Bioresource Technology 102 (2011) 10657–10662

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

The behavior of kinetic parameters in production of pectinase and xylanase by solid-state fermentation

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ARTICLE INFO

Article history:
Received 31 May 2011
Received in revised form 24 August 2011
Accepted 25 August 2011
Available online 8 September 2011

Keywords:
Solid state fermentation
Specific growth rate
Maintenance coefficient
Pectinases
Xylanase

ABSTRACT

Solid-state fermentation (SSF) is defined as the growth of microbes without a free-flowing aqueous phase. The feasibility of using a citrus peel for producing pectinase and xylanase via the SSF process by *Aspergillus niger F3* was evaluated in a 2 kg bioreactor. Different aeration conditions were tested to optimize the pectinase and xylanase production. The best air flow intensity was 1 V kg M (volumetric air flow per kilogram of medium), which allowed a sufficient amount of O_2 for the microorganism growth producing 265 U/g and 65 U/g pectinases and xylanases, respectively. A mathematical model was applied to determine the different kinetic parameters related to SSF. The specific growth rate and biomass oxygen yield decreased during fermentation, whereas an increase in the maintenance coefficient for the different employed carbon sources was concurrently observed.

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1. Introduction

Polygalacturonases, including endoPGases and exoPGases, comprise a family of enzymes termed pectinases that degrade pectin and pectic acid. They are predominantly used in the food industry to clarify wines and fruit juices and as an animal feed additive (Pedrolli et al., 2008; El-Sheekh et al., 2009). Xylanases comprise another important enzyme class that is added to feed for monogastric animals and used in paper production industries. They are one of the enzymes responsible for degrading the lignocellulose present in fibers (Tengerdy and Szakacs, 2003; Vafiadi et al., 2010; Song and Wei, 2010). In recent years, polygalacturonase and xylanase production by SSF has been studied extensively in the laboratory scale (Pandey et al., 1999; Fadel, 2001; Castilho et al., 2000; Heck et al., 2005; Negi and Banerjee, 2006; Favela-Torres et al., 2006; Botella et al., 2007; Mamma et al., 2008; Graminha et al., 2008; Chutmanop et al., 2008).

However, only a few of those studies focused on describing the processes through kinetic models representing SSF, and most of them considered a sole carbon source for facilitating microorganism growth. Submerged fermentation (SF) has been more thoroughly described by mathematical models. Kinetic models offer important mathematical representations of the processes, which enhance the understanding of them and allow for larger-scale simulations. Kinetic models aid in directing the fermentative process and reveal frequently ignored variables, such as specific growth rate, yields and maintenance coefficients, whose influence in the system representation is fundamental (Bhargav et al., 2008). Several difficulties are involved in measuring the growth parameters in SSF, including cellular growth analysis and substrate consumption determination, which are caused by the heterogeneous nature of the substrate, primarily an agroindustrial by-product, that is structurally and nutritionally complex (Pandey et al., 1999; Bhargav et al., 2008; Hashemi et al., 2011). Therefore, most of the solid-state kinetic studies, including those on enzyme production, are characterized by simple graphical descriptions of the product synthesis or by semi-qualitative descriptions, including statistical empirical models. Despite the utility of these approaches in fermenting repeated batches using the same conditions, it is useless for scaling up the SSF process. If the aeration is considered as air flow intensity, i.e. the average of rate air flow per kilogram (expressed as V kg M) a unit is obtained that is independent of the scale. However, most of the substrates used in SSF have more than one important carbon source, which is not taken into account in a lumped model.

Because citrus peel, an important agroindustrial by-product, offers several carbon sources for microorganism growth, it is adequate for simultaneous polygalacturonase and xylanase production by SSF (Mamma et al., 2008). Additionally, citrus peel is the major solid by-product that is generated by the citrus processing industry, which constitutes approximately 50% of the fresh fruit weight (Garzón and Hours, 1992; Patil and Dayanand, 2006;

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doi:10.1016/j.biortech.2011.08.106

Koubala et al., 2008). The disposal of this by-product poses a challenge for many factories, which often pelletize the peel and employ it as animal feed or use it as a pectin precursor. However, microorganisms readily use this substrate in fermentations due to its rich composition: 80% organic material, 19–30% free sugars, 20% pectin and 57% total dietary fiber (Chau and Huang, 2003).

Pectins are a family of complex polysaccharides that contain 1.4-linked α-d-galactosyluronic acid residues (Ptichkina et al., 2008; Mohnen, 2008; Vriesmann et al., 2011). Xylan, the main component of plant cell wall hemicelluloses, is another polysaccharide present in the natural fibers. It is a heteroglycan composed of a linear chain of xylopyranose residues bound by β-(1–4) linkages. A variety of substituents are linked to the main chain by glycosidic or ester linkages (Kabel et al., 2007; Komiyama et al., 2009; Oliveira et al., 2010). Both carbon sources are suitable substrates for fungal growth and for pectinase and xylanase production.

Aspergillus sp. is a microorganism that has been reported to produce a wide spectrum of enzymes using products such as pectinases, xylanases, α-amylases and cellobiases (Favela-Torres et al., 2006; Botella et al., 2007; Mamma et al., 2008; Graminha et al., 2008; Chutmanop et al., 2008).

The purpose of this work is to determine the kinetics of microbial growth related to pectinase and xylanase synthesis during the growth of *Aspergillus niger F3* in a SSF process of dried citrus peel. This analysis, in addition to considering the different carbon sources present in the growth medium and the ability of the microorganism to ferment them, will reveal the specific growth rate, maintenance coefficient and biomass/oxygen yield during the fermentation process.

2. Methods

2.1. Bioreactor

SSF and respirometric studies were carried out in a horizontal drum bioreactor, similar to the one employed by (Prado et al., 2004) charged with 2 kg of substrate and an initial moisture of 60% with a nutritive solution. As shown in Fig. 1, saturated air was supplied into the bioreactor via a compressor with a capacity of 6 ft^3/min. The drum was connected to a system with sensors to analyse the gas outlet composition, specifically the CO_2 and O_2 concentrations. The air was dried before being injection into the sensors.

2.2. Microorganism and strain manipulation

An *A. niger F3* strain from the Biotechnology and Bioengineering Division of the Federal University of Paraná was used for enzyme production. Periodic reactivation was made in Czapeck medium slants and incubated for 7 days at 30 °C. The fungus was maintained in Czapeck medium at 4–8 °C.

2.3. Inoculum preparation

The inoculum culture media was prepared by mixing 10 g dried citrus peel with 100 ml distilled water at 80 °C for 15 min to extract the sugars. After cooling to room temperature, the solution was filtered. The solution was diluted 1:10 with distilled water, NH_4NO_3 at 0.3% (w/v) was added, and the pH was adjusted at 5.0. The culture medium was autoclaved at 121 °C for 20 min. The primary inoculation was made with mycelium from the Czapek slant. The medium was incubated in a shaker at 30 °C at 120 rpm for 96 h.

2.4. Solid state fermentation

Dried, grinded and sifted citrus peel, where 75% of the peel exhibited a particle size from 0.8 to 2 mm, and 25% from 2 to 3 mm, was used as the substrate, supplemented with dried NH_4NO_3 at 0.43%, Na_2SO_4 at 0.021%, $MgSO_4.7H_2O$ at 0.077%, $ZnSO_4.7H_2O$ at 0.042%, KCl at 0.162%, and $Ca(OH)_2$ at 0.011%. The water volume needed to obtain 60% of initial moisture was calculated by mass balance, taking into account the natural moisture of citrus peel (about 12%) and moisture provided by the inoculum. Salts were dissolved in water and pH was adjusted at 5.0. The inoculation was made with 1:10 (v/w) of liquid inoculum, giving 2 kg of wet solid fermentation medium. All SSF medium was mixed outside the bioreactor. After that the bioreactor was charged with 2 kg of moist material and fermentations were performed at 30 °C. For aeration optimization, the fermentation time was fixed at 96 h and aeration intensity was tested at four levels, 0.5, 0.75, 1 and 1.25 V kg M, measured with a thermal dispersion flow sensor, Aalborg model GFM (Sturm, 2009). V kg M (L kg^{-1} min^{-1}) is defined as volumetric air flow per moist kilogram.

2.5. Respirometric studies in bioreactors

The production of CO_2 was measured by an infrared sensor (Vaisala model GMT, Brazil), and the consumption of O_2 by the cultures was measured by an electrochemical sensor O2-A2 (Alphasense, Great Nolley, UK) up to the end of CO_2 production and O_2 consumption. To accurately measure the O_2 balance, two sensors were used. One sensor was placed in the inlet and the other was located in the outlet of the bioreactor. To monitor and record the values, an acquisition data system was developed, which was used throughout the process. The O_2 consumption and CO_2 production were measured during 120 h. The accuracies of both sensors were less than 0.02% CO_2 plus 2% of reading value,

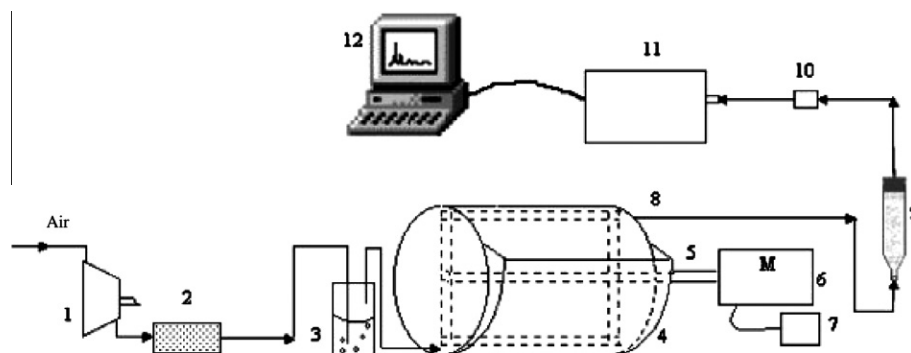

Fig. 1. Schematic representation of the fermentation and data collection system 1. compressor; 2. filter; 3. humidification; 4. bioreactor; 5. agitator; 6 and 7 motor; 8; gas outlet to bioreactor; 9. filter; 10. CO_2 and O_2 sensors; 11. control system; 12. computer.

including repeatability and calibration uncertainty. These values compared with certified factory references.

Kinetic parameters were calculated using FERSOL2, a software program developed for SSF analysis at Biotechnology and Bioengineering Division in UFPR (Sturm, 2009). Kinetic models are based on the O_2 and CO_2 balance of the process as described previously (Rodríguez-León et al., 2007).

2.6. Moisture content

The moisture content in the culture media and the *Aspergillus* fermented material was determined in an infrared balance, Sartorius model MA50, at 105 °C. Moisture content of solid fermentation medium has an error of ±0.5% comparing with theoretical values.

2.7. Enzyme assay

2.7.1. Exo-polygalacturonase activity

The pectinase activity was assayed by measuring the release of reducing sugars by the DNS method (Miller, 1959). D-galacturonic acid (Sigma) was used as the standard. In a test tube, 0.9 ml of 0.5% (w/v) pectin (Sigma) in 0.1 M citrate buffer (pH 4.0) was added to 0.1 ml of diluted enzyme solution. After 15 min incubation at 50 °C, the reaction was stopped by the addition of 1 ml of DNS and heated in boiling water for 5 min. Next, 5 ml of distilled water was added to each sample. Samples were read at 540 nm in a spectrophotometer, Spectrum Model SP2100. One unit of exo-polygalacturonase activity was defined as the activity that liberates 1 μmol of d-galacturonic acid per min at 50 °C, pH 4.0 (Botella et al., 2007).

2.7.2. Xylanase activity

Xylanase activity was assayed by measuring the release of reducing sugars by the DNS method (Miller, 1959). Xylose (Sigma) was used as the standard. Birchwood xylan (Sigma) was used as a substrate. A 1.0 ml reaction mixture, containing 0.5 ml of an appropriately diluted enzyme solution and 0.5 ml of a 1.0% (w/v) xylan suspension in 0.1 M citrate buffer, was prepared at pH 4.8. The mixture was incubated at 50 °C for 10 min, stopped by the addition of 1 ml DNS, and heated in boiling water for 5 min. Next, 5 ml distilled water was added to each sample, and the samples were read at 540 nm. A unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of reducing sugars per min at 50 °C, pH 5.4 (Botella et al., 2007).

2.8. Biomass analysis

The biomass produced during SSF was quantitatively analyzed by measuring the ergosterol present in the fermentation medium at different times. A sample of medium (1 g) from the bioreactor was collected after 24 h. To prepare the biomass standards, the strain of *A. niger F3* was grown in 500 ml Czapek liquid medium for 72 h. The biomass was separated by filtration, washed with ultra pure water and dried at 80 °C. The dry biomass was saponified, and the ergosterol was extracted with *n*-hexane and analyzed by High Performance Liquid Chromatography (HPLC) Varian ProStar) with a C18 column and a Photodiode Array (PDA) detector set to 282 nm (Carvalho et al., 2006).

3. Results and discussion

3.1. Study of airflow intensity

The intensity of air flow, expressed as V kg M, is an important and intensive factor in SSF, which, once optimized, may be maintained at any scale of work. Table 1 displays the results for pectinase and xylanase production by SSF at different air flow intensities in the bioreactor charged with 2 kg of moist material.

The best air flow intensity condition for the synthesis of both enzymes is obtained at 1 V kg M. Airflow intensity values above 1 V kg M do not improve pectinase and xylanase production. In order to corroborate this, the average quantities of enzymes produced at different air flow intensity conditions were compared employing a Tukey's test (data not shown) as statistical tool. In all cases there was a significant difference, but between condition at 1 V kg M and condition at 1.25 V kg M there was no significant difference at the confidence level of 95%. This means that the average quantities of enzymes produced in both conditions are statically equal, so working at higher values can be considered unnecessary and costly. In the fermentation conditions reported, only 0.2 V kg M airflow intensity is needed to oxidize the substrate. The amount of air needed can be calculated by considering the total oxygen demand to carry out the total oxidation of organic materials present in the medium. However, air is also responsible for removal of the heat generated in the oxidation, and 1 V kg M air flow intensity has been reported as the value necessary to remove metabolic heat produced by the SSF of citrus peel used as the carbon source by *A. niger* (Rodríguez-Fernández, 2009).

3.2. Kinetic analysis of the process

Once the adequate airflow intensity for the process has been defined, a 2 kg batch fermentation was launched and analysed for enzymes, biomass production, CO_2 production and O_2 consumption. Notably, natural SSF substrates primarily have more than one carbon source that can be employed by the microorganism for growth. Because the consumption rate for different substrates may be different, a deviation from classical growth curves (with one exponential growth phase) may appear. Additionally, the rate of synthesis for different enzymes may be markedly dissimilar, as shown in Fig. 2, where pectinase production is induced before xylanase.

The pattern of enzyme production by SSF, where the biomass production is a reference, reveals that the pectinase production rate is at its highest at 72 h, after which the rate declines, probably due to the consumption of most of the pectin present in the medium. However, xylanase production increases after 72 h, which is likely driven by the reduction of pectin available as carbon source, forcing the microorganism to use xylan as carbon source. Both enzymes are induced, and the microorganism can only produce them if there is pectin or xylan present in the growth medium. This production occurs on the absence of simple sugars or monomers in the medium, which are more easily fermentable and could act inhibiting the synthesis of the induced enzymes. Additionally, the stability of both enzymes in the medium is maintained, since their activities remain stable after synthesis.

The behavior of the respirometric coefficient, reported in Fig. 3a and b, illustrates that the microorganism began to grow after 20 h of fermentation, which is indicative of a lag or latency phase. The next 3 h exhibit a small increase in the respirometric coefficient, which dramatically increases up to 1 (mol CO_2/mol O_2) over the next 24 h, as is expected in respiration, and remains at this value

Table 1
Influence of the intensity of aeration in pectinase and xylanase production in a 2 kg bioreactor.

Air flow intensity (V kg M)	Pectinase (U/g) (d.b.)	Xylanases (U/g) (d.b.)
0.50	118.45 ± 1.77	24.23 ± 0.60
0.75	192.66 ± 2.31	50.40 ± 0.50
1.00	235.30 ± 2.72	65.38 ± 0.79
1.25	238.80 ± 2.57	64.72 ± 0.62

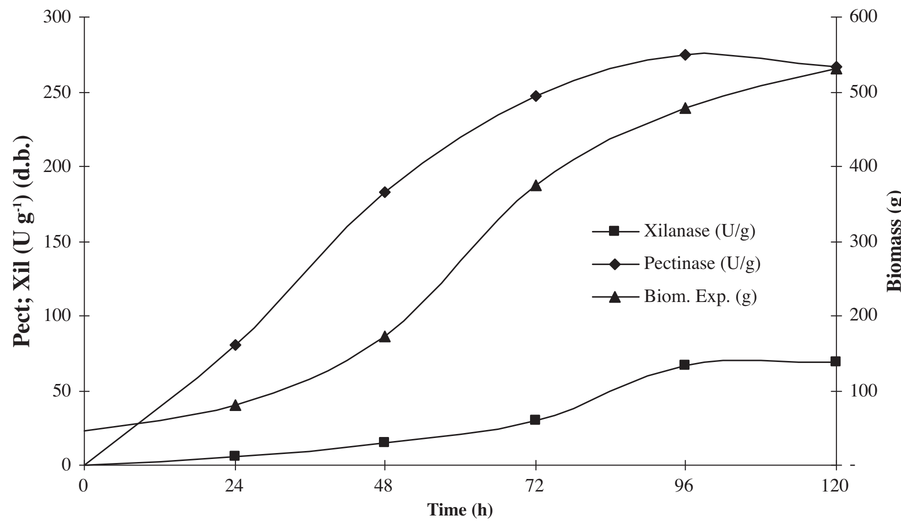

Fig. 2. The kinetic pattern of biomass, polimetylgalacturonase and xylanase production by SSF of dried citrus peel employing *A. niger F3* in a 2 kg bioreactor.

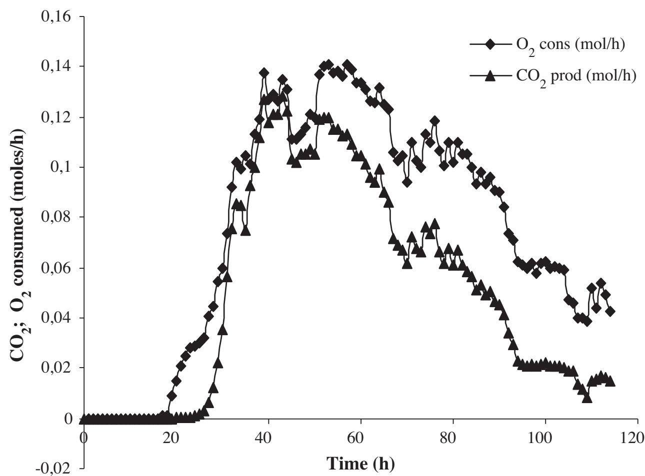

Fig. 3a. The pattern of O_2 consumed and CO_2 produced from growing of *A. niger F3* during the SSF of citrus peel in a 2 kg bioreactor.

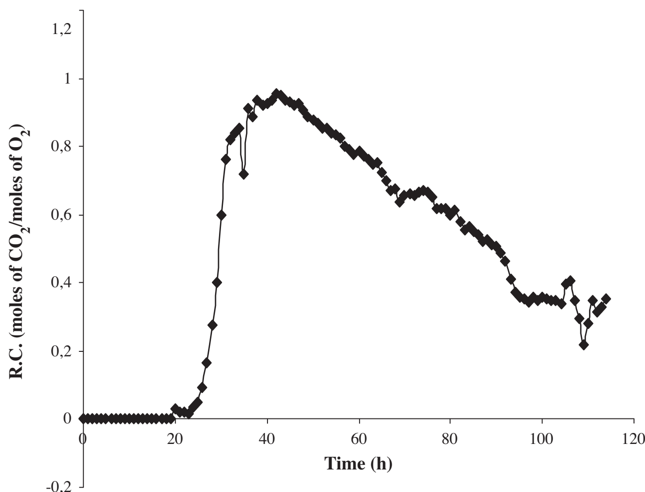

Fig. 3b. The evolution of the respirometric coefficient (RQ) from growing of *A. niger F3* during SSF of citrus peel in a 2 kg bioreactor.

between 38 and 48 h. After 48 h, the respirometric coefficient consistently decreases through the end of fermentation. Because maximum productivity occurs at 50 h for pectinase and at 96 h for xylanase, the ratio between the produced carbon dioxide and the consumed oxygen decreases throughout time. This decrease may be due to an increase in the maintenance coefficient due to the increased enzyme synthesis as shown in Fig. 2. Concurrently, there is a gradual increase of energy used in the endogenous processes of the microorganism, since digesting the carbon sources becomes gradually more difficult.

The specific growth rate (μ), defined as the velocity of biomass synthesis in a particular time interval related to the biomass concentration is expressed as (h^{-1}), while maintenance coefficient (m), defined as energy employed for the endogenous process is expressed as ($gg_{O_2}/g_{biomass}/h$) (Rodríguez-León et al. 2007), changes during the consumption of different carbon sources are reported in Table 2. These values were obtained by applying the Eq. (1) for the metabolic O_2 balance (Sato and Yoshizawa, 1988; Rodriguez-Leon et al., 1988):

$$\frac{dO_2}{dt} = \frac{1}{Y_{x/o}}\frac{dX}{dt} + mX \quad (1)$$

where dX/dt: biomass production rate ($g_{biomass}/h$); X: biomass synthesised during the time interval considered (g); $Y_{x/o}$: yield based on O_2 consumption for biomass synthesis ($g_{biomass}/\ g_{O_2}$); dO_2: differential O_2 consumed during the differential time interval (g_{O_2}); m:maintenance coefficient ($g_{O_2}/g_{biomass}/h$); dt: differential time interval (h).

The solution for Eq. (1), the biomass produced as a function of O_2 consumption, is given below (Eq. (2)):

$$X_n = \left(Y_{x_o}\Delta t\left(\frac{1}{2}\left(\left(\frac{dO_2}{dt}\right)t = 0 + \left(\frac{dO_2}{dt}\right)t = n\right) + \sum_{i=1}^{i=n-1}\left(\frac{dO_2}{dt}\right)t = i\right) + \left(1 - \frac{a}{2}\right)X_o - a\sum_{i=1}^{i=n-1} Xi\right) \Big/ \left(1 + \frac{a}{2}\right) \quad (2)$$

where (a) is defined as:

$$a = mY_{X/O}\Delta t$$

The specific growth rate could be calculated applying Monod model:

Table 2
Determination of the kinetic parameters μ (specific growth velocity), $Y_{X/O}$ (biomass yield based on oxygen consumption) and m (maintenance coefficient) by SSF of *A. niger F3* in citrus peel in a 2 kg bioreactor.

Time (h)	μ (h^{-1})	$Y_{X/O}$ ($g_{biomass}$ (g_{O_2})	m (($g_{O_2}/g_{biomass}$/h)	Average RQ (mol_{CO_2}/mol_{O_2})
20–48	0.069	3.55	0.0023	0.9
48–72	0.044	2.95	0.0038	0.8
72–96	0.025	2.01	0.0078	0.5
96–120	0.014	1.33	0.0112	0.4

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{3}$$

Eq. (2) was solved using a computational program, FERSOL2, where the respirometric coefficient values and the biomass produced were references for selected intervals. The computational program, FERSOL2 is a program that optimizes these parameters in order to match the biomass estimated by Eq. (2) to the biomass already determined analytically at selected points (Sturm 2009). The data in Table 2 indicate that the two kinetic parameters (μ, $Y_{X/O}$) are related to the maintenance coefficient in several ways. When μ and $Y_{X/O}$ are at their highest values, the maintenance coefficient is in its lowest value and *vice versa*. Higher maintenance coefficients correspond to the synthesis of a metabolite, a hydrolytic, substrate-induced enzyme in this scenario, which was previously not produced. The oxygen required (O_2) for the induced enzyme synthesis indicates a decrease in the oxygen employed for the biomass production, affecting the oxygen-based yield.

Table 2 indicates how the specific growth rate (μ) and the yield biomass/oxygen ($Y_{X/O}$) both correlate with the maintenance coefficient (m). From 20 to 48 h, μ and $Y_{X/O}$ are at their highest, while (m) is at its lowest.

This coincides with a high respirometric coefficient, which is consistent with the consumption of simple sugars that require a low energy demand for the endogenous process. Notably, energy consumed during fermentation is used by the microorganisms for two functions, reproduction and maintenance, and maintenance encompass the energy employed in the entire endogenous process.

After 48 h of fermentation, (μ) and ($Y_{X/O}$) began to decrease while the maintenance coefficient began to increase, which indicates a continuous increment in energy demand for synthesizing a metabolic enzyme complex that was able to degrade pectin and xylan. In contrast, *A. niger F3* demands less energy for pectinase than for xylanase production.

The fermentation productivities can be analysed by assessing the biomass produced and the two-enzyme complex synthesised. In Fig. 4a, the highest biomass productivity was reached at 72 h, and the highest pectinase productivity was reached at 48 h, which was well before the pectinase concentration peak. This observation could be explained by the following course of metabolic regulation. Pectinase synthesis began before 48 h, stimulated by the presence of the substrate and/or the decrease in free sugars. Approaching 48 h, when pectin was likely the main carbon source for the microorganism, the metabolism shifted the synthesis form pectinases to xylanases, which may have been due to the exhaustion of available free sugars.

The coefficient of maintenance and the energy that producing the enzyme and metabolising the pectin demands could also account for the metabolic switch. More sugars consumed from pectin are employed in endogenous processes because the microorganism needs more energy to consume free sugars present in the growing medium. Fig. 4b illustrates that the highest xylanase productivity was reached at 96 h, 24 h after biomass production peaked. This result indicates that xylan is a poorer source of carbon for energy and growth for *A. niger F3* compared with the previously consumed free sugars and pectinase. Those results reveal that the use of xylan

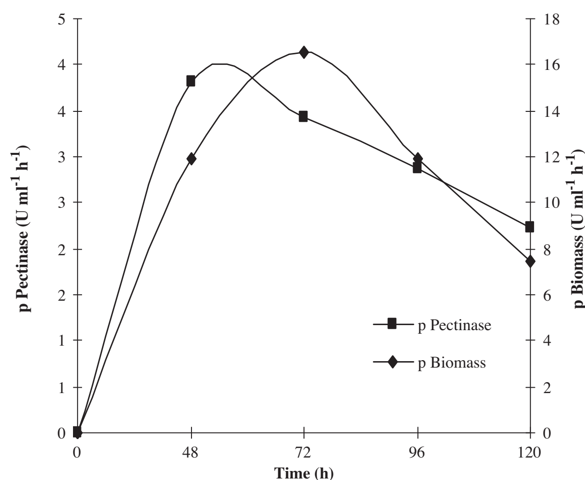

Fig. 4a. A comparison between the productivities of biomass and pectinase produced by the SSF process of citrus peel employing *A. niger F3*.

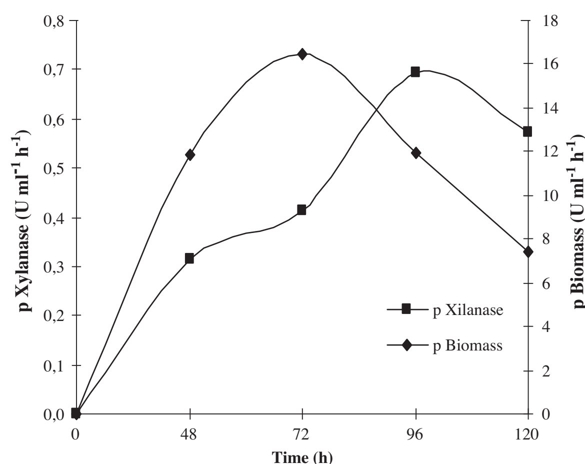

Fig. 4b. A comparison between the productivities of biomass and xylanase produced by the SSF process of citrus peel employing *A. niger F3*.

as a carbon source demands a greater amount of energy to synthesise xylanase. Most of the energy consumed using xylan as a carbon source by the microorganism is used in endogenous processes, indicating that the metabolic status of microorganisms is more stressed compared with consuming pectin. This stressed condition could be associated with the kind of simple sugar produced by pectin and xylan degradation. Galacturonic acid is the monomer produced by pectin and is a hexose, whereas xylose produced by the degradation of xylan is a pentose, which is more readily metabolised by many microorganisms than pentoses.

The defined kinetic parameters and the analysis allows to establish a proper fermentation termination point, which depends on the aim of the fermentation, such as what is being produced, which

is based mainly on the productivity of each metabolite or biomass production. If the total sugar consumed is not considered, the pectinase or xylanase yields for each enzyme will be different but still meaningful because the fermentation develops as a whole process.

4. Conclusions

The air flow intensity of 1 V kg M is the best condition for pectinase and xylanase production by SSF with citrus peel employing *A. niger F3* at 60% of initial moisture, initial pH 5.0 and 30 °C. The fermentation process with this complex substrate exhibits different synthesis phases, which can be monitored by the analysis of the respiratory quotient. The major amount of pectinase was produced in the first 72 h of fermentation, followed by an increase on the xylanase production in the next 24 h. The specific growth rate decreased throughout the entire fermentation process due to the consumption of different carbon sources. Xylan was the carbon source that demanded the most energy form the cell to its degradation.

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