African Journal of Biotechnology Vol. 9(20), pp. 2918-2922, 17 May, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1513
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Influence of nitrogen sources on production of β-galactosidase by *Aspergillus niger*

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Accepted 26 February, 2010

The study was undertaken to enhance the production of β-galactosidase using five organic nitrogen sources with wheat bran as a substrate under solid state fermentation. The microbial source Aspergillus *niger* and its DG-resistant mutant that were grown in medium with initial pH of 5.5 in 250 ml flasks at 30°C for 144 h and sample was harvested after every 24 h and analysed for substrate consumption, cell mass formation and enzyme production. All the nitrogen sources, ammonium sulphate, corn steep liquor, diammonium phosphate, fish meal and urea showed significant results. However, higher values of enzyme activity of 168.0 and 371.15 IU/l/h, parent and mutant, respectively, was obtained from sample in which corn steep liquor was used as a nitrogen source as compared to control (73.1 and 176.3 IU/l/h in parent and mutant, respectively). The effect of nitrogen sources was also found significant in both the organisms but higher in mutant organism (2.2 fold). It is concluded that enzyme production enhanced 2.7 fold by use of suitable production medium under optimum cultural conditions and that the mutant derivative of *A. niger* can be exploited for hyper production of this enzyme.

Key words: *Aspergillus niger*, wheat bran, corn steep liquor, β-galactosidase.

INTRODUCTION

Enzymes are organic biocatalysts, which govern, initiate and control biological reactions important for life processes. Enzymes are also important in the food processing industry for ingredient production and texture modification. Use of enzymes includes production of corn syrup, beverage clarification, baking, meat tenderization and low lactose milk preparation (Yoast et al., 1994). The advantages of applying microbial enzymes for industries are due to existence of different types of enzyme activities, the rapidity and stability of production through the inexpensive and reproducible microbial fermentation route.

The improvements in yield are easily obtained with genetic or protein engineering. Enzymes are sold on the basis of activity rather than weight or volume and thus the stability of enzyme preparation during storage is of prime importance. The strain must be able to give high yields of enzyme within shortest possible fermentation time. If possible, extracellular enzymes should be used because they are easier to produce and isolate, whereas intracellular enzymes must go through expensive disintegration processes. *Aspergillus niger* which produce extracellular enzyme previously used by various researchers for enzyme production was exploited in this study for β-galactosidase. β-Galactosidase (EC 3.2.1.23) is found in nature and are able to release wide range of compounds (Neustroeu et al., 1991; denHerder et al., 1992; Manzanares et al., 1998) and hydrolyzes lactose (milk sugar) to glucose and galactose in human for absorption

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Abbreviations: DG, Deoxyglucose; **p-NPG,** p-nitrophenyl β-D-galactopyranoside; **PDA,** potato-dextrose agar media; **SSF,** solid-state fermentation; **CLS,** corn steep liquor; **DAP,** diammonium phosphate; **BSA,** bovine serum albumin; **LSD,** least significant difference; **β –gal,** β- galactopyranoside.

into blood (Stred-ansky et al., 1993; Martins et al., 2002). It is reported that 50% of world population, including Orientals, Arabs, Jews, most Africans, Indians and Mediterranean has a low level of this enzyme (Rajoka et al., 2003). Consequently, much of the lactose in milk that they drink moves through their digestive tract to the colon, where its bacterial fermentation produces larger quantities of carbon dioxide, hydrogen and irritating organic acids. This results in painful digestive upset termed lactose intolerance (Seyis and Aksoz, 2004; Lavelace and Barr, 2005). Remediation of this genetic disorder consists in removing lactose from the diet or converting it into glucose and galactose by application of β-galactosidase or lactase ((Martins et al., 2002). Furthermore, this treatment could make milk a most valuable food available to a large number of adults and children intolerant to lactose (Novelin et al., 2005). Lactose is considered to be of less value as it causes sandiness in ice cream and has low sweetening power and fermentability. The enzymatic hydrolysis of lactose to glucose and galactose also constitutes the basis of the most biotechnological processes currently developed to exploit the sugar content of milk whey, a byproduct of cheese industry whose disposal now contributes to considerable pollution problems (Sienkiewier and Riedel, 1990; Castillo, 1990; Becerra and Siso, 1996).

The selection of an inexpensive and easily available substrate together with a suitable producer micro-organism, optimization of culture conditions and effective downstream processing are essential to reduce the cost of enzyme preparation (Muniswaran et al., 1994; Becerra and Siso, 1996; Qiae et al., 1997). In Pakistan, a total output of agricultural wastes viz. rice, wheat bran, rice polishing, bagasse, molasses, and corncobs alike is estimated to be over 50 - 60 million tons per year (Rajoka et al., 1997) and can be used for conversion to products of economic importance in the country. The enzyme activity can be increased using some nitrogen source with suitable production medium. The role of organic nitrogen was found important for production of β-galactosidase by *Aspergillus oryzae* strain (Baily and Linko, 1990) and *Lactobacillus bulgaricus* (Murad, 1998) and high stimulation of enzyme production was observed when whey-maize steep liquor was used as nitrogen source in growth media (Sridhar and Dutta, 1989). In this study, wheat bran an inexpensive and easily available carbon source was used and production was enhanced by using different locally available nitrogen sources under solid state fermentation and *A. niger* and its DG-resistant mutant was exploited for hyper production of this important enzyme.

MATERIALS AND METHODS

The present research work was conducted in collaboration with National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad. The substrate *p*-nitrophenyl β-D-galacto- pyranoside (*p*-NPG) was purchased from Sigma Chemical Company, USA. Corn steep liquor was obtained from Rafhan Maize Products (Pvt) Ltd, Faisalabad. Nitrogen sources and wheat bran were purchased from local market. All other chemicals were of analytical grade.

Microbial strain

The strain of *A. niger* NIAB 280 and its deoxyglucose resistant mutant were collected from NIBGE collection center, Faisalabad, Pakistan.

A. niger culture was maintained on potato-dextrose agar media (PDA) slants and petriplates. Plates and slants were stored at 4°C in a refrigerator and refreshed fortnightly. Potato-dextrose agar (PDA) contained 25% peeled potato, 1% glucose and 1.5% agar (Bisen and Verma, 1994). DG resistant mutant was grown on Vogel's medium contained 1% CMC and 0.5% DG in slants and stored at 4°C in a refrigerator for further use. Vogel's medium (w/v) was used for inoculum preparation. Before preparing inoculum, the purity of cultures were checked under stereomicroscope

General plan

For the achievement of an optimized cheap medium with wheat bran as a carbon source, different nitrogen sources were studied at flask level in solid-state fermentation (SSF). The best nitrogen source was evaluated in both parent and its DG resistant mutant derivative through the calculation of different kinetic parameters like substrate utilization, biomass estimation and product formation during time course studies.

Nitrogen sources like ammonium sulphate, ammonium nitrate, corn steep liquor (CSL), diammonium phosphate (DAP), fish meal and urea were added accordingly to the growth medium. These all were used at a rate to contain 0.246% nitrogen in the growth medium.

Growth conditions for enzyme productions

The solid-state fermentation studies were carried out in 250 ml flasks plugged with cotton and were incubated at 30 ± 2°C in an incubator. Each flask contained 2 g dry substrate, calculated quantity of nitrogen and was moistened with 4 ml of Vogel's medium of pH 5.5 containing all salts and 0.2% yeast extract. The whole contents were sterilized at 121°C for 15 min. After cooling to ambient temperature, the contents of flask were inoculated with 3 ml of cell suspension (Cruz and Park, 1982). During the SSF, flasks from each, parental and mutant were periodically sampled every day in triplicate for different analysis. Time course studies were done by harvesting the samples at 0, 24, 48, 72, 96,120 and 144 h periodically.

Isolation of galactosidase

After incubation, 50 ml of sterile distilled water and 0.2% (v/v) Tween-80 were added to each flask. After 30 min on shaker at 4°C, the mash was filtered through cheese cloth. The unutilized substance was collected, dried to a constant weight and weighed. The filtrate was centrifuged at 10,000 rpm; the cell free supernatant was preserved as crude enzyme for enzyme assay, protein and reducing sugars. The cell pellets was used for estimation of biomass.

β-Galactosidase assay

The reaction mixture for determination of β-galactosidase activity

Table 1. Effect of nitrogen sources on kinetic of β-galactosidase production by parent and DG resistant mutant derivative of *A. niger* following the growth on wheat bran under SSF.

Nitrogen sources	Strain	Q_p IU/l/h	$Y_{p/s}$ IU/g subs	$Y_{p/x}$ IU/g cells	q_p IU/g/h
Ammonium Sulphate	P	98.60	195.65	476.05	181.85
	M	205.25	480.39	762.38	324.03
Corn steep liquor	P	168.00	265.20	526.76	242.31
	M	371.40	631.00	968.89	514.48
Diammonium phosphate	P	81.50	206.47	463.43	177.96
	M	173.15	491.75	812.0	334.00
Fish meal	P	132.35	252.42	504.70	211.47
	M	290.20	578.66	873.68	397.52
Urea	P	115.50	234.27	494.31	213.54
	M	275.0	526.85	775.58	383.14
Control	P	73.1	165.06	380.66	84.16
	M	176.3	299.90	682.14	164.0
Standard Deviation	P	33.218	29.539	24.698	26.335
	M	77.528	62.88	84.697	75.968
	Pooled	94.374	170.293	191.344	111.362

P = Parent, M = mutant, Q_p = rate of β-galactosidase formation (IU l^{-1} h^{-1}), q_p = specific rate of enzyme production (IU g^{-1} cells h^{-1}), $Y_{p/s}$ = β-galactosidase yield (IU/ g^{-1} substrate utilized), and $Y_{p/x}$ = Specific yield of enzyme production (IU g^{-1} cells).

consisted of 0.1 ml of 5 mM *p*-nitrophenyl-β-D-galactopyranoside (*p*-NPG), 0.800 ml of 5 mM sodium acetate buffer (pH 5.5) and 0.1 ml of crude enzyme solution. The mixture was incubated at 40°C for 15 min. To stop the reaction, 2 ml of 1 M Na_2CO_3 solution was added and then held for 10 min and optical density was noted at 400 nm on spectrophotometer (LaboMed, INC). One unit of β-galactosidase is defined as the amount of enzyme that releases 1 µmol of nitrophenol per min under the defined assay conditions (Kotwal et al., 1998).

Protein determination

Total proteins in the crude enzyme were estimated by method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Biomass estimation

Time course study was done on different nitrogen sources. The samples for both parent and mutant in triplicate were harvested at regular time incubations from separate flasks. To each flask, 50 ml deionized water and 0.2% (v/v) Tween 80 were added. After 30 min on a shaker at 4°C, the mesh was filtered through cheese cloth. The filtrates were centrifuged at 10,000 rpm for 15 min at 4°C. The cell pellets were suspended in autoclaved 0.89% saline solution and recentrifuged as above. The supernatant was discarded and the pellet was suspended and dried in a preweighed dry crucible in oven. After sufficient drying in few days they were weighed to get dry biomass of the organism.

Substrate utilization

During the time course study, the amount of substrate utilized or that remained was also determined. The sample was squeezed from cheese cloth. The biomass left was carefully dried in an oven using pre-weighed crucible. The amount was deducted from the total substrate weight to determine the utilized one.

Determination of growth kinetic parameters

Kinetics of growth was determined as described by Pirt (1975). The maximum enzyme production (Q_p) was determined by maximum slope in plot of substrate (g/l) and enzyme produced (IU/l) versus time of fermentation.

Product yield coefficient with respect to cell mass ($Y_{p/x}$) = dP/dx
Product yield coefficient with respect to substrate ($Y_{p/s}$) = dP/ds
Specific rate of product formation (q_p) = μ x $Y_{p/x}$

Statistical analysis

The data obtained was entered in computer using Mstat software. Proper tabulations were made and means were worked out. Analysis of variance was applied to see the significance of nitrogen sources and organisms. The comparison of means was made by LSD-test (Steel et al., 1997).

RESULTS AND DISCUSSION

Among the various nitrogen sources (ammonium sulphate, diammonium phosphate, urea, fish meal and corn steep liquor) added at 0.08 g nitrogen/per flask to medium containing wheat bran (2 g/flask). Corn steep liquor, one of the byproduct of starchy industry and the least expensive nitrogen source favoured maximum β-Gal (168.0, 371.15 IU/l/h) production in wild and mutant cultures, respectively. This was followed by fish meal, and urea, whereas ammonium sulphate and diamminium phosphate were found to be good sources of nitrogen. As enzyme production that attained its maximum value 2 g corn steep liquor per flask, was retained in all subsequent experiments.

Table 2. The sum of squares for effect of nitrogen sources on kinetics of β-galactosidase production by parent and DG resistant derivative mutant of *A. niger* following growth on wheat bran under SSF.

S.O.V	DF	Q_p	$Y_{p/s}$	$Y_{p/x}$	q_p
N.sources (A)	4	31196.513**	8421.2**	11182.5**	10518.1**
Organisms (B)	1	90203.37**	483381.1**	96668.2**	173361.51**
AxB	4	2432.84**	12.31.4**	4378.9**	2559.7**
Error	10	51.237	45.28	41.99	3.282
Total	19				

**Highly significant.

Table 3. Values for effect of nitrogen sources on kinetic of β-galactosidase production by parent and DG resistant mutant derivative of *A. niger* following the growth on wheat bran under SSF.

Nitrogen sources	Q_p	$Y_{p/s}$	$Y_{p/x}$	q_p
A.sulphate	151.9c	338.0e	619.2d	252.9d
CSL	269.5a	448.1a	747.9a	378.4a
DAP	119.6d	349.1d	637.7c	253.5d
Fish meal	181.5b	415.5b	689.2b	304.5b
Urea	195.3b	380.6c	634.9c	298.3c
Control	99.7c	247.5e	586.9d	124.1e

Means having different letters differ significantly at 5% probability level.

Table 4. Mean values for effect of organisms on parent and DG resistant mutant of *A. niger* on β-galactosidase production on wheat bran supplemented with different nitrogen sources under SSF.

Organisms	Q_p	$Y_{p/s}$	$Y_{p/x}$	q_p
Parent	119.03b	230.8b	493.0b	204.42b
Mutant	259.88a	541.7a	838.7a	390.63a

The results pertaining to β-galactosidase production are presented in Table 1 and the analysis of their variance in Table 2. The sum of squares showed that the effect hof different nitrogen sources on all product parameters was found to be highly significant. The difference was also found to be highly significant among tested organisms in respect of all product formation parameters. The interactive effect of tested organisms and nitrogen sources was found highly significant with all parameters. The mean values for Q_p (Table 3) ranged between 119.6-269.5 IU/l/h, recorded maximum for corn steep liquor (CSL) containing substrate and minimum with diammonium phosphate (DAP) supplemented substrate as compared to control (99 IU/l/h). Product yield coefficient ($Y_{p/s}$) and specific yield ($Y_{p/x}$) were recorded to be significantly higher with CSL containing media and minimum with DAP containing media. Specific product rate (q_p) ranged between 252.9-378.4 IU/l/h and was recorded maximum with CSL and minimum with ammonium sulphate. *A. niger* mutant strain exhibited significantly improved production of all kinetic parameters over parent strain (Table 4).

The comparison of mean values (Table 5) for tested organisms (parent and mutant) indicated that both organisms showed maximum values of Q_p, $Y_{p/s}$ and $Y_{p/x}$ on media supplemented with CSL as nitrogen source. Similarly, maximum values of q_p was recorded with same nitrogen source in both strains. The highest volumetric productivity for β-galactosidase (269.5 IU/l/h) was recorded with wheat bran-CSL medium that was 2.7 fold more than wheat bran alone. This was significantly higher than the calculated values (80.0 IU/l/h) reported previously by Rajoka and Yasmeen, (2005) in β-galactosidase production from *Kulyveromyces marxianus* grown on lactose-corn steep liquor. Sridhar and Dutta (1989) reported higher stimulation of β-galactosidase production from *Streptococcus cremoris* when grown on maize steep liquor supplemented whey media that confirmed these findings. Pandey et al. (1994) found that corn steep liquor increased the glucoamylase production while urea (0.25% w/v) favoured maximum pectinase production in *Streptomyces* sp. RCK-SC (Khuad et al., 2004). This may have occurred due to the positive regulation of global nitrogen metabolism regulator, AreA (Perez-Gonazalez et al., 1998) in corn steep liquor or ammonium sulphate in the culture medium. Enhanced substrate consumption and product formation kinetic parameters due to addition

Table 5. Interactive effect of nitrogen sources and organisms on parent and DG-resistant mutant of *A. niger* on kinetics of β-galactosidase production grown on wheat bran under SSF.

Nitrogen sources	Q_p IU/l/h		$Y_{p/s}$ IU/g subs		$Y_{p/x}$ IU/g cells		q_p IU/g/h	
	P	M	P	M	P	M	P	M
A.sulphate	98.6bc	205.3c	195.6c	480.4	476.0c	762.4d	181.9c	324.0e
Corn steep liquor	168.0a	371.09a	265.2a	631.0a	526.8a	968.8a	242.3a	514.5a
DAP	81.0cd	158.1d	206.5c	491.8d	463.4c	812.0c	173.0d	334.0d
Fish meal	73.02d	290.0b	252.4a	578.7b	504.7b	873.7b	211.5b	397.5b
Urea	115.5b	275.0b	234.3b	526.8c	494.3b	775.6d	213.5b	383.1c
Control	73.1 e	176.3 e	195.1 c	299.9 b	391.7 c	782.1 d	84.16e	164.0d

9y8aMeans having different letters in columns differ significantly at 5% probability level.

of corn steep liquor as nitrogen source suggested that the mutant organism may be exploited for hyper production of β-galactosidase.

REFERENCES

Baily MJ, Linko M (1990). Production of β-galactosidase by *Aspergillus oryzae* in submerged bioreactor cultivation. J. Biotechnol. 16: 57-66.

Becerra M, Siso MIG (1996). Yeast β-gal in solid state fermentation. Enzyme Microb. Technol. 19: 39-44.

Bisen PS, Verma K (1994). Lab exercise on fungi. In: Hand Book of Microbiology (eds. P. S. Bisen and K. Verma), p. 174, CBS Pub. Delhi-110-032 Indian.

Bradford MM (1976). A rapid and sensitive method for the quantification of micro-organism quantities of proteins utilizing the principles of protein dye-binding. Anal. Biochem. 72: 248-254.

Castillo FJ (1990). Lactose metabolism by yeasts. In: Yeast biotechnology and biocatalysis (eds. Verachtert H and De Mot. R.), pp. 297-320, Morcel Dekker, N.Y.

Cruz R, Park YK (1982). Production of fungal α-galactosidase and its application to the hydrolysis of galacto-oligosaccharides in soybean milk. J. Food Sci. 47: 1973-1975.

Den-Herder IF, Mateo-Rosells AM, Vanzuilen CM, Punt PJ, Vanden-Hondel CAMJ (1992). Cloning and expression of a member of *Aspergillus niger* gene family encoding α-galactosidase. Mol. Gen. Genet. 233: 404-410.

Kotwal SM, Gote MM, Sainkar SR, Khan MI, Khaire JM (1998). Production of α-galactosidase by thermophilic fungus *Humicula* sp. in solid state fermentation and its application in soymilk hydrolysis. Process Biochem. 33: 337-343.

Khuad RC, Kapoo M, Rustagi RE (2004). Enhanced production of an alkaline pectinase from Streptomyces sp. RCK SC by whole cell immobilization and solid state cultivation. World J. Microbiol. Biotech. 20: 35-37

Lavelace HY, Barr SI (2005). Diagnosis, symptoms and calcium intakes of individuals with resorting lactose intolerance. J. Am. Coll. Nutr. 24: 51-57.

Manzanares P, De-Graff Lh, Visser J (1998). Characterization of galactosidases from *Apergillus niger*: purification of a novel α-galactosidase activity. Enzyme Microb. Technol. 22: 383-390.

Martins DBG, De-Sougz CG, Simoes DA, De-Morais MA (2002). The β-galactosidase activity in *Kluyveromyces marxianus* CBS 6556 decrease by high concentration of galactose. Curr. Microb. 44: 379-382.

Muniswaran PKA, Selvokumar P, Charyuly NCI (1994). Production of cellulases from coconut core pith in solid state fermentation. J. Chem. Technol. Biotechnol. 60: 147-151.

Murad HA (1998). Utilization of ultrafilteration permeate for production of β-galactosidase from *Lactobacillus bulgaricus*. Milchwissenchaft. 53: 273-276.

Neustroeu KN, Krylov AS, Abroskina ON, Firsoy LM, Nasonoy VV, Yakhorlin A, (1991). Isolation and properties α-galactosidase from *Aspergillus awamori*. Biochem. (Mascow). 56: 288-296.

Novelin S, Neuhaus W, Kulbe KD (2005). A new innovative process to produce lactose-reduced skim milk J. Biotechnol. (E pub ahead of print).

Pandey A, Ashkumary L, Selvakumar P, Vijavakshmi KS (1994). Influence of water activity on growth and activity of *Aspergillus niger* for glycoamylase production in solid-state fermentation. World J. Microbiol. Biotechnol. 10: 485-486.

Perez-Gonazalez JA, Vanpeij NN, Bezoen A, Mac-Cabe AP, Ramon D, De-Graff LH (1998). Molecular cloning and transcriptional regulation of the *Aspergillus nidulans xyn*D gene encoding a β-xylosidase. Appl. Environ. Microb. 64: 1412-1419.

Pirt SJ (1975). Princioles of Cell Cultivation. Blackwells Scientific, London. ISBN 0-63208150-3.

Qiae D, Tang G, Zhong L, Xi Q, Yang K (1997). Studies on regulation of glucoamylase gene (glaA) expression in *Aspergillus niger*. Wei Sheng. Wu Xue Bao. 37: 349-354.

Rajoka MI, Bashir A, Malik KA (1997). Mutagenesis of *Cellulomonas biazotea* for enhanced production of xylanases. Bioresour. Technol. 62: 99-108.

Rajoka MI, Khan S, Shahid R (2003). Kinetics and regulation studies of the production of β-galactosidase from *Kluyveromyces marxianus* grown on different substrates. Food Technol. Biotechnol. 41: 315-320.

Rajoka MI, Yasmeen A (2005). Induction and production studies of a novel glucoamylase of *Aspergillus niger*. World J. Microbial. Biotechnol. 21: 179-189.

Sienkiewier T, Riedel CL (1990). Whey and whey utilization. Verlag Th. Manu, Germany.

Seyis I, Aksoz N (2004). Production of lactose by *Trichoderma* sp. Food Technol. Biotechnol. 42: 121-124.

Sridhar N, Dutta SM (1989). Factors affecting production of β-galactosidase of *streptococcus cremoris* (H) grown in whey. Indian J. Dairy Sci. 42: 538-542.

Steel RGD, Torrie JH, Dieky DA (1997). Principals and Procedures of Statistics. 3rd Ed. McGraw Hill Book Co, Inc., New York, USA.

Stred-Ansky M, Tomaska M, Sturdik E, Kremnicky L (1993). Optimization of β-galactosidase extraction from *Kluyveromyces marxianus*. Enzyme Microb. Technol. 15: 1093-1065.

Yoast S, Adoms RM, Mailer SE, Moon K, Palombella AL, Schmidt BF (1994). Generation and chacterization of environmentally sensitive variants of β- galactosidase from *Lactobacillus delbrucckii* subsp. *bulgaricus*. Appl. Environ. Microbiol. 60: 1221-1226.