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PRODUCTION OF ß-GLUCOSIDASE BY *PENICILLIUM PURPUROGENUM*

A.B. Dhake; M.B.Patil*

Post Graduate Teaching Department of Biochemistry, L.I.T. Premises, Nagpur University, Nagpur, India

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ABSTRACT

The fungus *Penicillium purpurogenum* was found to produce intracellular ß-glucosidase. Maximum activity of ß-glucosidase was observed on sucrose. Various cultural parameters of cultivation of *P. purpurogenum* for production of ß-glucosidase were optimized. Maximum enzyme content was observed after 96 hours of cultivation at 30°C. Addition of amino acids histidine and cysteine induced ß-glucosidase synthesis to certain extent. The optimum temperature and pH for ß-glucosidase activity was 50°C and 5.5 respectively. ß-glucosidase of *P.purpurogenum* shows stability at pH 2 thus it could be an ideal enzyme for debittering in fruit juice and wine industries.

Key words: ß-glucosidase, *Penicillium*

INTRODUCTION

ß-glucosidase or ß-glucoside glucohydrolase [EC 3.2.1.21] has been widely used in ethanol production using various cellulosic agricultural residues such as corn stover, straw and bagasse (1,14,26) and in the synthesis of useful glucosides (5,21). In the flavor industry, ß-glucosidases are also the key enzymes in enzymatic release of aromatic compounds from glucosidic precursors present in fruits and fermentation products (6,11,23). ß-glucosidase is also useful in the process of deinking of printing ink from wastepaper (4,9,24).

Many bacteria, fungi and yeast have been shown to produce the enzyme ß-glucosidase. The bacteria like *Bretlanomyces bruxellensis* (11), *Oenococcus oeni* (11) and *Clostridium thermocellum* (7), the fungi like *Aspergillus oryzae* (16), *Fusarium oxysporum* (2), *Trichoderma reesei* (19), and the yeasts like *Candida peltata* (18), *Candida curvata* (20) are also reported to be the main producers. *Penicillium purpurogenum* is among the few fungi, which produced intracellular ß-glucosidase. The objective of the present investigation was to optimize the cultural parameters for maximum production of ß-glucosidase by *P. purpurogenum.* Effect of initial pH, agitation and different carbon sources on enzyme production has been studied. The effect of additives like amino acids, detergents and vitamins are also determined. The catabolite repression by glucose on enzyme production has also been observed.

MATERIALS AND METHODS

Organism, maintenance and culture condition

P. purpurogenum was isolated from the decaying wood in our laboratory and identified by IMT, MTCC, Chandigarh, India. The isolate was maintained on potato dextrose agar (PDA), pH 5.2 to 5.8, at 4°C, by periodic transfer. All the chemicals used were of analytical grade. The culture was transferred to new slants at every two months to keep it viable.

The medium used by Riou *et al*. (16) with composition of [0.2% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.1% NH_4NO_3, 0.1% $NH_4H_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% yeast extract; pH 6.0] supplemented with an appropriate carbon source at a concentration of 0.5% (W/V) was used. The medium used for production of ß-glucosidase by Saha and Bothast (18) had the following composition (per liter): 10 mL of solution A, 10 mL of solution B, 100 mL of solution C, 10 g of yeast extract, and 10 g of glucose. Solution A was a trace mineral solution having the following ingredients (per liter): 1.1 g of CaO, 0.4 g of ZnO, 5.4 g of $FeCl_3.6H_2O$, 0.25 g of $CuSO_4.5H_2O$, 0.24 g of

*Corresponding Author. Mailing address: Post Graduate Teaching Department of Biochemistry, L.I.T. Premises, Nagpur University, Nagpur-440 033, India. E-mail: mbpatil1956@sify.com

$CoCl_2.6H_2O$, 0.06 g of H_3BO_3, and 13 mL of concentrated HCl. Solution B (per liter) was composed of 10.1g of MgO and 45 mL of concentrated HCl. Solution C (per liter) contained 64 g of urea, 12 g of KH_2PO_4, and 1.8 g of Na_2HPO_4. Substrates were sterilized separately. Czapek-Dox medium [0.6% NaCl, 1% Sucrose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, 2.5% $NaNO_3$, pH 5.5] used by Patil and Shastri (13) was found to be best for ß-glucosidase production. The flasks with 1/5 liquid medium of its volume were inoculated with loopful spores from slants of *P. purpurogenum* and incubated at 30ºC for 4 to 5 days under static condition.

Extraction of intracellular enzyme

After desired period of incubation the mycelial mass was collected by filtration, washed with distilled water, and crushed in cold distilled water. The mycelial extract was centrifuged at 5,000g for 20 min at 4ºC. The supernatant was used as the source of enzyme.

Enzyme assay

ß- glucosidase assay was based on the procedure described by Riou *et al*. (16) by mixing 0.1 mL of 5 mM P-nitro phenyl ß-D-glucopyranoside (pNPG) and 0.4 mL 0.1 M Sodium acetate buffer at pH 5.5. After incubation for 10 min at 50ºC, the reaction was stopped by addition of 2 mL of 1 M sodium carbonate and the liberated P-nitro phenol was monitored at 420 nm. One unit of ß-glucosidase activity corresponded to the release of 1 µmol of *p*-nitro phenol min^{-1} under the assay conditions. ß-glucosidase activity was reported to be units/g/min of the mycelium.

Effect of environmental variables on ß-glucosidase production

The effect of various carbon sources was studied by replacing sucrose in the production medium with different carbon sources. Similarly, the sodium nitrate was replaced with various nitrogen sources. The starting pH of the medium was varied between 3 and 6.5. The effect of different concentrations of NaCl was also studied. The effects of various additives were also studied on 0.5% concentration level on ß-glucosidase production. The effect of different glucose in 25mM to 75mM concentration by supplementing glucose at regular interval of 24 hours up to 72 hours starting from the time of inoculation was also studied on enzyme production.

Characterization of ß-glucosidase

ß-glucosidase activity was measured at different temperatures (30 to 70ºC). The enzyme activity was measured from pH 2 to 7 by using the buffers of various pH in the standard protocol as mentioned above. The buffers used were HCL-KCL (0.1M, pH 2), Sodium citrate (0.1M, pH 3), Sodium acetate (0.1M, pH 4 to 6) and Citrate-Phosphate buffer (0.1M, pH 6 to 7). The ß-glucosidase activity was measured at 420 nm.

RESULTS AND DISCUSSION

The capacity of microorganisms to produce enzymes is influenced by environmental conditions such as temperature, pH, agitation, and the addition of inducers or repressor. *P. purpurogenum* was found to produce intracellular ß-glucosidase. *Trichoderma reesei* has also been reported to be the producer of intracellular ß-glucosidase (19).

Optimization of incubation period

P. purpurogenum starts ß-glucosidase synthesis within 24 hours of inoculation and the ß-glucosidase level reaches to its maximum within 96 hours (4 days) of incubation (Fig. 1). The time required for ß-glucosidase production by *C. peltata* was 4 days (18), and that for *A. oryzae* it was reported to be 14 days (16).

Optimization of medium constituents

The concentration of carbon and nitrogen sources, as well as the concentration of NaCl exerts a high influence on ß-glucosidase production. Inclusion of NaCl in the cultivation medium at 0.6% could improve the ß-glucosidase production. Similarly varying the concentration of medium constituents for maximum ß-glucosidase production by *P. purpurogenum* has a great effect. In order to determine the optimum concentration of the medium constituents, Oneway analysis of variance and the critical difference analysis was carried out by using SPSS-7 software. All the results were also analyzed by applying single linear regression.

The analysis for productivity suggested that there is a significant difference in the production for different carbon sources with $p < 0.001$. In order to determine, which of the sources used have significant effect on β-glucosidase production as compared to others, the critical difference (C.D.) analysis was carried out by the following expression:

$$C.D. = \sqrt{2S_e^{\,2}/r} \times t_{0.05}$$

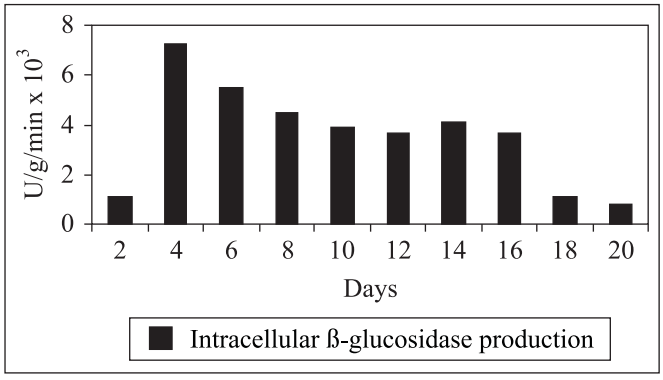

Figure 1. Effect of incubation time on ß-glucosidase production by *P. purpurogenum*.

Where S_e^2 is the mean square error and r is the number of replications, which are 3 in the present exercise. The mean productivity in the descending order has been shown in Table 1. Also, the table shows the difference between the consecutive productivity levels. The critical difference analysis resulted into a value of 439.28 ($t_{0.05}$ for 14 d.f. is 2.15). The comparison of this value with the differences in Table 1 reveals that the productivity for sucrose, cellobiose and maltose does not differ significantly, since the observed differences (d_i) are less than the C.D. However, this set differs significantly from the rest as the difference in the productivity of this set and the others exceed critical difference. Amongst the first three, since sucrose yielded maximum productivity, this carbon source was selected.

Carbon is a part of all compounds of living organisms. Breakdown of carbon source liberates energy, which is utilized by the organism for growth and development. The effect of different carbon sources was also studied by Riou *et al.* (16) for *A. oryzae* maximum ß-glucosidase production was found on quercetin. For *C. peltata* Saha and Bothast (18) optimum ß-glucosidase production were reported on xylose. Sandhu *et al.* (20) reported arabutin to be the best carbon source for optimum ß-glucosidase production by *C. curvata.*

Using different concentrations of sucrose, ß-glucosidase production was measured. In order to determine the optimum sucrose concentration, one-way analysis of variance was carried out. The analysis revealed significant difference in the productivity for different concentrations with $p < 0.001$. The critical difference analysis resulted into a value 654.09 ($t_{0.05}$ used for 10 d.f.), while the difference between the productivity for 1 and 2% was 860.85. Since, the difference exceeded the C.D. and the productivity for 1% was the highest, this sucrose concentration was selected for further studies (Table 2).

Organism requires nitrogen for the synthesis of compounds like proteins, nitrogenous compounds, vitamins and nucleic acids. Mainly the nitrates are use as a nitrogen source in the medium. In the present investigation for different nitrogen sources, there was significant difference in the production of ß-glucosidase with $p < 0.001$. The C.D. analysis was carried out resulting into a value 829.83 ($t_{0.05}$ used for 6 d.f.). The difference between the average production for $NaNO_3$ and KNO_3 was greater than the C.D. indicating that the difference is statistically significant. Since $NaNO_3$ showed maximum productivity, it was identified as the best-preferred nitrogen source by *P. purpurogenum* for ß-glucosidase production (Table 3).

Accordingly, six different concentrations of $NaNO_3$ were used and the production was observed for each concentration. The difference was found to be significant with $p < 0.001$. The critical difference analysis yielded a value 664.73 [$t_{0.05}$ used for 12 d.f.]. The difference between the production for 1, 2.5, 2 and 1.5% $NaNO_3$ concentrations was less compared to the C.D. indicating insignificance of difference in production at these concentrations. However, the productivity for these concentrations differed significantly than those for 0.5 and 3% $NaNO_3$ concentrations. Since, 2.5% yielded maximum β glucosidase, this concentration was selected for further experiment (Table 4).

The analysis of variance for eight different initial pH concentrations revealed that there is significant difference in the production of β glucosidase obtained at different pH levels with $p < 0.001$. In order to determine the pH level giving significantly higher productivity, the critical difference analysis yielded a value 555.878 ($t_{0.05}$ used for 16 d.f.). On comparing this value with the difference of the average productivity values for different pH levels, it was observed that the difference between the productivity for pH 5.5, and that for pH 5, which is next

Table 1. Effect of various carbon sources on production of ß-glucosidase by *P. purpurogenum.*

Carbon source (1%)	Mean productivity (U/g/min)	Observed Difference (*d*)
Sucrose	7459.97	
Cellobiose	7199.35	260.62
Maltose	6939.61	259.74
Lactose	6274.66	664.95
Rutin	6062.49	212.17
Xylose	5643.66	418.83
Glucose	4282.28	1361.38

Table 2. Effect of different concentration of sucrose on production of ß-glucosidase by *P. purpurogenum.*

Sucrose concentration (%)	Mean productivity (U/g/min)	Difference
1	7367.95	
2	6507.1	860.85
3	6126.24	380.86
5	5743.58	382.66
4	5345.19	398.39

Table 3. Effect of various nitrogen sources on production of ß-glucosidase by *P. purpurogenum.*

Nitrogen sources (2.5%)	Mean productivity (U/g/min)	Difference
$NaNO_3$	7386.847	
KNO_3	6379.737	1007.11
$(NH_4)_2NO_3$	5948.023	431.714

Table 4. Effect of different concentration of $NaNO_3$ on production of ß-glucosidase by *P. purpurogenum.*

$NaNO_3$ concentration (%)	Mean productivity (U/g/min)	Difference
2.5	7472.513	
1	7025.287	447.2267
2	7022.123	3.163333
1.5	6830.77	191.3533
0.5	6496.257	334.5133
3	6172.617	323.64

lowest, is 1455.47. This difference is greater than the critical difference value of 555.878 indicating that the production of β glucosidase at pH 5.5 is significantly higher than that for other pH values. The enzyme units at pH 5, 4.5 and 6 did not differ significantly as observed through critical difference analysis. But the productivity of this group differed significantly than that for pH 4, 6.5, 3.5 and 3 since the difference was much greater (i.e. 1599.72 and more) than critical difference. Since, pH 5.5 yielded maximum β glucosidase production was significantly different from others, this pH level was selected subsequently (Table 5). The same experiment was also performed by Sandhu *et al*. (20) for *C. curvata* by varying initial pH, where pH 5 was found to be optimum initial pH.

P. purpurogenum failed to grow in submerged condition hence no enzyme production was observed.

The analysis of NaCl concentrations revealed that there is significant difference in the production of ß-glucosidase with $p < 0.05$. The critical difference resulted into a value 640.14 ($t_{0.05}$ used for 8 d.f.). The difference between the β glucosidase units at concentrations 0.6 and 0.4% did not differ significantly as the difference is less that the C.D. However, the productivity for 0.6 % Nacl is significantly higher than that of concentrations 0.2 and 0.8 % and hence was selected for next experiments. Maximum enzyme production was obtained at 0.6% level in the cultivation medium (Table 6). The presence of sodium ions in the surrounding environment has proved to be essential for effective transport through membranes. The use of NaCl by Damaso *et al*. (3) has also indicated the significant impact of NaCl on production of xylanase.

Comparison of media for ß-glucosidase production

For selection of fermentation medium for ß-glucosidase production by *P. purpurogenum* three different media were used. The analysis of variance for these media indicated that the productivity in different media is statistically significant (p <0.001). The critical difference analysis gave a value of 397.47 ($t_{0.05}$ for 6 d.f. is 2.45). By comparing this value, it is evident that the difference between productivity in the medium used by Czapek-dox (13) and Riou *et al.* (16) is statistically significant since the difference is greater than the C.D. Thus, Czapek-Dox medium was selected which found to be best for ß-glucosidase production (Table 7).

Influence of additives on ß-glucosidase production

Addition of different additives in cultivation medium has an impact on ß-glucosidase production. The ANOVA for 16 additives suggested significantly large differences in the productivity (p<0.001). The C.D analysis yielded a value 515.576 ($t_{0.05}$ used for 32 d.f.). The productivity due to additives Histidine and Cysteine does not differ significantly, as their difference is less than C.D. However, the productivities due to

Table 5. Effect of initial pH on production of ß-glucosidase by *P. purpurogenum.*

Initial pH	Mean productivity (U/g/min)	Difference
5.5	7399.15	
5	5943.68	1455.47
4.5	5755.23	188.45
6	5694.03	61.194
4	4094.31	1599.72
6.5	4004.79	89.522
3.5	3999.11	5.684
3	2405.49	1593.62

Table 6. Effect of different NaCl concentration on production of ß-glucosidase by *P. purpurogenum.*

NaCl Concentration (%)	Mean productivity (U/g/min)	Difference
0.6	7453.622	
0.4	6948.469	505.153
0.2	6649.777	298.692
0.8	6349.774	300.003

Table 7. Effect of various mediums on production of ß-glucosidase by *P. purpurogenum.*

Medium	Mean productivity (U/g/min)	Difference
Czapek-Dox	7545.642	
Riou *et al.*	6663.591	882.051
Saha *et al.*	5982.563	681.028

all other additives differ significantly from these two. Addition of amino acids like serine, proline, norvaline, tryptophan, cystine, leucine, alanine, glutamine did not induce the enzyme production where as cysteine and histidine could increase the enzyme activity markedly (Table 8).

Addition of vitamins like ascorbic acid and thiamine had no effect on β-glucosidase production and detergents like SDS, tween 20 and tween 80 completely inhibited the growth of *P. purpurogenum* hence no enzyme production was observed (Table 8).

Activity of ß-glucosidase was determined at different pH levels. The analysis revealed that there is significant difference in the activity with $p < 0.001$. The critical difference analysis reveled that the activity at pH 5.5 was significantly higher than the rest of the pH levels as the difference between the activity at pH 5.5 and the next lowest 6 (575.687) exceeded the critical difference of 233.09 (Table 9). All the enzymes have a definite pH range for activity. The pH of the medium in which the enzyme is secreted and exposed affects the ionization state of its amino acids, which dictates the primary and secondary structure of the enzyme, thus controlling its activity (8). The influence of pH of the cultivation medium may be related directly with the stability of enzyme (25). *P. purpurogenum* was found to grow and produce ß-glucosidase at a quite low pH (even at pH 2). Effect of pH on activity was amazing in the sense that the ß-glucosidase of *P. purpurogenum* was highly active and 83% activity was recorded at pH 2 (Table 9). Both Sandhu *et al*. (20); Saha and Bothast (18) have reported optimum activity at pH 5, where as Rudick *et al*. (17) recorded optimum activity at pH 4.5 in case of *A. fumigatus*.

β-glucosidase activity was also determined at different temperatures, which suggest that the activity varies significantly with temperature. The activity was found to be significantly higher at temperature of 50ºC as revealed by critical difference analysis. The difference between the activities for 50ºC and 40ºC respectively was 1151.36, which is much larger than the critical value of 195.55; hence temperature of 50ºC was selected for determination of ß-glucosidase activity (Table 10). Riou *et al*. (16) in case of *A. oryzae* reported optimum activity at 50ºC. Saha and Bothast (18) also reported 50ºC as optimum temperature for ß-glucosidase produced by *C. peltata*, Where as Parry *et al*. (12) reported the optimum temperature to be 80ºC in case of *Thermoascus aurantiacus* ß-glucosidase.

Catabolite repression by glucose

It has been generally found that addition of glucose to the fermentation medium affects enzyme synthesis, (22). This type of relationship occurs not only in bacteria but also in fungi like *A. niger*, leading to interruption of production of polygalacturonase and pectin esterase (10) and in *P. expansum* reduction in synthesis of polygalacturonase has been observed (15). The inhibition of ß-glucosidase production by

Table 8. Effect of different additives on production of ß-glucosidase by *P. purpurogenum.*

Additive (0.5%)	Mean productivity (U/g/min)	Difference
Histidine	8657.53	
Cysteine	8476.421	181.109
Leucine	7539.274	937.147
Ascorbic acid	7377.747	161.527
Control	7367.955	9.792
Glutamine	7018.945	349.01
Alanine	6926.718	92.227
Lysine	6564.494	362.224
Cystine	6471.104	93.39
Thiamine	6260.308	210.796
Serine	5601.542	658.766
Valine	5198.831	402.711
Threonine	4734.053	464.778
Tryptophan	4676.211	57.842
Norvaline	4251.587	424.624
Tyrosine	4186.825	64.762

Table 9. Effect of pH on activity of ß-glucosidase by *P. purpurogenum.*

pH	Mean productivity (U/g/min)	Difference
5.5	3694.287	
6	3118.6	575.687
2	2974.68	143.92
3	2974.68	0
4	2974.68	0
5	2974.68	0
7	1583.45	1391.23

Table 10. Effect of temperature on activity of ß-glucosidase by *P. purpurogenum.*

Temperature	Mean productivity (U/g/min)	Difference
5.5	3694.287	
50	3694.28	
40	2542.92	1151.36
30	2015.21	527.71
60	1727.37	287.84
70	1247.63	479.74

glucose has also been reported in *Candida curvata* (20). Addition of different concentration of glucose to the cultivation medium of *P. purpurogenum*, from 25 mM to 75 mM concentration inhibited ß-glucosidase synthesis. Glucose was added at 25, 50 and 75 mM concentration to the cultivation medium at a regular interval of 24 hours from 0 to 72 hours; it was noticed that although the addition of glucose did not stop the growth of organism but enzyme production was partially arrested. From the Fig. 2 it was found that the higher the concentration of glucose added, more was the inhibition of the enzyme production as the concentration decreases the inhibition was also less. This might be because when glucose was added at 24, 48 and 72 hours there was already some amount of ß-glucosidase produced by organism for utilizing sucrose as carbon source that was not the case with addition of glucose at 0 hours. The organism has readily available glucose to be used as carbon source. Thus the catabolite repression of ß-glucosidase production by glucose was observed in *P. purpurogenum* (Fig. 2).

The properties (pH and temperature stability) exhibited by ß-glucosidase of *P. purpurogenum* are unique pointing out the great potential in store for application of this enzyme in biodebittering of fruit juices, and for improvement of flavors in the wine industry as this enzyme appears to withstand the highest stress of acidity and sugar concentration which is normally found in fruit juices and wines.

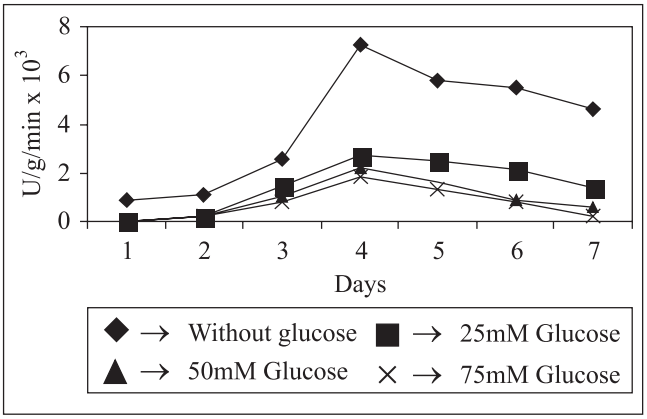

Figure 2a. ß-glucosidase production in absence of glucose and when glucose added at 0 hours at 25, 50 and 75 mM concentrations.

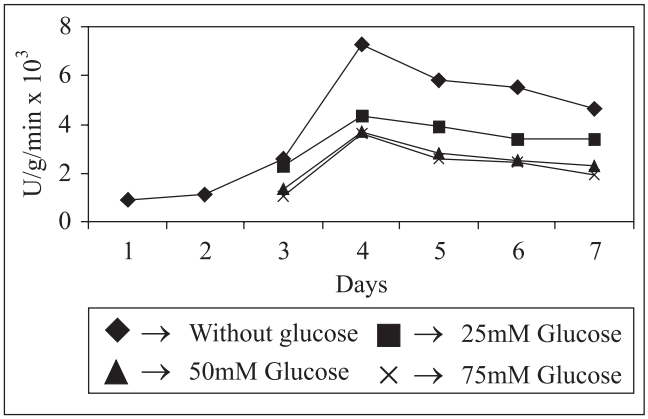

Figure 2b. ß-glucosidase production in absence of glucose and when glucose added at 24 hours at 25, 50 and 75 mM concentrations.

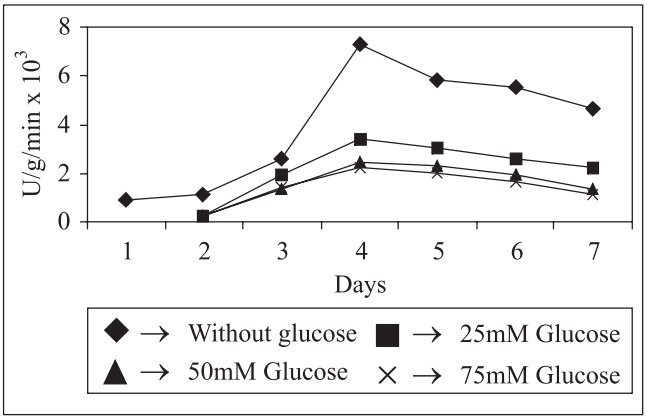

Figure 2c. ß-glucosidase production in absence of glucose and when glucose added at 48 hours at 25, 50 and 75 mM concentrations.

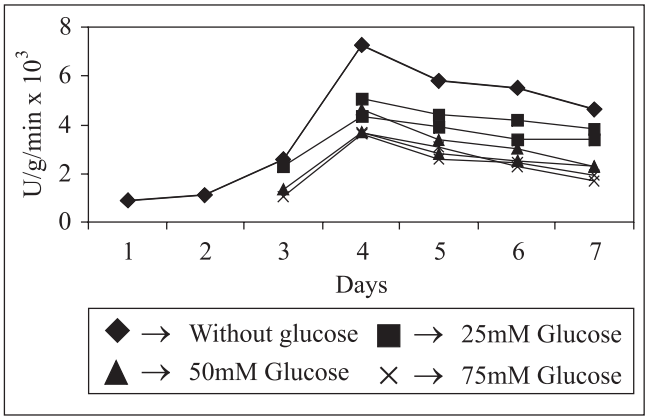

Figure 2d. ß-glucosidase production in absence of glucose and when glucose added at 72 hours at 25, 50 and 75 mM concentrations.

Fig. 2 (2a, 2b, 2c and 2d). Catabolite repression of ß-glucosidase production by glucose in *P. purpurogenum.*

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RESUMO

Produção de ß-glucosidase por *Penicillium purpurogenum*

Verificou-se que *Penicillium purpurogenum* foi capaz de produzir ß-glucosidase intracelular, com atividade máxima sobre a sacarose. Vários parâmetros culturais para produção da enzima foram otimizados. Verificou-se que a produção máxima da enzima ocorria após 96 h de cultivo a 30ºC. A adição dos amino-ácidos histidina e cisteína induziram a síntese da enzima até certo ponto. A temperatura e pH ótimos para atividade da enzima foram 50ºC e 5,5, respectivamente. A ß-glucosidase de *Penicillium purpurogenum* foi estável em pH 2,0, o que torna a enzima ideal para uso na indústria de sucos de frutas e vinhos.

Palavras-chave: ß-glucosidase, *Penicillium purpurogenum*

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