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Bioconversion of agroindustrial wastes to pectinases enzyme via solid state fermentation in trays and rotating drum bioreactors

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ABSTRACT

In this work, production of polygalacturonases through solid-state fermentation of orange pomace by *A. niger* was studied. Bench-scale pectinase productions in tray and rotating-drum bioreactors were compared. Tray bioreactor was more suitable for pectinases production; despite the fact that moisture control was much better in rotating-drum bioreactor, while agitation and watering was carried out in the fermentation. Exo- and endo-pectinases production yields in tray bioreactor were 45 and 37% higher than rotating-drum bioreactor, respectively. Moreover, to solve dryness in tray bioreactor, sugarcane bagasse was studied as a water preservative material. Comparing the obtained water sorption isotherms for bagasse and orange pomace indicated that addition of bagasse to orange pomace for preservation of water in the fermentation was a great help. This issue was experimented in tray bioreactor and it was proved that bagasse enhanced pectinases production from orange pomace. The percentage of bagasse for preservation of moisture in pectinases production was optimized. The obtained results demonstrated that addition of bagasse (40 g bagasse. g^{-1}dry solid) to orange pomace led to 17 and 23% enhancement in exo- and endo-pectinase activities, respectively. Finally, enzyme kinetics for exo- and endo-pectinases were investigated.

1. Introduction

Due to the favorable condition for filamentous fungi, solid state fermentation (SSF) is going to become a useful technology for enzyme production in the future. Several studies have proved that enzyme production in submerged fermentation (SmF) faces a barrier called catabolic repression that puts a limit for achieving high levels of productivity. However, such limitation can be improved by using controlled feeding strategies; while, SSF shows suitable resistance against catabolic repression and represents a suitable technology for the production of commercial enzymes (Behera and Ray, 2016; Viniegra-González and Favela-Torres, 2006). Furthermore, it was demonstrated that specific gene expression are involved in SSF which leads to a 500 fold higher total protein secretion (total protein production is related to specific gene) in SSF compared to SmF (Oda et al., 2006). Based on future projection for the bioconversion of various kinds of solid substrates to extracellular enzymes, these facts have turned SSF to a promising technology (Ding et al., 2018; Salgado et al., 2014; Khanahmadi et al., 2018).

In addition to the biological predominance of SSF over SmF for enzyme production, SSF has gained importance due to several advantages over SmF, including simple design of bioreactors and also capable of using low costs raw material and agrowaste material, reduced energy requirements and minimum amount of wastewater output (Andreaus et al., 2016; Meghavarnam and Janakiraman, 2017, Manzanares et al., 2012).

Several types of bioreactors are used for SSF process, the most well-known configurations are including tray bioreactor, rotating-drum bioreactor, and packed-bed bioreactor. There are specific preferences for the application these types of bioreactors (Mitchell et al., 2006; Ashok et al., 2017). The selection of the most appropriate bioreactor for a specific SSF process changes from case to another case and depends on several factors such as rigidity of the substrate, type of microorganism, product property and other process parameters (Mitchell et al., 2006; Dey and Banerjee, 2012).

Tray bioreactor is suitable for cultivation of filamentous fungi, especially in production of fermented foods like *tempeh* or soy sauce *koji* (Couto and Sanromán, 2006). This type of reactor is very simple and does not require any complicated design. Moreover, the scale-up is relatively easier in this bioreactor. It can be simply done by increasing the number of trays. However, this bioreactor has some drawbacks such as the requirement of a large room and labor as well as inability in precise control of temperature in a too thick bed. Furthermore, tray bioreactor suffers from the serious problem of dryness, since it is not

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possible uniformly to add water to the static bed (Mitchell et al., 2006). Another consequence of scale-up might be to define the undesired size and uneconomic scale to operate.

Recently, special attention has been paid to rotating-drum bioreactor for SSF process (Rodríguez-Jasso et al., 2013). In this type of bioreactor, mixing should provide better temperature and moisture control in compare to static tray bioreactors. However, mixing may damage microorganism, especially in case of filamentous fungi. Basically, one should compare static bed operation with rotating-drum operation; the basis of the comparison would be the rate/level of production of the desired product. However, this cannot be generalized to all cases as it depends on the sensitivity of the selected organism to the shear forces as well as the rigidity of the selected substrate (Mitchell et al., 2006).

SSF process was implemented for production of enzyme (Hölker et al., 2004. Among commercial enzymes, polygalacturonases (pectinases) are important groups of hydrolytic enzymes produced by microorganisms, especially fungi (Rodríguez-Jasso et al., 2013). Pectinases are important industrially, especially in the fruit juice industry, not only to increase juice extraction yield but also to improve juice's quality (increasing clarity and decreasing viscosity). These enzymes have other applications in food industry such as extraction of vegetable oils, pretreatment of fermenting tea, coffee and sugar extraction from date fruits (Bahramian et al., 2010). SSF and SmF processes are most suitable for enzyme production. Pectinases like almost all fungal enzymes can be obtained by both SmF and SSF. However, SSF is frequently advocated by experts over SmF for pectinases production, as it is much more efficient in the production of one unit of pectinases. Moreover, SSF process can potentially use low cost pectic-agroindustrial wastes, such as citrus pomace as raw feed for production of useful enzyme (Diaz-Godinez et al., 2001; Ruiz et al., 2012).

Several researches have been conducted to investigate on production of pectinases in a specific bioreactor (summarized in Table 1). However, a few studies have been carried out to compare pectinases production in packed and mixed beds. Therefore, additional studies are required to evaluate the effect of mixing on the production of pectinases. This study aims to compare tray and rotating-drum bioreactors for the production of pectinases in bench scale.

Furthermore, in our recently published research, a new model was introduced for controlling the water activity in agitated SSF (Mahmoodi et al., 2016). By the projection of the proposed model, it is possible to calculate the amount of water that should be added to compensate the lost water along the process. Accordingly, the water activity of the bed at the optimum point was fixed. However, employing this model is only possible for agitated beds, since mixing is necessary for uniform distribution of water through the bed. In order to avoid drastic reduction of water activity in the static bed, the motivation of doing a complementary investigation about retaining of water activity in the tray bioreactor is discussed in the last section of this paper.

2. Materials and methods

2.1. Chemicals

Dinitrosalicylic acid (DNS), potassium sodium tartarate and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Citrus pectin was purchased from Sigma Chemical Company (MO, USA).

2.2. Analytical methods

The colorimetric absorptions were determined by means of a spectrophotometer (2100 SERIES, UNICO, U.S.A.). Viscosity was measured with an Ostwald viscometer (Gallenkamp, Germany). Additionally, all microorganism observations were done under a Nikon YS 100 microscope (Nikon, Japan).

2.3. Microorganism isolation

A wild type of *Aspergillus niger* was harvested from a rotten orange as described previously (Mahmoodi et al., 2016). The stock cultured was monthly revived and grown on slant media for further use.

2.4. Preparation of spore suspension

The spore suspension was prepared by washing a 5-day incubated agar culture on petri-dish (plate agar) with an isotonic saline solution (Martínez-Trujillo et al., 2011). The spores were added to the sterilized medium to reach a final concentration of 10^7spores.$g^{-1}$of dry solid matter (Acuña-Argüelles et al., 1995).

2.5. Substrate preparation

Fresh orange pomace with initial moisture content of 30% (on wet basis) was obtained from a local factory and used as the main carbon source. Then combination of ammonium sulfate and yeast extract (1:1) were added as nitrogen sources to reach a total carbon to nitrogen ratio of C/N = 10 (initial carbon to nitrogen ratio of orange pomace was C/N = 32). Then, the total moisture content of the mixture was fixed at 70% The solid medium consisted of orange pomace, as a source of pectin (Alemzadeh et al., 2005) and pectinases production stimulator was added (Naidu and Panda, 1998). The sole carbon source was mixed with $(NH_4)_2SO_4$ and yeast extract as nitrogen sources (Mahmoodi et al., 2017). The optimum medium composition was used for pectinases production (Mahmoodi et al., 2016).

2.6. Enzyme extraction

The produced pectinases were extracted by washing the 96 h incubated culture (incubator temperature set at 30°C) using 0.1M acetate buffer solution (pH 4.5), according to method reported by Mrudula and Anitharaj (2011). The extracted solution was filtered and centrifuged at 4000 rpm for 20 min to separate suspended spores, and the supernatant was kept at 3°C for further analysis.

2.7. Determination of water isotherms for orange pomace and sugarcane bagasse

Orange pomace and sugarcane bagasse were placed in sealed containers above NaCl solutions with different defined concentrations and then incubated at 30°C for 25 days until equilibrium was achieved. Then the samples were dried at 60°C and the content of water was calculated. The water activity related to each concentration of salt was

Table 1
Different bioreactors employed for cultivation of pectinases producing fungi under different conditions.

Ref.	Substrate	Type of reactor	Fungus
Pitol et al. (2016)	Wheat bran + sugarcane bagasse	Packed-bed bioreactor	*Aspergillus niger*
Ruiz et al. (2012)	Lemon peel pomace	Column-tray bioreactor	*Aspergillus niger*
Abbasi and Fazaelipoor (2010)	Pectin	Surface culture bioreactor	*Aspergillus niger*
Huerta et al. (1994)	Sugarcane bagasse + pectin + sucrose	Packed-bed bioreactor	*Aspergillus niger*
Demir and Tari (2016)	Wheat bran	Tray bioreactor	*Aspergillus sojae*

obtained from the literature (Chirife and Resnik, 1984). The experiments were carried out in triplicates and the average values were reported.

2.8. Enzyme assay

The endo pectinase activity was assayed in a reaction mixture containing 1 mL of extract and 18 mL of 2% pectin in acetate buffer (0.1 M, pH 4.5). The reduction in viscosity was determined by an Ostwald viscometer at 30°C. One unit of endo pectinase activity was defined as the amount of enzyme required to reduce the viscosity by 50% in 1 min (Acuña-Argüelles et al., 1995).

The exo-pectinase activity was assayed in a reaction mixture containing 0.3 mL of suitable dilution of extracted enzyme and 0.7 mL acetate buffer (0.1 M, pH 4.5) along with 1 mL of 0.9% pectin in acetate buffer. The concentration of reducing sugars was determined by DNS method by reading the absorbance at a wavelength of 540 nm. The calibration curve for the reducing sugar was prepared according to equivalent amount of D-galacturonic acid. One unit of exo-pectinase activity was defined as the amount of enzyme that catalyzes the formation of one μmol of galacturonic acid per minute (Solis-Pereira et al., 1993).

2.9. Moisture optimization procedure

The moisture of culture media was optimized in flasks based on the levels of produced endo and exo-pectinases activities. For this purpose, five similar samples of the substrate (containing 5 g dry solid matter) were prepared and all parameters except moisture were optimized according to method introduced by Mrudula and Anitharaj (2011). The samples were inoculated with the fungal spores and the moisture of samples was set at 60, 65, 70, 75 and 80% (wet bases) and the produced enzymes were extracted.

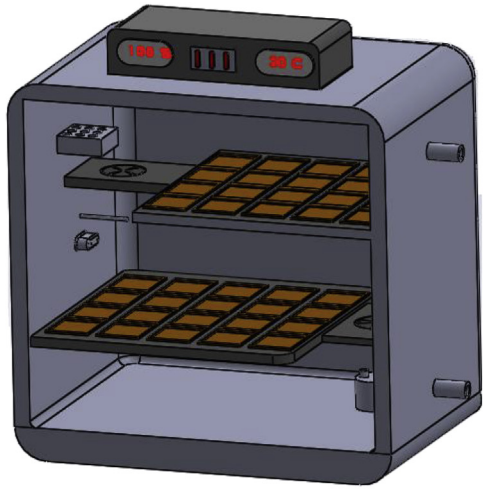
2.10. Tray bioreactor design and configuration

Tray bioreactor shown in Fig. 1(A) and (B) was for the production of pectinases. As it is shown, air entered the chamber from the bottom and passed around the trays. Eventually, air went out from the top. The bottom of the trays was uniformly perforated to accelerate the diffusion of oxygen into the bed. The depth of the bed was small enough (approximately 1.5 cm) to avoid any temperature gradient across the bed. Similar set was conducted by Rajagopalan and Modak (1995) for enzyme production using different organism. The temperature in the chamber was set at 30°C and the air moisture was approximately 100% to minimize water loss caused by evaporation through the bed. Water was not added within the process. The trays were filled with 285 g of dry substrate; the sample was taken in every 10 h and the exo- and endo-pectinase activities were determined for each sample. The duration of the fermentation process was 96 h.

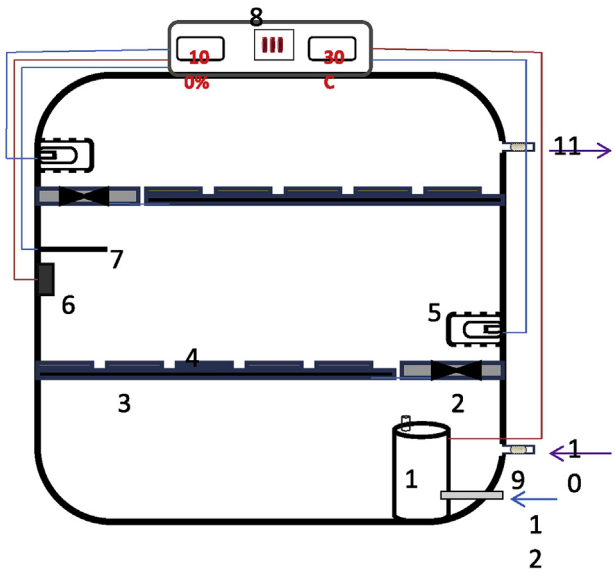
2.11. Rotating-drum bioreactor design and configuration

Agitated cultivation of *A. niger* on orange pomace was carried out in a 15.8 L rotating drum bioreactor (see Fig. 2(A) and (B)). The bed volume was approximately 30% of total volume (4.7 L). The drum was constructed with a piece of pipe having ID: 24 cm, length: 35 cm and thickness of 5 mm. The rotating-drum was rotated at a constant rotational speed of 1.14 rpm and aerated at a constant air flow rate. Four baffles (baffle width of 15 mm) were placed inside the drum to improve the mixing (Fung and Mitchell, 1995). Water was added directly to the bed through a sprinkler. The temperature was well controlled using the inlet air.

The drum was filled with 285 g of the solid substrate. The moisture content of the bed was measured in every 10 h by sampling from the bed and the amount of water was calculated according to the model



(A)



(B)

Fig. 1. Three-dimensional (A) and two-dimensional (B) diagrams of bench scale tray bioreactor (1: Humidifier; 2: Fan; 3: Perforated tray; 4: Bed; 5: Heater; 6: Moisture sensor; 7: Temperature sensor; 8: Moisture & temperature controller; 9: Air filter; 10: Air inlet; 11: Air exit; 12: Sterilized water).

previously discussed (Mahmoodi et al., 2016). The drum was simultaneously rotated for 5 min with each injection of water to the bed. The fermentation process lasted for 96 h.

2.12. Determination of kinetic parameters

For determination of kinetic parameters, enzymatic reactions were carried out at the same conditions as described for the determination of exo- and endo-pectinases activities, using a constant amount of enzyme concentration of 5.2 and 15% v/v for endo- and exo-pectinases activities, respectively. Initial pectin concentrations were set at 0.463, 4.63, 13.9, 23.7 and 46.36%, mM for determination of exo-pectinase activity and 5.426, 54.25, 238, 579.89 and 644.3 mM for endo-pectinase activity. Kinetic parameters for exo- and endo-pectinases activities were expressed by determination of the initial velocity at different concentration of substrate; then, Michaelis-Menten and Logistic equations

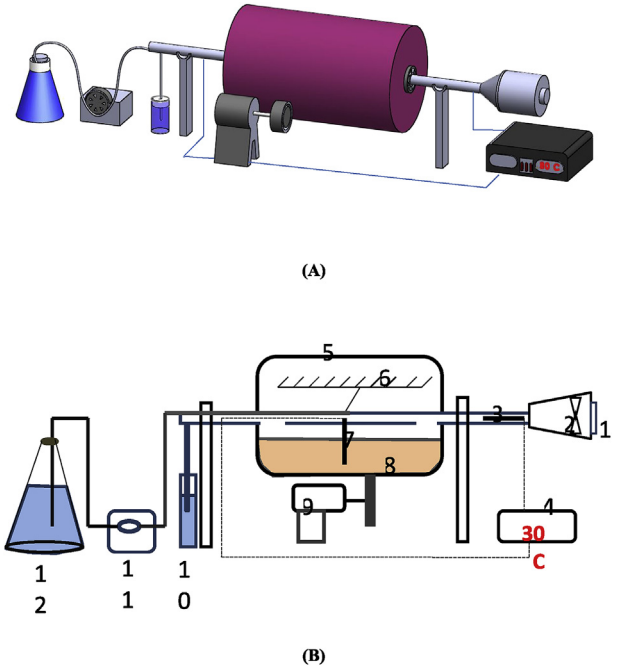


Fig. 2. Three dimensional (A) and two-dimensional (B) diagrams of bench scale rotating drum bioreactor (1: Air filter; 2: Fan; 3: Heater; 4: Temperature controller; 5: Drum; 6: Water sprinkler; 7: Bed; 8: Temperature sensor; 9: Motor; 10: Exhaust air; 11: Peristaltic pump; 12: Sterilized water tank).

were applied for the experimental data using MATLAB software (2013).

3. Results and discussion

3.1. Moisture optimization

Based on obtained results, *A. niger* showed maximum pectinases production was performance at the moisture level of 70% (Fig. 3). It is very well known that the moisture content of substrate affected on growth and enzyme production. The present data indicate that substrate moisture optimization is a simple approach of obtaining more concentrated enzyme extracts. The obtained data on moisture optimization for the production of pectinases by *A. niger* through SSF of soy and wheat bran are in agreement with the data reported literature (Castilho et al., 2000).

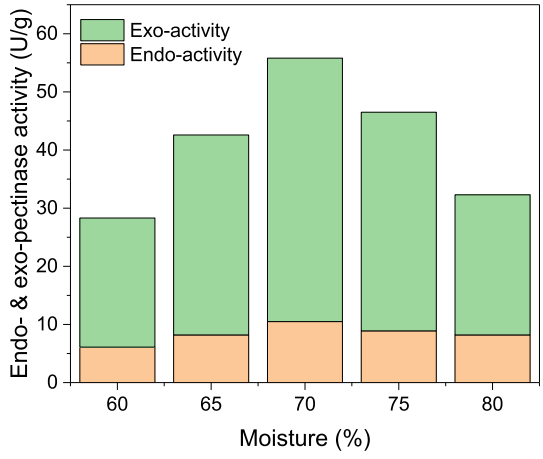


Fig. 3. Moisture optimization for pectinases production in term of exo- (□) and endo- (■) pectinases activity (after 96 h incubation at 30°C).

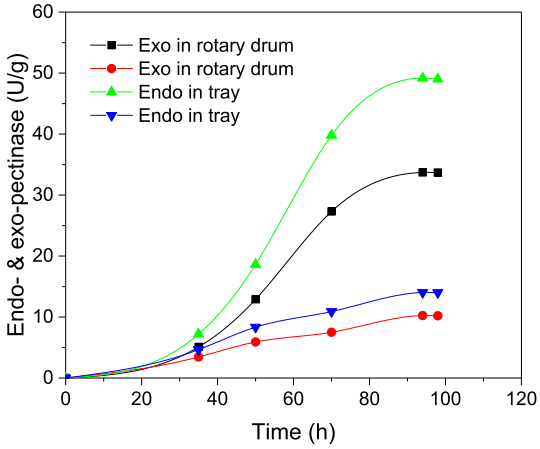


Fig. 4. The comparison of endo and exo-pectinases activities obtained from the tray and rotating drum bioreactors along the process at the same condition (Exo in a rotating drum (■), Exo in a tray (▲), Endo in a rotating drum (□), Endo in a tray (●)).

3.2. Comparative production of pectinases in the tray and rotating-drum bioreactors

Despite the fact that moisture and temperature control in rotating-drum bioreactor was much better than tray bioreactor; while, pectinases production was significantly higher in tray bioreactor. As shown in Fig. 4, exo- and endo-pectinases production yields are 45 and 37% higher in tray bioreactor, respectively. This confirms the rate of enzyme production reported by other researchers that filamentous fungi had better performance for enzymes production in a static bed (Mitchell et al., 2006).

In general, rotating-drum bioreactor has great potential to improve biological fermentation while, the system can be well aerated and avoid support clogging and improving heat removal from the bed (Hardin et al., 2000; Stuart et al., 1999). On the contrary, in the stationary fermentation, mass and heat transfers are not uniform within the bed and on the surface layer of the substrate may be easily dried in compare to the inner layers of the substrate (Mitchell et al., 2006). Nonetheless, rotating-drum bioreactor has great chance to replace stationary fermentation in most industries; in contrary, the shear forces are very destructive and damage filamentous fungi should not be underestimated.

The disadvantage of mixing on cellulase production in SSF of wet corn distillers grain using *Trichoderma reesei* NRRL 11460 has been reported (Flodman and Noureddini, 2013). However, they provided values for a reduction in cellulase activity (5–10%) is lower than the values obtained in this study for reduction in exo- and endo-pectinase activities (45 and 37%, respectively). The significant gap may be due to the physical properties of the orange pomace compared to corn grain. In other word, for the cases when the substrate is soft, like orange pomace, mixing may lead to forming a doughy and adhesive bed which would decrease the void fraction of the bed and accordingly reduces the oxygen diffusion within the bed (Mitchell et al., 2006). Therefore, this work clearly demonstrates rotating-drum bioreactor may not be a suitable option for SSF of orange pomace.

3.3. Improved tray bioreactor performance via developing a technique for retaining the bed moisture

As discussed previously, the main drawback in employing tray bioreactor in SSF is its inefficiency to control the bed moisture, since water cannot be uniformly distributed in to a static bed. Of course, the degree of evaporation is directly related to relative humidity of atmosphere in contact with the bed. However, there are a few techniques to

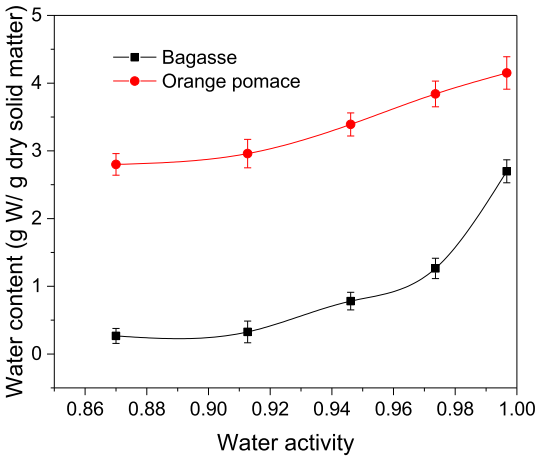


Fig. 5. Sugarcane bagasse (▲) and orange pomace (■) water sorption isotherms at 30 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

retain the initial moisture and bed's water activity without addition of water to the bed. Oriol et al. (1988) suggested that supplementing the bed with water reserving materials, such as sugarcane bagasse, can help the bed to retain its water activity. To examine such idea, water sorption isotherms were plotted for both bagasse and orange pomace and their water preserving abilities were compared. As illustrated in Fig. 5, there is a significant difference between water activities of bagasse and orange pomace for defined water content. Results showed that bagasse shall provide the necessary water activity required for the suitable growth ($a_w = 0.9 - 1$) even in low water contents of W = 0.26–2.6 g water/g dry solid matter. Thus, from the viewpoint of water activity detainment, addition of sugarcane bagasse to orange pomace is expected to improve the performance of SSF process. However, the effect of the chemical composition of bagasse in pectinases production should not be disregarded.

Several studies showed that orange pomace is an inducer for pectinases production, due to the existence of pectin chains in its chemical structure (Naidu and Panda, 1998; Flodman and Noureddini, 2013). In contrary, bagasse is mainly composed of cellulose, lignocelluloses, and lignin, none of which has been reported to be a stimulator for pectinases production. The typical compositions of bagasse and orange pomace are summarized in Table 2. It is thus expected that pectinases production could be decreased with replacing a portion of orange pomace with bagasse. In fact, improvement in pectinase production yield is related to the addition of sugarcane bagasse to orange pomace is definitely attributed to the moisture preservation of the natural bagasse.

To investigate the effect of sugarcane bagasse on pectinases production, the initial concentration of bagasse in the substrate was set at 20% (w/w) and pectinases production was compared to the situation when pure orange pomace was used as the sole substrate. The results indicated that the obtained exo- and endo-pectinases activities in mix media of orange pomace and bagasse were slightly higher (7 and 8%, respectively) compared to pomace fermentation media. Fig. 6 (A) depicts the presentation of the obtained data.

Table 2
Typical compositions of orange peel pomace and sugarcane bagasse.

Component	Composition (% dry matter)	
	Orange peel pomace (Santi, 2012)	Bagasse (Rezende et al., 2011)
Aqueous extractive	30.80	Nd*
Toluene-ethanol extractives	1.33	Nd
Ash	7.43	20.90
Lignin	1.95	22.20
Cellulose	26.10	35.20
Hemicelluloses	11.88	24.50
Pectin	16.96	Nd
Total nitrogen	0.89	Nd
Phenol	1.19	Nd
Tannins	0.65	Nd
Condensed tannins	0.22	Nd

*Nd refers to “not determined"

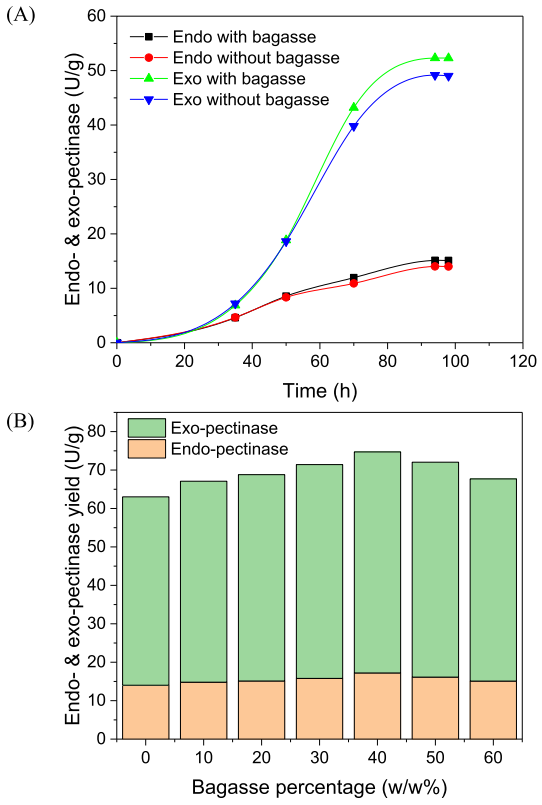


Fig. 6. (A) The effect of the addition of sugarcane bagasse (20% w. w^{-1}) to orange pomace on pectinases production by *A. niger* in tray bioreactor (Exo with bagasse (■), Exo without bagasse (▲), Endo with bagasse (□), Endo without bagasse (●)). (B) Optimization of the percentage of sugarcane bagasse in the substrate, containing orange pomace as the main constituent, on pectinases production by *A. niger* in tray bioreactor (Exo pectinase (□) yield, Endo pectinase yield (■)). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In a recently conducted research (van Bruggen et al., 2017), it was demonstrated that bagasse application significantly enhanced the decomposition of sweet orange leaves. It was expressed that the main reason for this effect was the maintenance of water when the leaves were covered by a layer of bagasse.

3.4. Optimizing the effect of sugarcane bagasse as a water reservoir

As previously discussed, into some extent, addition of 20% bagasse to substrate has improved pectinases production. However, one has to keep in mind that addition of an excess amount of bagasse as substrate and elimination of orange pomace from substrate may lead to a reduction of pectinases production; since a stimulator for pectinase production is eliminated and a non-stimulator material is added. Therefore, an experimental study was performed to determine the optimum percentage of bagasse in the substrate to enhance pectinases production. For this purpose, pectinase production was monitored at different percentages of bagasse including 10, 20, 30, 40, 50 and 60 g bagasse. g^{-1} dry solid matter. The obtained results indicated that the highest exo- and endo-pectinases activities were achieved with the initial concentration of 40 g bagasse. g^{-1} dry solid matter; the achieved data are

shown in Fig. 6(B).

3.5. Determination of kinetic parameters

Kinetic equations are useful tools to study the quality of the extracted enzymes for the enzymatic reactions. In addition, in industrial application, these equations may assist us to design enzymatic reactors more precisely.

In order to find a relationship between the substrate concentration (s) and enzymatic reaction rate (r_s) Michaelis-Menten and Logistic equations, the most well-known equations for describing enzymatic reactions, experimental data were fitted (as explained in "materials and methods"). These rate equations are defined, stated as follows:

$$r_s = \mu_{max} \frac{S}{K_S + S} \left(\text{Michaelis} - \text{Menten equation} \right) \quad (1)$$

$$r_s = \mu_{max} s \left(1 - \frac{S}{S_{max}}\right) \left(\text{Logistic equation} \right) \quad (2)$$

where s is the concentration of substrate (citrus pectin) in the reaction mixture and μ_{max}, K_S and S_{max} as maximum substrate concentration are constants. The illustrated data are shown in Fig. 7(A) and (B) and obtained constants were calculated as follows for both mentioned models:

For Michaelis-Menten equation: $\mu_{max} = 771.7\ \mu M.\ min^{-1}$ and. $K_S = 31.91\ mM$

For Logistic equation: μ_{max}=0.01887$min^{-1}$and.$S_{max} = 97.24\ mM$

As can be inferred from the very high regression coefficient (0.998), Michaelis-Menten model was suitable for describing the kinetics of exo-pectinase activity.

Similarly, kinetic parameters for endo-pectinase activity were determined by quantifying the initial reaction rate of pectinases in the reduction of viscosity of different concentration of pectin. Experimental data were fitted to Michaelis-Menten and Logistic models, as shown in Fig. 8(A) and (B), respectively. The kinetic parameters were calculated as follow:

For Michaelis-Menten equation: $\mu_{max} = 48.19\ mP.\ min^{-1}$ and. $K_S = 478.3\ mM$

For Logistic equation: $\mu_{max} = 4.438 \times 10^{-5} P.\ M.^{-1}\ min^{-1}$and. $S_{max} = -1.776 \times 10^{4} mM$

Results indicated that Michaelis-Menten model was very appropriate to describe the kinetics of endo-pectinase activity as implied by the high regression coefficient ($R^2 = 0.9992$).

4. Conclusion

In this study, production of pectinases using two different bioreactors was successfully carried out in solid state fermentation. The results indicated that tray bioreactor was more suitable for the production of pectinases than rotating-drum bioreactor. Furthermore, the effect of adding sugarcane bagasse as a water reservoir to the pectic substrate was investigated. It was concluded that bagasse can improve pectinases production by retaining water activity along the process.

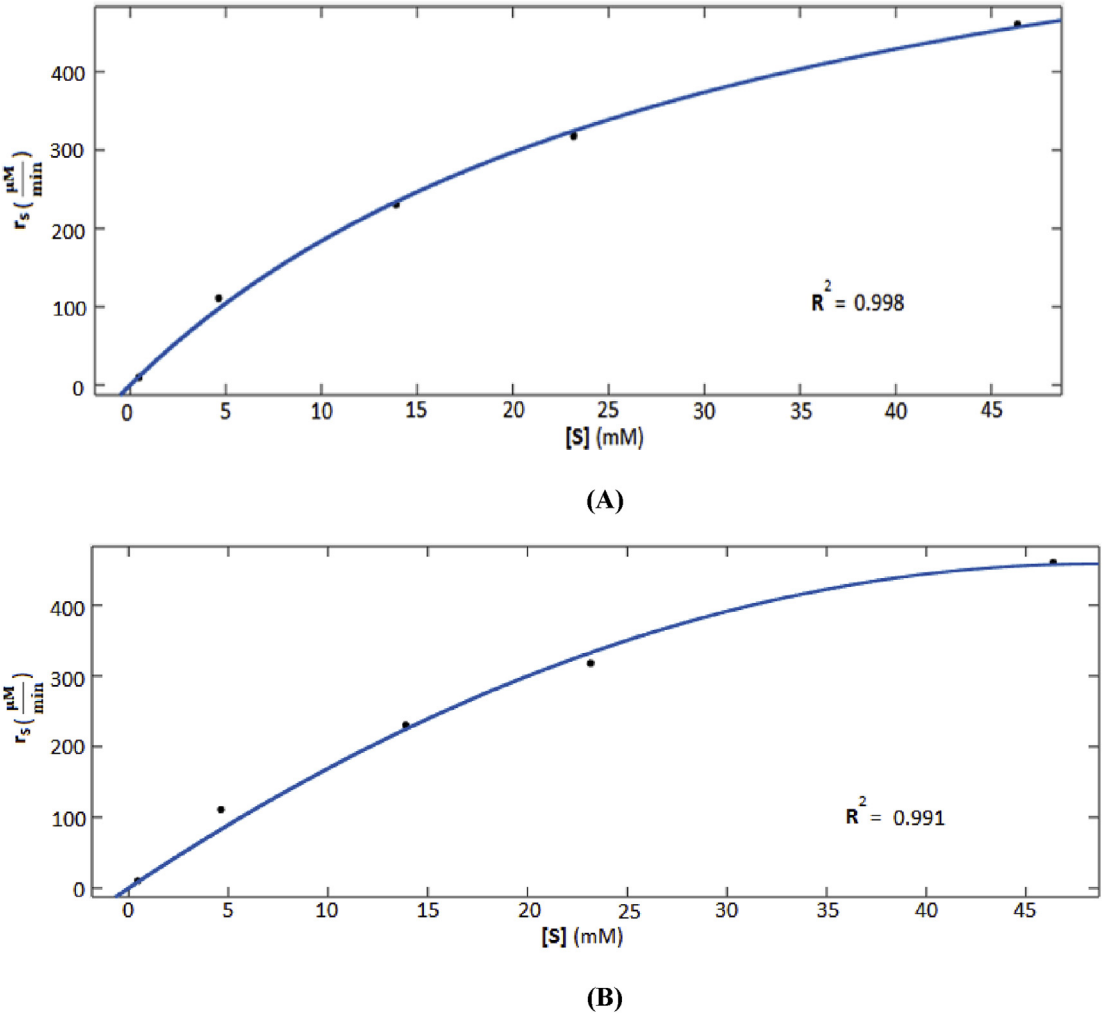


Fig. 7. (A). Kinetic parameters for Michaelis-Menten rate model of exo-pectinase activity. (B) Kinetic parameters for Logistic rate model of exo-pectinase activity.

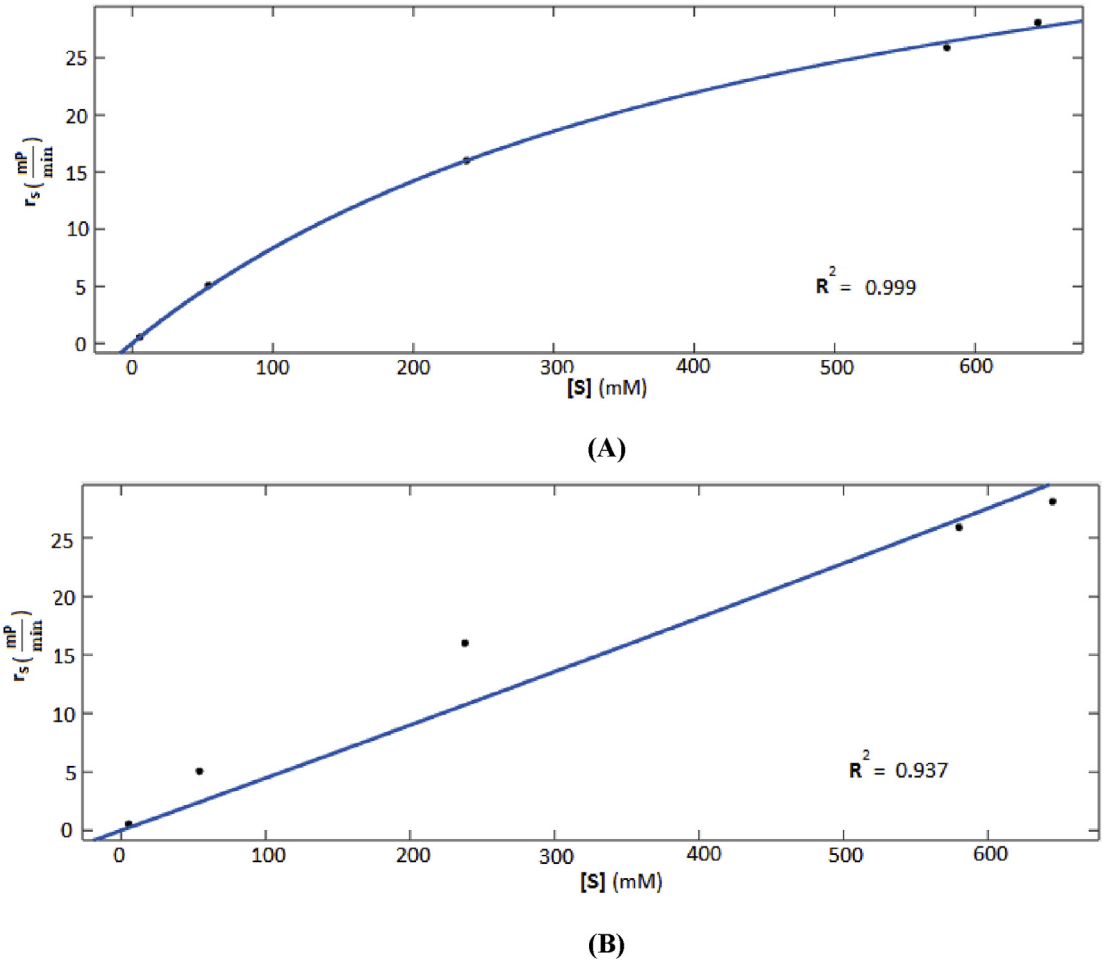


Fig. 8. (A) Kinetic parameters for Michaelis-Menten rate model of endo-pectinase activity (B) Kinetic parameters for Logistic rate model of endo-pectinase activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101280.

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