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Effect of Media Composition and Growth Conditions on Production of β-Glucosidase by *Aspergillus niger* C-6

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Abstract

The hydrolytic activity of fungal originated β-glucosidase is exploited in several biotechnological processes to increase the rate and extent of saccharification of several cellulosic materials by hydrolyzing the cellobiose which inhibits cellulases. In a previous presentation, we reported the screening and liquid fermentation with *Aspergillus niger*, strain **C-6** for β-glucosidase production at shake flask cultures in a basal culture medium with mineral salts, corn syrup liquor, and different waste lignocellulosic materials as the sole carbon source obtaining the maximum enzymatic activity after 5–6 d of 8.5 IU/mL using native sugar cane bagasse. In this work we describe the evaluation of fermentation conditions: growth temperature, medium composition, and pH, also the agitation and aeration effects for β-glucosidase production under submerged culture using a culture media with corn syrup liquor (CSL) and native sugar cane bagasse pith as the sole carbon source in a laboratory fermenter. The maximum enzyme titer of 7.2 IU/mL was obtained within 3 d of fermentation. This indicates that β-glucosidase productivity by *Aspergillus niger* **C-6** is function of culture conditions, principally temperature, pH, culture medium conditions, and the oxygen supply given in the bioreactor. Results obtained suggest that this strain is a potential microorganism that can reach a major level of enzyme production and also for enzyme characterization.

Index Entries: Sugar cane bagasse pith; *Aspergillus niger*; β-glucosidase; corn syrup liquor; oxygen supply.

Introduction

The enzymatic conversion of cellulose is catalyzed by a multiple enzyme system. β-Glucosidase is one of the essential enzymes in the enzymatic

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conversion of cellulose. Stenberg *(1)* indicates that the *Trichoderma* enzyme system has been studied extensively and has insufficient β-glucosidase activity for the practical saccharification of cellulose. High levels of β-glucosidase are important for the complete conversion of cellulose owing to the inhibition, by cellobiose, of the cellobiohydrolase and endoglucanase activities *(2,3)*.

One of the potential uses of β-glucosidase is based on the fact that it is one of the components of the cellulose enzyme complex and is therefore important in complete breakdown of cellulose to glucose. The existing literature on the utilization of the cellulosic materials indicates that the problem of effective utilization and conversion of these renewable carbon sources to useful products is not yet solved. This is because successful utilization of the lignocellulosics depends on the development of economically feasible processes for enzyme production as well as its utilization. In the process of converting cellulosic materials into useful products, the production of the enzyme is the most expensive part. Its cost may be considerably reduced using cheaper substrates *(4)*.

Aspergillus species are known to be good producers of β-glucosidase *(5)* and *Aspergillus niger* is the most efficient producer of β-glucosidase activity *(6)*. Because β-glucosidase activity in different cellulase preparations including *Trichoderma viride* is suboptimal for the saccharification process *(1,7)*, it is essential to find an alternative for supplying additional β-glucosidase to such reactions.

In a previous work *(8)*, we showed that sugar cane bagasse pith, which is available as an agricultural residue in large quantities, can be used as a substrate under modified culture conditions for cellulose and β-glucosidase, as well as for xylanase production. In view of the potential uses of the β-glucosidase, it is desirable to evaluate the production of this enzyme from different microbial sources.

The goal of this work was evaluate the production of β-glucosidase with the strain of *Aspergillus niger* **C-6** in submerged bioreactor cultivation. This *Aspergillus* strain was isolated from soil samples obtained from a sugar cane factory located at Zacatepec, Morelos, México.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma Chemical Co. (USA). Solid culture media used for strain propagation were obtained from Bioxon (México).

Microorganism

The filamentous fungi strain was supplied by and maintained at the UPIBI/IPN microbial culture collection. This microbial collection supplies

different microbial strains to the research projects and also to practical courses at the Professional Unit of Biotechnology of the National Polytechnic Institute in México.

Culture Conditions

All cultures were grown on potato dextrose agar slants (12 mL) at 29°C for 5–6 d and then exposed to daylight for 24 h to encourage sporulation. Slants were maintained at 4°C and subcultured at about monthly intervals. Spore suspensions were produced by adding sterile water (10 mL) to a slant and gently rubbing the mycelium with a sterile loop. Clumps of spores were dispersed by adding a drop of Tween 80 to the suspension. Inocula were prepared by harvesting spores from 5 – 7 d old PDA slants in the proportion 3:1 in distilled water with Tween 80. Fermentor and Erlenmeyer flask were inoculated with this spore suspension to give 10^6–10^7 spores/mL.

β-*Glucosidase Production*

Culture Media

The nutrient medium composition in g/L of tap water was: sugar cane bagasse pith, 20; corn syrup liquor (CSL), 20; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.1; the pH value was adjusted to 5.5 before sterilization, 2 mL/L of Tween 80 was added after sterilization.

Shake Flask Cultures

One-hundred fifty milliliters of medium in 500-mL Erlenmeyer flasks in a shaking water bath (180 rpm) at 29°C and 1 mL conidia suspension of the above prepared inoculum was added to each flask. For the experiments about pH effect, the initial pH was adjusted to the follow values: 4.4, 5.5, and 8.0; samples of 10 mL were periodically removed aseptically (after 2, 3, 6, 10 days) and analyzed for pH, dry weight, soluble protein, β-glucosidase and FP enzymatic activities after centrifugation at 5000 rpm for 15 min to obtain cell-free filtrates.

Fermentation Cultures

Fermentor cultivations were performed in a 2-L Microferm fermentor (New Brunswick Scientific Co.), total working volume of 1.2 L. Standard fermentation conditions were: temperature, 29°C; initial pH, 5.5 (uncontrolled); 240 h of fermentation time. Three agitation speeds (100, 180, 250 rpm) and three aeration rates (0.2, 0.5, and 1.0 vvm) were combined giving nine conditions for enzyme production. These conditions were selected only to evaluate the minimum and maximum possibilities for cultivation the *Aspergillus* strain. During the fermentation time, samples of 10 mL for analysis were collected periodically from the fermentor for pH, dry weight, and soluble protein determinations. The β-glucosidase and FP enzymatic activities were determined after centrifugation at 5000 rpm during 15 min to obtain the culture filtrates.

Agitation and aeration was performed according to equipment specifications, medium (1200 mL) was inoculated with 50 mL conidia suspension prepared as described. The temperature was maintained at 29°C. The initial pH of 5.5 was not adjusted.

Determination of Fermentation Parameters

Specific Rates

Specific growth rate (μ_x) and product formation (μ_p) were determined by a computational program based on the method of LeDuy and Zajik *(9).*

Volumetric Oxygen Mass Transfer (K_La)

The K_La values were measured by a static gassing-out method based on the procedure described by Stanbury & Whitaker *(10)*.

Analytical Methods

β-Glucosidase Activity

The β-glucosidase activity was determined as an aryl-beta-glucosidase *(11)*, according to the following procedure. The substrate was 10 m*M* *p*-nitrophenyl β-D-glucopyranoside (Sigma, St. Louis, MO) in 0.05 *M* acetate buffer, pH 4.8. A 1.4 mL substrate solution and a 0.1 mL sample were incubated at 50°C, and the reaction was terminated after 10 min with 3 mL of 0.1 *M* NaOH. The absorbance was read at 410 nm. A standard curve was obtained with *p*-nitrophenol (Sigma).

Filter Paper Activity

Filter paper activity (FPA) was determined by the method of Mandels *et al. (12)* as follows: 0.5 mL of culture supernatant was added to 1 mL of 0.075 *M* citrate buffer, pH 4.8, and filter paper (50 mg, Whatman #1) at 50°C for 60 min. The enzymatic reaction was terminated by addition of 3 mL dinitrosalicylic acid reagent.

Soluble Protein Determination and Reducing Sugars Concentration

These assays were performed with the cell-free supernatant. Soluble protein content was determined without precipitation according to Lowry *et al.* (*13*) in an aliquot of cell-free culture filtrate after ±4°C overnight dialysis, using bovine serum albumin (Sigma) as standard. All reducing sugar determinations were performed by the 3, 5-dinitrosalicylic (DNS) method *(14).* Results were expressed as the mean of three independent readings.

Dry Weight Determination

Samples of 5 mL were filtered on a dried and pre-weighed filter (Whatman GF/F) and washed thoroughly with cold distilled water. The filter with mycelium was then dried for 24 h at 105°C and weighed.

The determinations of growth by dry weight were expressed as the mean of three independent readings.

Results and Discussion

The approach used here was to search for another microbial source of β-glucosidase using one filamentous strain isolated by us and evaluate its production in the fermentation equipment, studying different culture conditions including the influence of aeration and agitation rate on enzyme production.

Figures 1 and 2 show the *Aspergillus niger* **C-6** grew rapidly in basal medium and maximum cellulase production in the culture filtrate was observed on the sixth day of growth. β-Glucosidase activity appeared in the culture filtrate on the first day, obtaining maximum activity on the third day of cultivation followed by FPA activity after the second day.

There was a close relationship between the secretion of extracellular soluble protein and production of enzymatic activities (Figs. 1 and 2) while measurement of growth (as mycelial protein) indicated that maximum enzymatic activities were detected during the stationary phase. The amount of soluble protein present in the supernatants from filtrate culture reached a maximum level after 3–4 d of growth and then declined slowly until the final time of fermentation.

Similarly to Gokhale et al. *(15),* the levels of β-glucosidase were highest when CSL was used at the culture medium composition and native sugar cane bagasse pith as the sole carbon source. This shows that not only the overall amount of enzyme, but also the balance of the cellulose components, can be modified by variation of the medium composition.

The addition of Tween 80 at the culture medium doubled the yields of β-glucosidase in shake-flask cultures, similarly to Stenberg *et al. (16).*

Figures 1 and 2 show the pH response also. The highest β-glucosidase activities were found in cultures whose final pH was near 5.6.

Cultivation of *Aspergillus niger* **C-6** in shake flask was not far more successful than growth in a stirred-tank fermentor. A comparison of β-glucosidase activity indicated that in the culture fluid in the shake flasks, activity was on average similar to that found in the fermentor broth. Intensive agitation altered the morphology of *Aspergillus* strain **C-6** and reduced β-glucosidase productivity; shearing forces may disrupt fragile microbial tissue and have a marked influence on enzyme production. This effect and differences in morphology due to higher agitation rates have been reported for other filamentous fungi (*17–19*).

To study the effect of temperature on enzyme production, the organism was grown at four temperatures: 25, 29, 33, 37°C. The production of the enzymatic activities was monitored up to 10 d of incubation, and the values obtained are given in Table 1. The optimum temperature for the production of β-glucosidase was 29°C, whereas growth of the organism at higher temperatures resulted in a decrease in enzyme production. The effect of initial

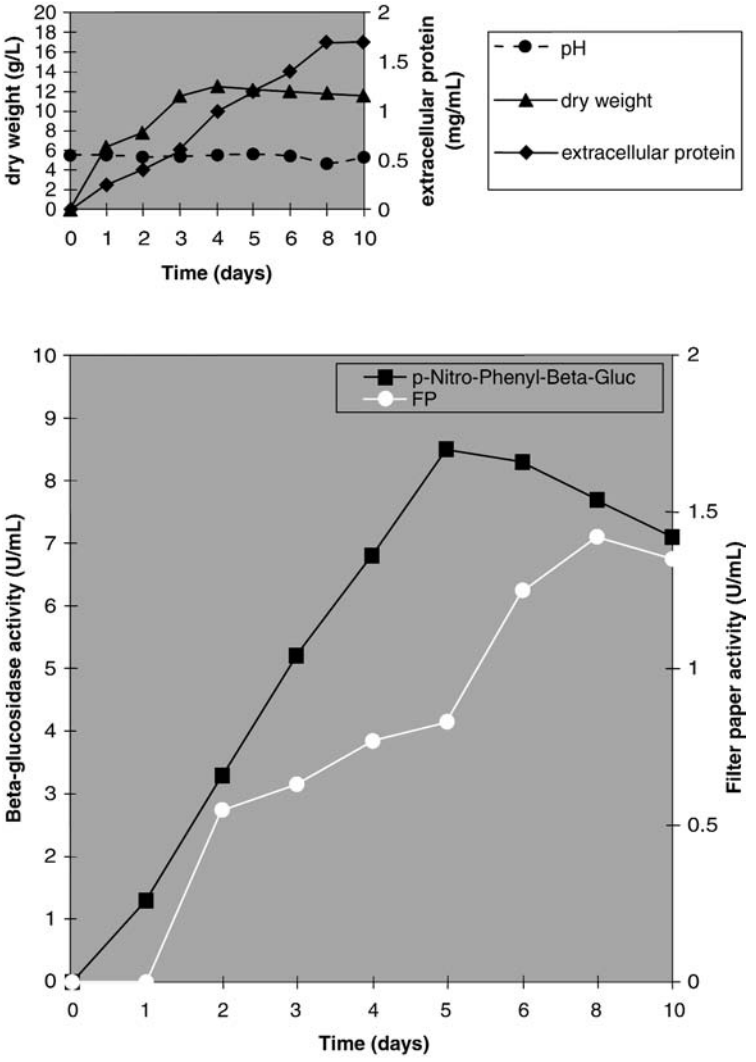
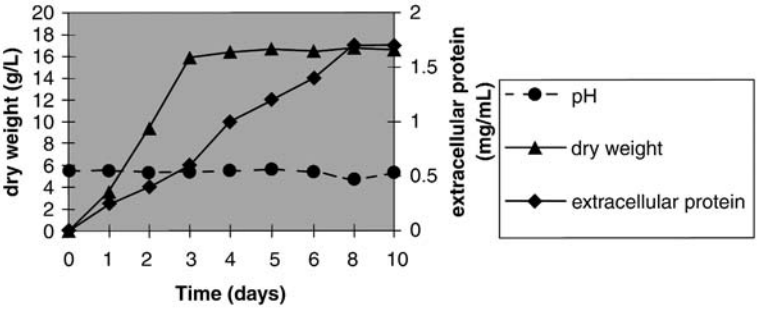


Fig. 1. Time course for production of extracellular activities of β-glucosidase (■) and FPA (❍) by *A. niger* **C-6** grown on sugar cane bagasse pith as sole carbon source at 29°C in shaking flask (150 rpm), medium pH (●), dry weight (▲), and extracellular protein (♦) during enzyme production.

pH after sterilization of the culture medium also is given in Table 1 and by this reason the initial pH in the bioreactor cultivation was 5.5.

Different agitation speeds (100, 180, and 250 rpm) and different aeration rates (0.2, 0.5, and 1.0 vvm) were studied in the bioreactor for evaluate only different ranges (Figs. 3 and 4). An increase in the aeration rate for a agitation speed to 180 rpm did not necessarily reduce the enzymatic activity. Excessive agitation (250 rpm) resulted in low enzyme activity at all aeration conditions studied (Fig. 5). The fermentations carried out at the aeration rate of 0.2 vvm showed that an increase in stirrer speed decreased



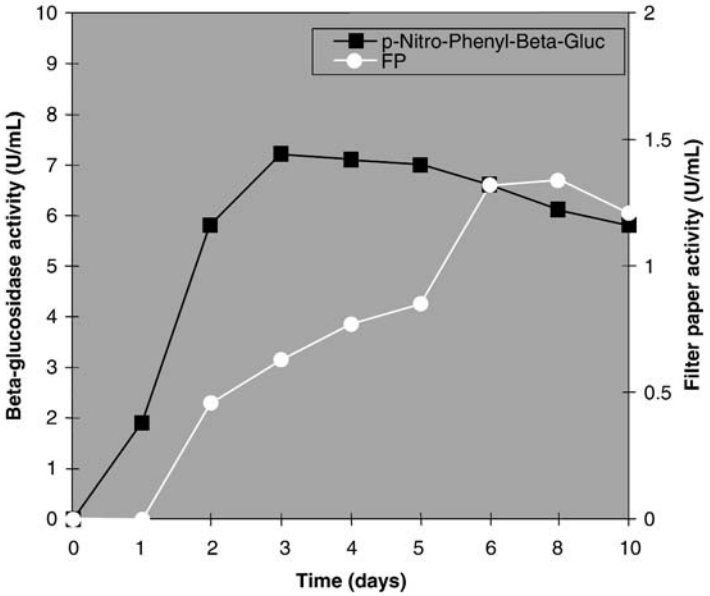


Fig. 2. Time course for production of extracellular activities of β-glucosidase (■) and FPA (❍) by *A. niger* **C-6** grown on sugar cane bagasse pith as sole carbon source at 29°C in an stirred-tank fermentor. The working volume was 1200 mL, medium pH (●), dry weight (▲), and extracellular protein (♦) during enzyme production.

Table 1
Effect of Temperature and pH on β-Glucosidase Production at Shake Flask Cultures by *A. niger* **C-6**

	Yield(U/mL) at				Yield(U/mL) at		
Enzymatic activities	25°C	29°C	33°C	37°C	pH 4.0	pH 5.5	pH 6.0
β-Glucosidase	4.9	8.6	7.2	5.5	6.1	8.6	8.0
Filter paper cellulase	0.55	1.37	1.0	0–72	0.38	1.29	1.1

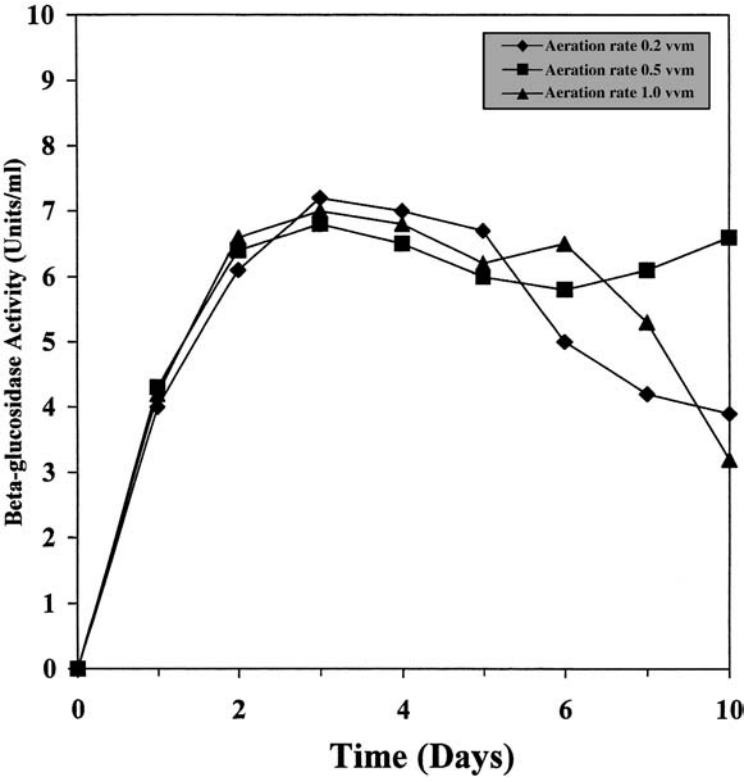


Fig. 3. β-Glucosidase activity at 180 rpm agitation speed in the sugar cane bagasse pith fermentation from *A. niger* **C-6**.

the enzyme activity (Fig. 6). These results are consistent with observations made by Wase *et al.* (*20*) for cellulase production by *Aspergillus fumigatus* in agitated vessels. From the data presented it can be noted that best air supply conditions were an agitation speed of 180 rpm and 0.2 vvm of aereation rate.

Cultivation of *Aspergillus niger* **C–6** in shake flasks at different agitation speeds indicated that 150 rpm was the best agitation rate for β-glucosidase production (data no shown). Separate culture fermentations carried out for 164 h at 180 rpm were more successful than in the stirred-tank fermentor (*21*).

Table 2 presents the results of all fermentor runs. Enzyme activities obtained at aeration rates of 0.2 vvm (experiments I, IV, VII) suggest that minimum aeration levels lead to higher productivity. Maximum enzymatic productivity (100 U L h^{-1}) was obtained with a medium value of $K_L a$.

From the curves of growth and β-glucosidase activities, the specific growth rate (μ_x) and β-glucosidase formation (μ_p) were calculated using the method of Le Duy and Zajik (*9*). The value of the maximum μ_x increased with increasing $K_L a$, a sign that growth was dependent on oxygen supply. There was no such correlation between μ_p and the $K_L a$. It was observed that

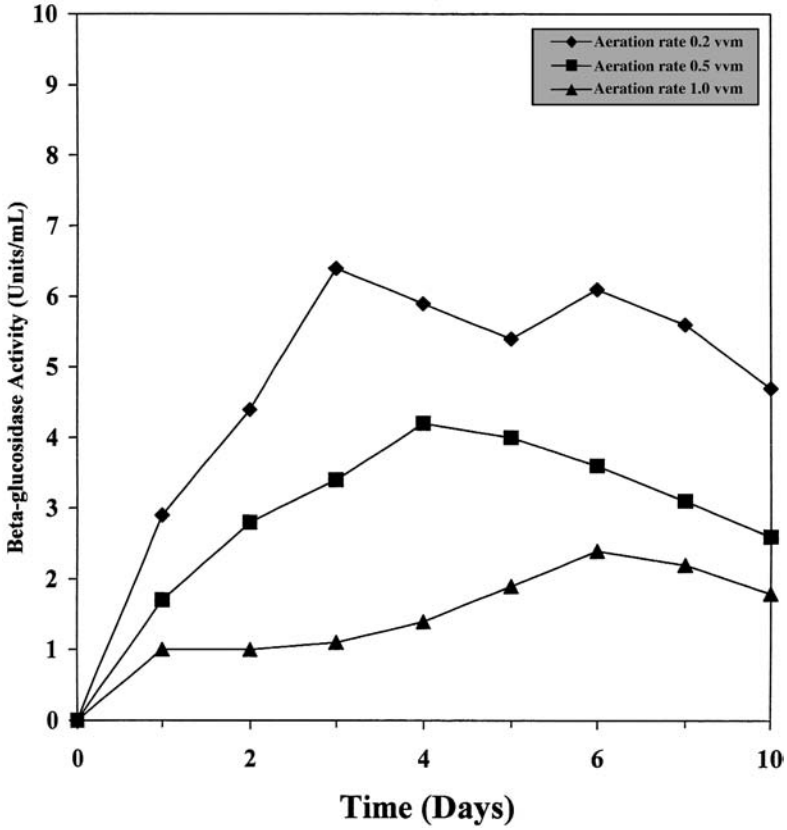


Fig. 4. β-Glucosidase activity at 100 rpm agitation speed in the sugar cane bagasse pith fermentation from *A. niger* **C-6**.

β-glucosidase specific rates varied even at similar $K_L a$ values and data from experiments III and IV or V and VII indicated that is possible to produce different specific production rates with the same initial $K_L a$. Product formation is therefore not solely a function of $K_L a$ and vigorous agitation clearly influences this behavior.

In the fermentor, β-glucosidase activity decreased with increases in oxygen concentration. This effect could be due to the action of oxygen inactivation of the enzymes similarly to results obtained by Robinson (*22*) and Rodríguez *et al.* (*23*).

With regard to filter paper activity, it was used as a reference during fermentation, because FPA activity of a cellulose preparation is strongly dependent on its β-glucosidase activity (*24*). In stirred-tank fermentations maximum enzyme yield occurs around 72 h. During the rest of fermentation, the mycelial enzyme apparently is released into the medium. It would be desirable to stop the fermentation as soon as possible and treat the culture to encourage the release of the mycelial-associated enzyme (*25*).

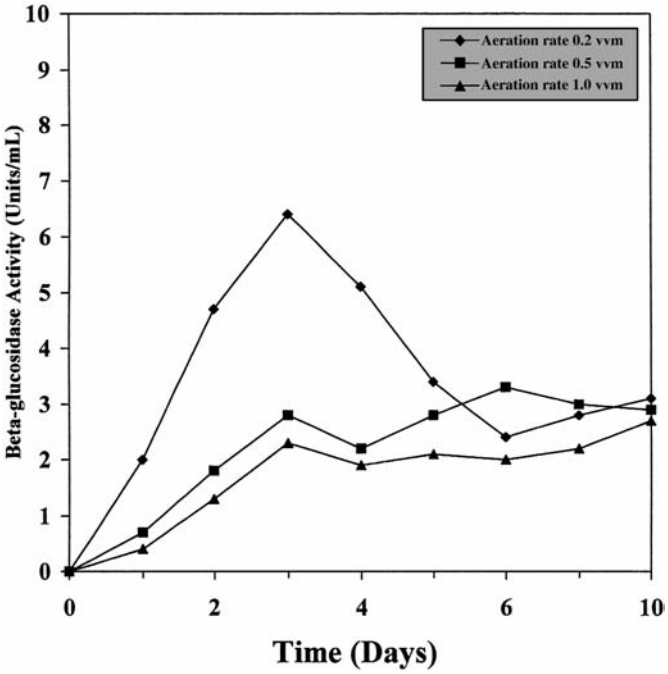


Fig. 5. β-Glucosidase activity at 250 rpm agitation speed in the sugar cane bagasse pith fermentation from *A. niger* ***C-6***.

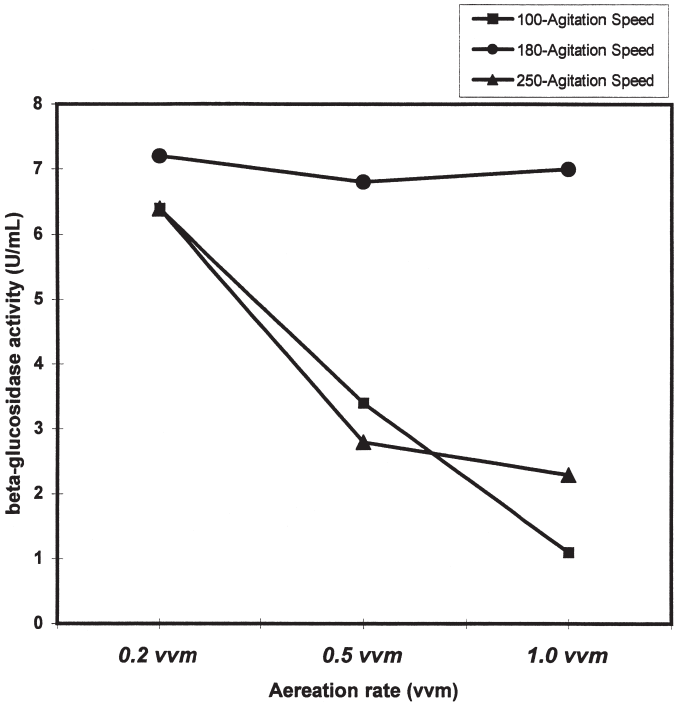


Fig. 6. Effect of aereation rate and agitation speed on the production of β-glucosidase activity in a 2-L stirred-tank laboratory fermenter.

Table 2
Results Obtained for the Growth of *A. niger* **C-6** on Sugar Cane Baggasse Pith Medium at Different Air Supply Conditions

	pH	Typical $K_L a$ (h^{-1})	Fermentation time (h)	Fermentation time of maximum activity (h)	Cell mass (g/L)	β-Glucosidase (U/mL)	Productivity (U/L/h)	μ_{pmax}	μ_{xmax}
I	5.6	1.05	240	96	17.1	6.4	88.8	0.86	0.07
II	5.4	2.19	240	96	16.3	4.2	43.8	0.18	0.11
III	5.2	3.21	240	144	18.7	3.1	21.5	0.25	0.11
IV	5.2	3.15	240	72	17.3	6.8	94.4	1.39	0.11
V	5.5	5.87	240	72	15.9	7.2	100.0	1.57	0.14
VI	5.6	7.04	240	72	17.9	7.0	97.2	1.63	0.18
VII	5.7	5.7	240	72	19.2	6.3	87.5	0.92	0.24
VIII	5.1	9.4	240	72	18.4	2.8	38.9	0.61	0.63
IX	4.8	11.07	240	72	18.1	2.1	29.2	0.08	0.72

I, 100 rpm/0.2 vvm; II, 100 rpm/0.5 vvm; III, 100 rpm/1.0 vvm; IV, 180 rpm/0.2 vvm; V, 180 rpm/0.5 vvm; VI, 180 rpm/1.0 vvm; VII, 250 rpm/0.2 vvm; VIII, 250 rpm/0.5 vvm; IX, 250 rpm/1.0 vvm.

Conclusions

The experimental results obtained support the following conclusions: it was possible to obtain 7.2 IU/mL of β-glucosidase activity using a stirred tank reactor with the *Aspergillus niger* **C-6** strain using a basal culture medium with corn syrup liquor. As reported by Kerns *et al. (26)*, it was possible to consider this raw material as a good nitrogen source for the production of β-glucosidase and cellulase activities. The optimum pH and temperature for production of β-glucosidase using this strain were found to be 5.5 and 29°C, respectively.

As shown in Fig. 6, aeration and agitation therefore both influence the β-glucosidase production of *Aspergillus niger* **C-6,** growth was dependent on oxygen supply. The best conditions obtained for enzyme production in a 2 L stirred-tank fermentor were 0.2 vvm and 180 rpm of aereation rate and agitation speed, respectively.

Acknowledgments

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