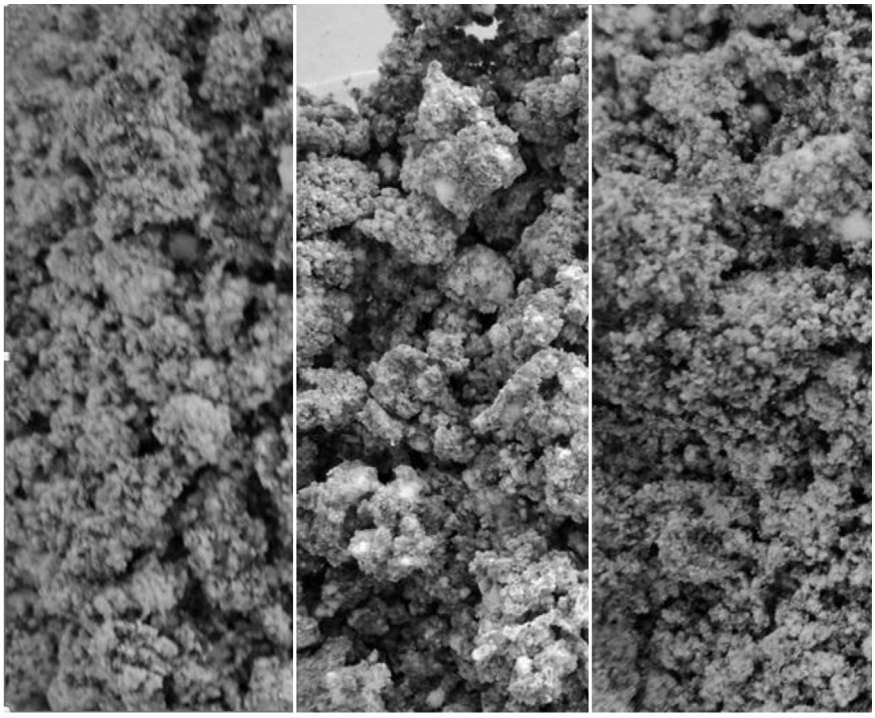

Fig. 2. Close up of tray batches with (a) 8 mm, (b) 11 mm and (c) 14 mm thicknesses (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content, substrate particle size: 100–250 μm, relative humidity: 90%).

3.2. Combined effect of relative humidity and solid substrate thickness on PG activity

In the previous section (3.1) it was observed that the thickness of the substrate has a significant effect on the PG production capacity of *A. sojae* mutant at 90% RH. Many studies showed that RH of the environment also plays an important role in the SSF (Madeira et al., 2011; Sekar and Balaraman, 1998; Ito et al., 2011). Lu et al. (2003) clearly stated that the RH of the chamber controls the moisture of the fermentation medium and indirectly the product yield in the koji type of fermentation. In the case of the current study, an interaction was expected between the thickness of the bed and RH of the environment. Therefore, experimental design and statistical analysis tools were utilized.

For this purpose, a factorial D-optimal design was used with two factors; relative humidity of the environment (A) and thickness of the bed (B) at 3 levels. Each run was conducted in identical glass trays (Section 2.4). The runs of the experimental design and related PG activity responses were given in Table 1.

The results of the experimental design were analyzed with the DesignExpert (version 7.0.0) software and the ANOVA table was given in Table 2. According to this analysis, a significant (p-value < 0.05) model was constructed including the terms A, B and AB. The p-values of these terms indicated that RH itself did not

Table 2
ANOVA table of the PG activity results in the tray type SSF.

Source	Sum of squares	df	Mean square	F-value	p-value
Model	100360.5	8	12545.1	30.5	0.0086
A-R. humidity	5272.9	2	2636.5	6.4	0.0826
B-S. thickness	54835.9	2	27418.0	66.6	0.0033
AB	28504.1	4	7126.0	17.3	0.0206
Pure error	1234.5	3	411.5		
Cor total	101595.0	11			
Std. dev.	20.3		R-squared	0.9878	
Mean	155.5		Adj R-squared	0.9554	
C.V. %	13.0		Pred R-squared	N/A	
PRESS	N/A		Adeq precision	14.9	

Table 1
D-optimal design and actual PG activity values.

Std	Run	A: Relative humidity (%)	B: Substrate thickness (mm)	PG activity (U/g substrate)
4	1	70	11	298
1	2	70	8	114
5	3	80	11	237
3	4	90	8	217
2	5	80	8	264
8	6	80	14	37
10	7	70	8	136
9	8	90	14	68
12	9	90	14	37
11	10	80	11	269
6	11	90	11	116
7	12	70	14	73

affect the PG activity production between 70 and 90% levels; however it had a strong interaction with the substrate thickness as expected. As observed from the interaction plot (Fig. 3), runs of the 14 mm thickness gave the lowest PG activities. On the other hand, as the RH decreased, the PG activity increased at the 11 mm thickness. Maximum PG activity (298 U/g substrate) was obtained at the 70% RH with 11 mm thickness. The pictures of the 1st, 10th and 11th runs (Table 1) with 11 mm batches fermented at 70, 80 and 90% RH conditions were presented in Fig. 4a, b and c, respectively. When, the top views of the trays fermented at 70, 80 and 90% RH were examined, relatively large sized substrate-fungus aggregates in the tray fermented at 90% RH (Fig. 4c) compared to the 70 and 80% RH batches (Fig. 4a, b) were observed. The fermentation media of 70 and 80% RH batches were more homogenous in shape with smaller aggregates. This might be one of the reasons of achieving higher PG activities with 70 and 80% RH batches. On the other hand, Fig. 4a and b presented some dried parts on the surface of the fermentation media which was far less in the Fig. 4c. The close view showed that (not given) the 90% RH batch had a different morphology with the presence of denser white aerial hyphae grown on the wheat bran. The presence of more aerial hyphae and production of low PG activity was similarly observed at the flask scale (48 h and 72 h SmF inoculated SSF runs) as published in Demir and Tari (2014).

As a result, maximum obtained PG activity could be enhanced by 31% compared to the first tray type SSF given in Section 3.1. Additionally, 11 mm thickness and 70% relative humidity conditions were selected for the kinetic tray SSF study in order to monitor the process. The approach to the tray-type bioreactor presented in this part of the study produced reproducible and encouraging results for a further optimization under pilot-scale conditions. Furthermore, 70% relative humidity condition is also beneficial in controlling contamination problems faced by many industrial fungal fermentation processes. In fact this condition not only reduces the risk of contamination but also reduces the operational costs by using less water supply.

3.3. Kinetic study on the PG production

The production process of PG from *A. sojae* mutant in the trays were monitored for 4 days under the conditions of 10^7 spore/g substrate inoculum size, 37 °C, 62% initial moisture content, water as moistening agent and 70% RH loaded with 150–250 μm particle sized wheat bran to the thickness of 11 mm. Two of the trays were withdrawn each day and analyzed immediately for their PG activity, final pH, moisture content, water activity, specific PG activity and consumption of total carbohydrate during the 4 days of the incubation. The averages of the measurements of two trays are presented in Fig. 5.

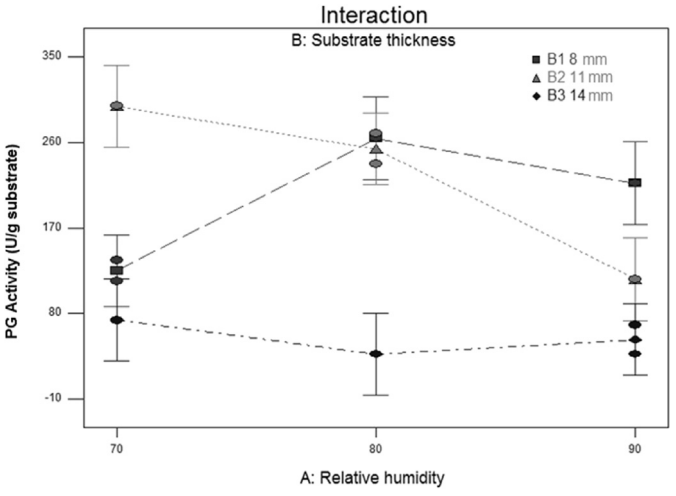

Fig. 3. The interaction plot of relative humidity and substrate thickness.

A relatively high PG activity was obtained on the third day of the fermentation (Fig. 5a) unlike the flask scale SSF with the maximum PG activity observed on the fourth day of the fermentation (Demir and Tari, 2014). Contrary to the flask scale profile, the PG activity decreased on the 4th day. It is thought that reason for the reduction in PG activity on the 4th day might be due to the significant decline in the moisture content and water activity on the same day as can be seen in Fig. 5a. The water activity of the fermentation medium was preserved at 0.998 even the moisture content reduced to 37% at the end of the 3rd day. However on the 4th day, water activity sharply decreased to 0.862 probably due to the large contact area of the tray exposed to air. The air was circulated in the chamber in order to maintain uniform temperature and humidity distribution automatically by the incubator. It was unfortunate that the incubator did not allow the user to control the rate of the air circulation. This fact was probably the reason for the reduction in the moisture content and water activity on the last day of the SSF. This result indicated the importance of aeration rate in the PG production by SSF as Ruiz et al. (2012) observed that forced aeration helped the removal of process heat and improved performance of the SSF in a vertical column – tray bioreactor. It is thought that the difference in the PG activity of the batches fermented under the same conditions (4th day of the current experiment and 1st run of the experimental design in Table 1) was due the compositional variations between the wheat bran batches. The fluctuation occurred on the 4th day of the fermentation was also reflected to the final pH, specific PG activity and consumption of carbohydrate profiles given in Fig. 5b.

The productivity of the process conducted under optimized conditions was calculated as 3.41 U/g substrate^{-1} h^{-1} for the 3rd day of the SSF. For an approximate comparison with the literature; in a similar scale-up study (Bhavsar et al., 2011) performed from flask scale to tray scale, the productivity of the phytase from *A. niger* fermented on the 28 × 24 × 4 cm trays with 100 g of wheat bran moistened with an optimized medium was 1.55 IU/g substrate^{-1} h^{-1}. On the other hand, the maximum PG activity obtained in the current study (298 U/g substrate) is thought to be promising as it is significantly higher than the maximum PG activity produced by *A. niger* CCT0916 using cashew apple bagasse and tray type bioreactor (Alcantara and Silva, 2012). It should be considered that the process conditions should be optimized in a more controlled bioreactor to reach the PG activity level of 387 U/g dried medium obtained by an *A. niger* in a fixed-bed column bioreactor (Linde et al., 2007).

4. Conclusion

The results of this study showed that *A. sojae* mutant -a high PG activity producer strain at the flask scale-also has the potential to produce PG with a relatively high enzymatic activity in the trays similarly using wheat bran without the addition of any nutritive supplement. A maximum PG activity of 298 U/g substrate could be achieved as the substrate thickness and relative humidity factors were optimized. It is believed that the results of this study will provide a basis for the production of PG in tray type SSF bioreactors. The kinetic study revealed the reduction in the water activity during the process, indicating the process can be improved using a bioreactor with the control of temperature, moisture and air flow rate. Overall from the industrial point of view, *A. sojae* mutant is a promising strain for the production of polygalacturonase enzyme in tray type bioreactors with the support of optimization of process

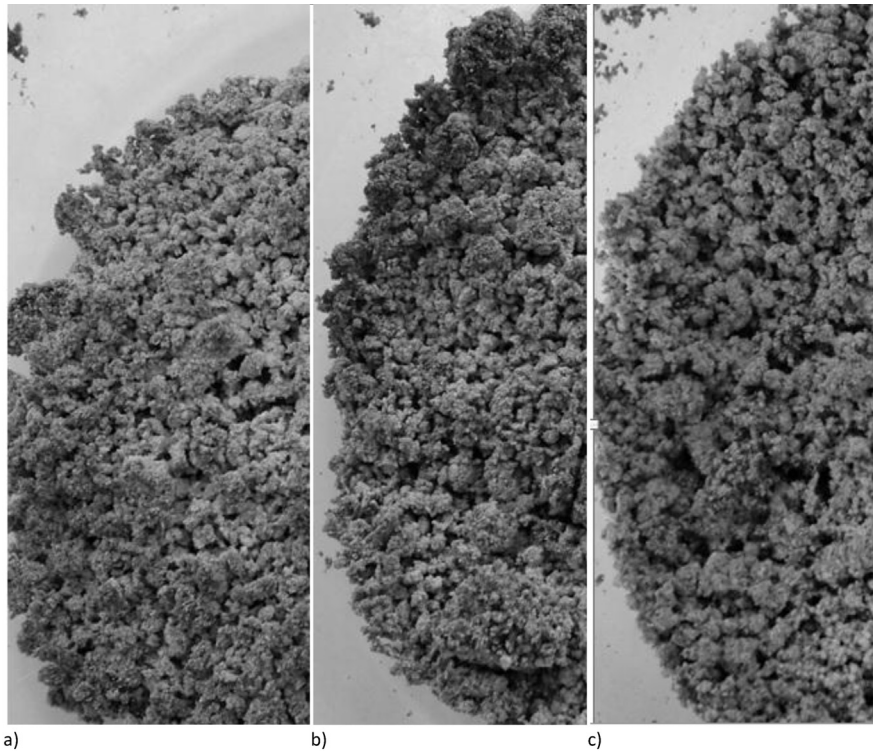

Fig. 4. Top view of tray batches of 11 mm bed thickness fermented at (a) 70%, (b) 80% and (c) 90% relative humidity levels (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content, substrate particle size: 100–250 μm).

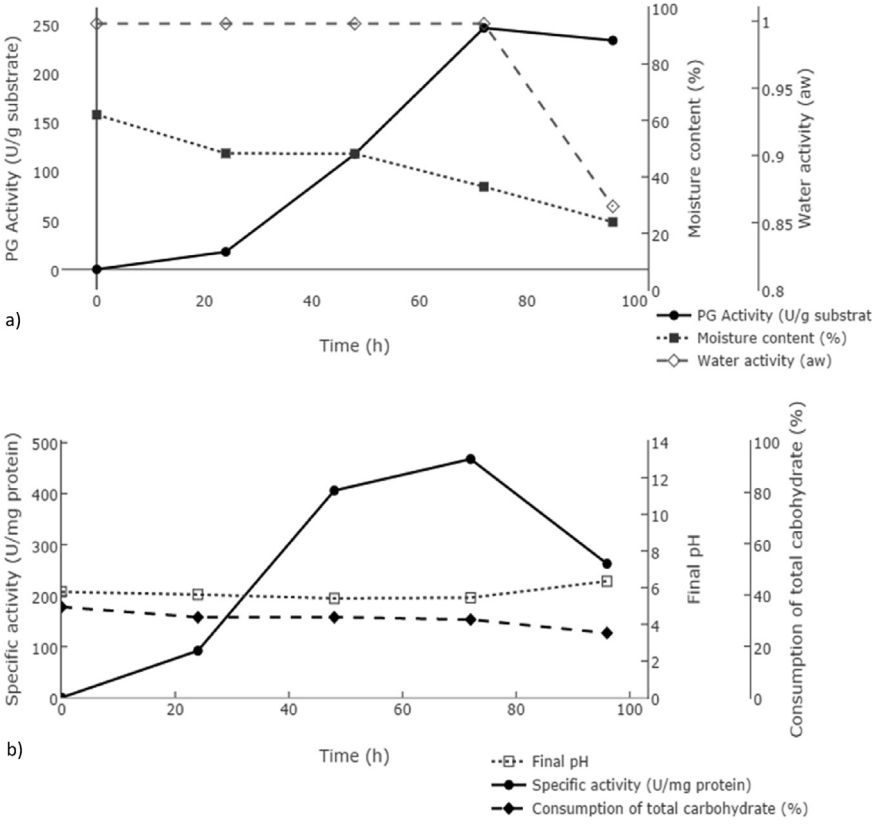

Fig. 5. Kinetic profiles of (a) PG activity, moisture content and water activity, (b) final pH, specific activity and consumption of total carbohydrate profiles of *Aspergillus sojae* mutant by SSF conducted in trays (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, substrate particle size: 100–250 μm, 11 mm substrate thickness, 70% RH).

factors.

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