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Gamma radiation induced mutagenesis in *Aspergillus niger* to enhance its microbial fermentation activity for industrial enzyme production

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Abstract α- and β-Galactosidases find application in food processing, health and nutrition. *Aspergillus niger* is one of the potent producer of these enzymes and was genotypically improved using gamma-ray induced mutagenesis. The mutant-derivative produced two-fold higher α- and β-galactosidases. For testing genetic variability and its relationship with phenotypic properties of the two organisms, DNA samples of the mutant and parental strains of *A. niger* were amplified with 28 deca-nucleotide synthetic primers. RAPD analysis showed significantly different pattern between parental and mutant cultures. The mutant derivative yielded homogeneous while parental strain formed heterogeneous amplification patterns. Seven primers identified 42.9% polymorphism in the amplification products, indicating that these primers determined some genetic variability between the two strains. Thus RAPD was found to be an efficient technique to determine genetic variability in the mutant and wild organisms. Both wild and mutant strains were analyzed for their potential to produce galactosidases. Comparison of different carbon sources on enzyme yield revealed that wheat bran is significant ($P < 0.01$) effective producer and economical source followed by rice bran, rice polishing and lactose. The mutant was significantly better enzyme producer and could be considered for its prospective application in food, nutrition and health and that RAPD can be effectively used to differentiate mutant strain from the parental strain based on the RAPD patterns.

Keywords Induced mutagenesis · DNA polymorphism · *Aspergillus niger* · Microbial fermentation · Kinetics · Thermodynamics

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Introduction

α- and β-Galactosidase are found in nature and both have different physiochemical and kinetic properties [1]. α-Galactosidase EC (3.2.1.22) (alpha-D-galactoside galactohydrolase) has a number of food processing and medical applications [2]. It is used in beet refining and in processing of legume foods for upgrading their nutritional value and enhancing their digestion [3]. In paper and pulp industry, biobleaching can be improved by adding galactosidases in combination with xylanases. Infants normally have the intestinal enzyme β-D-galactosidase (EC 3.2.1.23) or lactase that hydrolyzes lactose to glucose and galactose for

absorption into blood [4–6]. But 50% of world population, including Orientals, Arabs, Jews, most Africans, Indians and Mediterraneans has a low level of this enzyme [7] and have digestion problem, termed lactose intolerance [8]. Remediation of this genetic disorder consists in removing lactose from the diet or converting it into glucose and galactose by application of β-galactosidase [7]. The hydrolysis product is relatively sweeter, more soluble and shows higher biodegradability [5].

These enzymes are found in microbes, plants and animals but microorganisms are the most promising source [2]. Among various groups of microorganisms, the filamentous fungi have been the preferred sources of these enzymes [4]. Extensive work has been reported on efficient production of galactosidases (α- and β-galactosidases) and efficient inducers for large scale enzyme production [7]. Due to high cost of currently used technology of enzyme production, there is a dire need for developing low cost process for their production. Application of cheap substrates through solid-state fermentation (SSF) could meet this objective. Solid state fermentation technique is well accepted and has been used by many workers [1–3] for its simplicity, cheapness, less energy intensiveness, high product formation rate and less catabolite repression [2].

Pakistan needs to take up commercial production of such valuable biological products by technology improvement, establishment of industrial scale setup and genetic improvement of industrially important microbes. *A. niger* is a well known and better organism for its faster growth, wider acceptance of enzyme source for food and feeds and its GRAS nature [4, 5]. It is amenable to mutagenesis [9] and can be improved using γ-ray mutagenesis as reported earlier for other system [7, 9–11]. This study was therefore planned to use gamma irradiation to produce desirable mutant of *A. niger* and compare their ability with wild and other organisms for α- and β-galactosidases biosynthesis. Genetic variability between de-oxy-glucose-resistant mutant and parental cultures is presented as reported earlier [12, 13] for fingerprinting of different fungal strains.

Materials and methods

Procurement of materials and organism

The substrates *p*-nitrophenyl α-D-galactopyranoside and *p*-nitrophenyl β-D-galactopyranoside (*p*-NPG) were purchased from Sigma Chemical Company, USA whereas other reagents were of analytical grade. Bagasse was obtained from Crescent Sugar Mills, Faisalabad. Corncobs and corn steep liquor (CSL) were from Rafhan Maize Products (Pvt) Ltd, Faisalabad. All other agro-industrial waste substrates, used as a carbon source, were purchased from the local market. Lignocellulosic substrates were treated with alkali (2% NaOH) in an autoclave (15 min) keeping biomass alkali solution ratio 1:10 [10]. Treated substrates were autoclaved for 15 min, washed to neutralize and again dried in an oven at 80°C for 48 h. Moisture content in dry biomass was determined by further drying at 110°C until constant weight. NIAB 280 strain of *A. niger* was collected from NIBGE culture collection center, Faisalabad, Pakistan. Rice bran and rice polishing were purchased from a rice Sheller.

Culture maintenance, inoculum preparation and gamma radiation mutagenesis

The culture was maintained on potato-dextrose agar media (PDA) slants and Petri-plates [1]. Inoculum was prepared by suspending the spores from a PDA slant by adding 10 ml of sterile saline and contained 1×10^6 spores/ml. This fungal population was used for inducing mutation and to inoculate the growth media.

The spore suspensions (in 30 ml McCartney vials) were exposed to different doses (0.4–1.2 kGy with increment of 0.1 kGy) of gamma rays in gamma cell radiation chamber (Mark-V) as described previously [10]. Mutant derivatives were selected, and characterized in solid culture as described previously for β-fructofuranosidase [11, 14] and β-xylosidase [15]. Briefly, the survivors from 1.2 kGy treatment were plated on Vogel solid medium containing 1% guar gum, 0.45% oxgall and 1% (w/v) Deoxy-D-glucose (DG) and incubated at 30°C for 5 days. Single colonies were tested for production of β-galactosidase in plates tests as described earlier for β-xylosidase [15]. Among 500 colonies, one DG resistant mutant produced larger yellow zone among three putative mutants, was grown on Vogel's solid medium containing 1% galactose and 0.5% DG in slants and stored at 4°C in a refrigerator for further use.

Solid-state fermentation

Experiments were carried out in 250 ml Erlenmeyer flasks containing 2 g substrate. The substrate was moistened with 4 ml of Vogel's medium (initial pH 5.5) and thoroughly mixed. When soluble carbon sources were used polymeric resin DIAIO HP 20 from M/S Mitsubishi Japan (10 g; 150–250 μm particle size) was added as inert support material (that higher weight of resin which did not permit free water in SSF was used) to supply anchorage to the microorganisms and impregnated with 4 ml Vogel's medium. After autoclaving at 121°C for 15 min and cooling to ambient temperature, the flasks were inoculated with 3 ml of spore suspension and incubated statically in a 40-l incubator where temperature and humidity could be controlled automatically. Control temperature was 30°C and

relative humidity was 90%) as described earlier [1]. During SSF the flasks from each of the parental and mutant strains were periodically sampled at 0, 24, 48, 72, 96, 120, 144 and 168 h in triplicate for analyses. Unless mentioned otherwise, these conditions were maintained throughout the experiments.

After optimizing carbon source (wheat bran) nitrogen sources and optimum pH, temperature and inocula size were evaluated (by varying one parameter at a time approach) by observing their responses on product yield in both organisms in time course studies. To investigate the effect of pH on production process, initial Vogel's media pH was adjusted at different pH (3.0–9.0) with 0.1 M HCl or 0.1 M Na OH. To investigate the effect of inoculum size, 1–6 ml vegetative conidia were used. To study the effect of temperature, fermentation temperature in incubator was varied between 25 and 50°C.

Extraction of enzymes

For recovery of galactosidases from solid media, 50 ml of sterile distilled water (chilled) and 0.2% (v/v) Tween-80 were added to each flask and shaken for 30 min at 4°C on an orbital shaker [4] and the mash was filtered through cheese cloth. The filtrate from above was centrifuged at 10,000 rpm; the cell free supernatant was preserved for enzyme assays.

Assay of galactosidases

The reaction mixture for determination of α-galactosidase activity consisted of 0.1 ml of 5 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*-NPG), 0.80 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. The reaction was terminated; 3 ml of 1 M Na_2CO_3 solution was added and held for 10 min. Optical density was recorded at 400 nm on a spectrophotometer (Labo Med, INC).

β-Galactosidase activity was determined under the same conditions as described for α-galactosidase with the only difference that 5 mM *p*-nitrophenyl-*β*-D-galactopyranoside was used in place of *p*-nitrophenyl-α-D-galactopyranoside (*p*-NPG). One unit of α or *β*-galactosidase is defined as the amount of enzyme that releases 1 µmol of *p*-nitrophenol per min under the defined assay conditions [7]. Both enzyme activities under SSF were expressed as IU/g dry substrate (gds). Each sample was tested at least in triplicate.

Analytical methods

Total protein in the crude enzyme was estimated by the Bradford method [16] using enzyme filtrate, and bovine serum albumin (BSA) as standard. Routinely sugars in fermentation mash were determined using DNS reagent [17]. Total cellulose and hemicellulose were determined as done by Shirlaw [18]. Product yield expressed as IU/g dry solid gds (IU/gds) was determined as done by Pirt [19].

Total genomic DNA isolation for genetic variability

Total genomic DNA was isolated separately from 3 to 4 days grown culture of parent and mutant strains of *Aspergilus niger* using a modification of the cetyltrimethyleammonium bromide (CTAB) procedure [20, 21]. After RNase treatment, the DNA concentration was measured by flouremeter (Hoefer). The quality of DNA was assessed by running 8 ng of DNA on a 0.8% agarose gel. The total genomic DNA was diluted in double distilled water to a concentration of 1 ng/µl for PCR analysis.

RAPD analysis

PCR reaction was performed in 50 µl reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, dTTP, 30 ng of primer, 0.001% gelatin, 1 ng of genomic DNA and 2 unit of *Taq* polymerase (MBI Fermentas, Pakistan). For RAPD analysis, a total of 28 random decamer primers (OPB-12, OPH-05, OPI-13, OPJ 05, OPJ-07, OPJ-14 OPK-14, OPK-20, OPP-01, OPP-02, OPQ-02, OPH-03, OPI-02, OPJ-02, OPP-10, OPP-16, OPQ-03, OPQ-04, OPQ-10, OPQ-15, OPQ-17, OPR-04, OPS-20, OPU-01 OPU-10, OPU-18, OPV-06, OPX-01) purchased from Operon Technologies Inc., Aalamada, CA, USA were used. Amplification was performed in a thermal cycler (Eppendorf, Germany) using the cycling program: one cycle of 94°C for 5 min; 40 cycles of 94°C for 1 min, 36°C for 1 min; 72°C for 2 min; followed by one cycle of 72°C for 10 min and then held at 20°C until the tubes were removed [12, 13].

PCR products were resolved on 1.2% agarose gels and detected by staining with ethidium bromide. RAPD amplification was repeated at least two times and only reproducible, unambiguously scorable fragments were recorded [18]. For genetic diversity analysis, all polymorphic loci were scored as present/absent. The bi-variate 1–0 data were used to estimate similarity on the basis of the number of shared amplification products as done by [21, 22].

Results

Selection and screening of mutant strains of *A. niger*

After exposure to 1.2 kGy dose of *γ*-rays, survivors were permitted to grow on guar gum in the presence of DG

(1.0%) and aspartate (150 μg/ml) and genotypes resistant to both compounds were isolated on solid selection plates as described in materials and methods. Among 500 genotypes, well-developed yellow zone on *p*NPGa-agar plates appeared around three colonies. Semi-quantitative plate studies on *p*-nitrophenol β-galactoside (*p*-NPGa)–DG-solid media revealed that DG resistant mutant (M_1) produced a promising yellow zone [15] among three candidate mutants and was selected for all further experiments. Other mutants were moderate to low producers of α- and β-galactosidases and were discarded. Preliminary studies indicated that mutant strain produced two-fold more α- and β-galactosidases.

Genetic variability studies

Samples of genomic DNA of parental and mutant strains were analyzed by RAPD-PCR method to verify that the mutant isolate was genetically different from the parental organism or not by knowing whether the mutant strain gave homogeneous or heterogeneous banding pattern of amplified fragments. A total of 28 primers were employed ("Materials and methods" section). A total of 121 loci were amplified, with an average of ~4 loci per primer. Of these, 10.74 were polymorphic. Out of the 28 primers, eight primers, OPP-02, OPP-09, OP-16, OPK-17, OPK-20, OPB-12, OPQ-04 and OPJ-14 amplified polymorphic DNA fragments (Online supplementary data Table 1). Of these polymorphic primers, three produced polymorphic loci in parent strain and five primers produced polymorphic loci in mutant strain of *A. niger*. Thus mutant strain (higher enzyme producer) showed nearly uniform RAPD pattern, whereas the parental strain (lower enzyme producer) gave heterogeneous amplification pattern (results not shown). Thus amplification pattern gave us rough estimate about the mutant strain of the organism. A total of seven fragments were amplified with a primer OPJ-14 while the minimum number of fragments (02) was amplified by OPP-16 and OPP-17 each (Online supplementary data Table 1). The size of DNA fragments amplified varied from 0.5 to 2.0 kb.

Selection of substrate for production of α- and β-galactosidase

The results on the effect of different substrates including mono-saccharide, disaccharide homo-polysaccharides and complex lignocellulosic substrate (PS), from among various respective substrates (Online supplementary data Table 2) are reported as a mean of three sets of experiments (Online supplementary data Table 2). Quantity and nature of carbohydrates had direct effect on enzyme production. Mono-saccharides did not cause catabolite repression of both enzymes in SSF. But they were inferior to disaccharides, and complex polysaccharides ($P = 0.0007$). The analysis of variance (Online supplementary data Table 2) showed that the effect of carbon sources on product yield of β-galactosidase was profoundly significant ($P < 0.01$). Similarly highly significant difference was also observed between tested organisms (parent and mutant of *A. niger*). *A. niger* mutant showed improved product yield over parental strain. Product yield coefficient ($Y_{p/s}$) of and both α- and β-galactosidase were higher on wheat bran and minimum with glucose (Table 1; Online supplementary data Table 2). The representative kinetics of product formation by the parent and mutant cultures from galactose, lactose, rice bran and wheat bran (Online supplementary data Fig. 1) indicated that the activity in both strains attained its peak value after 120–144 h. DNA was extracted after growth of organisms on different carbon sources and was analyzed by RAPD-PCR method as mentioned above but we did not observe any change in the amplification products using different primers and results were not presented.

Table 1 Effect of carbon and nitrogen sources on product yield ($Y_{P/S}$) of α- (α-Gal) and β-galactosidases (β-Gal) by *A. niger* and its DG-resistant mutant derivative under SSF

	Strain	$Y_{P/S}$ (IU α-Gal/gds)	$Y_{P/S}$ (IU β-Gal/gds)
Carbon sources			
Monosaccharides	P	15.9 ± 3.2d	45.9 ± 4.3d
	M	33.6 ± 2.2bcd	97.4 ± 2.3bcd
Disaccharides	P	18.1 ± 3.2cd	62.6 ± 2.3cd
	M	43.5 ± 3.2b	133.9 ± b
Polysaccharides (PS)	P	15.8 ± 2.2d	59.7 ± 15cd
	M	33.2 ± 0.4gbcd	96.9 ± 0.64bcd
Complex PS	P	38.2 ± 14.9bc	111.9 ± 44.3bc
	M	72.9 ± 28.4a	223.8 ± 86.7a
Nitrogen sources			
Control	P	64.0 ± 2c	195.1 ± 3.1d
	M	135.3 ± 3b	299.9 ± 10.2b
NH_4 salts	P	66.6 ± 9.1c	230.5 ± 15cd
	M	149.3 ± 13.6ab	555.7 ± 23a
Organic sources	P	77.9 ± 12.4c	250.6 ± 15.3c
	M	170..1 ± 25a	578.9 ± 52.1a

Each value is a mean value of n = 3 experiments. Means in each column followed by different letters differ significantly at $P \leq 0.05$

The results obtained on α-galactosidase production and its statistical analysis showed that the effect of carbon sources and organisms was highly significant on product yield. The interaction between carbon sources × organism was non-significant ($P < 0.225$). The comparison of mean values for different carbon sources revealed that product yield ($Y_{p/s}$) of α-galactosidase was significantly ($P < 0.01$)

higher on complex polysaccharides (wheat bran was superior) and was selected for subsequent experiments.

Effect of nitrogen sources on production of α- and β-galactosidases

Nitrogen sources like ammonium sulphate, ammonium nitrate, CSL, fish meal, diammonium phosphate (DAP) and urea were added to the Vogel's medium (replacing ammonium sulphate and ammonium nitrate in the basal medium) to enhance growth and enzyme production. All these sources were used at the rate to contain 0.246% nitrogen (as present in the basal Vogel's medium with ammonium sulphate, and ammonium nitrate). Product yield ($Y_{p/s}$) of β-galactosidase and α-galactosidase was recorded significantly higher with CSL. When other nitrogen sources were added, α-galactosidase and β-galactosidase responded differently. α-Galactosidase was favoured by urea while β-galactosidase by fishmeal. Inorganic nitrogen sources supported lower values of both α- and β-galactosidases (Table 1; online supplementary data Table 3).

Effect of inoculum size on production of galactosidases

The comparison of mean values (Fig. 1) on effect of inoculum size on production of both enzymes displayed that significantly higher values of $Y_{p/s}$ were recorded with wheat bran inoculated with 3 ml (2.5×10^6/ml) culture followed by 4 ml culture. The mean values (Fig. 1) for organisms depicted that mutant strain showed higher values for product yield than the parental strain at all levels of inoculum size.

Effect of initial pH of the medium on production of galactosidases

The influence of initial pH of culture on α and β-galactosidase production was studied in the range of 5.0–9.0 (Fig. 2.). It was concluded that maximum product yield of enzyme occurred at pH 5–6. After that enzyme production dropped slowly. The studies indicated that in the absence of pH control, initial pH of 5.5 is regarded as optimal for α and β-galactosidase production in SSF.

Effect of fermentation temperature on α and β-galactosidase production

To evaluate the optimum temperature for maximum enzyme production, parental and mutant strains of *A. niger* were grown at different temperatures ranging from 22 to 40°C with an increment of 2 (Fig. 3). The growth of test organisms at higher and lower temperature than optimum

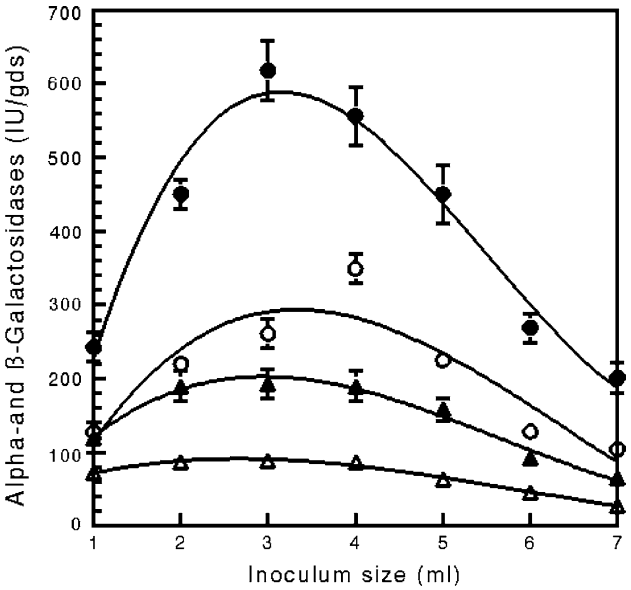

Fig. 1 Effect of inoculum size on production of α- and β-galactosidases by parental and mutant organisms following growth on wheat bran-CSL solid media (initial pH 5.5). The media were inoculated with different volumes of conidial suspension and incubated at 30°C in a controlled incubator. Each value is a mean of $n = 3$ readings. *Error bars* show standard deviation among three readings. *Open triangle* α-galactosidase (α-gal) by parent; *filled triangle* α-gal by mutant; *open circle* β-gal by parent; *filled circle* β-gal by mutant

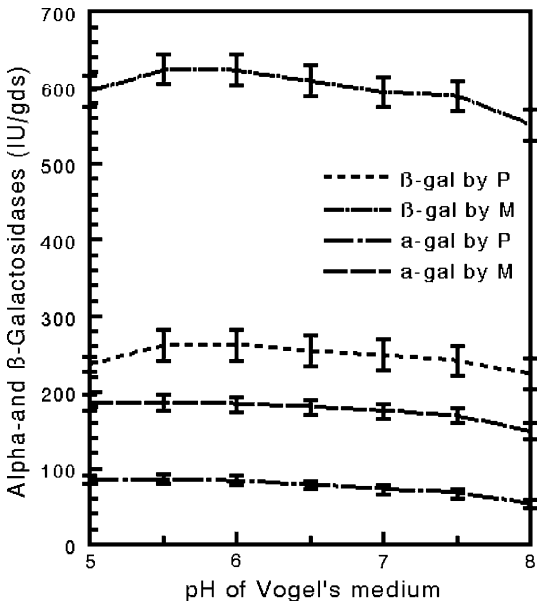

Fig. 2 Effect of initial pH of the Vogel's medium on production of α- and β-galactosidases by parental (P) and mutant (M) organisms following growth on wheat bran-solid media supplemented with CSL. The media were inoculated with 3 ml of conidial suspension and incubated at 30°C in a controlled incubator statically. Each value is a mean of $n = 3$ readings. *Error bars* show standard deviation among three readings

resulted in a decrease in enzyme activity. The optimum temperature was 30°C for biosynthesis of both α- and β-galactosidase activities. Product yield ($Y_{p/s}$) was

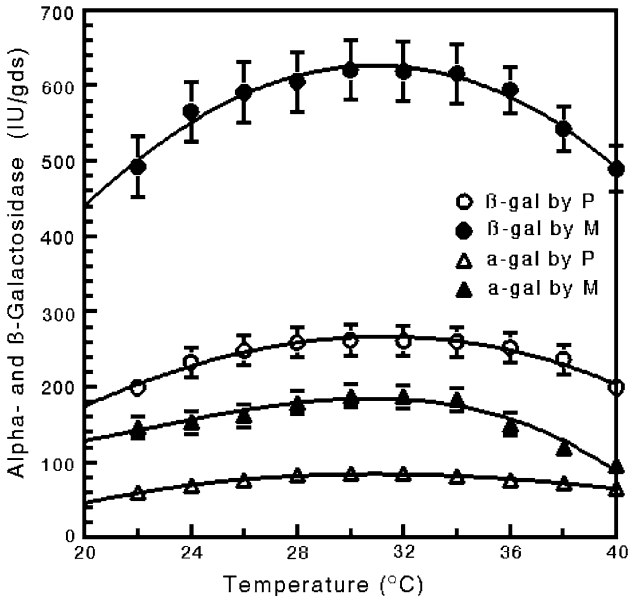

Fig. 3 Effect of fermentation temperature on production of α- and β-galactosidases by parental (P) and mutant (M) organisms following growth on wheat bran-solid media (initial pH 5.5) supplemented with CSL. The media were inoculated with 3 ml conidial suspension and incubated at 30°C in a controlled incubator. Each value is a mean of $n = 3$ readings. *Error bars* show standard deviation among three readings

maximum at 30°C and minimum at 40°C. The production is also high at 28° and 32°C but relatively lower than that at 30°C. The enzyme activities of DG-resistant mutant derivatives were higher (1.5–2.0 fold) than those of its parent at all temperatures, these showing that the enzyme production process by the mutant was more resistant to high temperature than that of wild strain.

Discussion

The method of choice for enzyme production has predominantly been fungal systems [1–3]. In previous studies several fungal strains, namely *Humicola* sp., *Penicillium* spp. and *Aspergillus* spp. [4, 25] were used. *A. niger* [25] is a potent producer of galactosidases. This study was envisaged to explore potential of the wild and its catabolite-resistant mutant derivative for the production of both α- and β-galactosidases in SSF and establish relationship of their genetic variability and enzyme productivity.

RAPD is a powerful tool to study variation and relatedness between species [20]. However, reproducibility in amplification of RAPD markers is a major concern [20]. In the present study, major/bright DNA fragments were scored to overcome the problem of reproducibility. Minor fragments were not scored because of random priming nature of the PCR reaction [13]. Random primers have been successfully applied to differentiate isolates of *A. niger* and other strains worldwide [20–22]. This technique was capable to readily differentiate between parent and mutant strain (online supplementary data Table 1) and production pattern of the tested strains. Both strains with some primers showed a correlation of genetic variability with enzyme production. Similar RAPD markers were also used to verify genetic diversity and phenotypic correlation in other studies [20–22]. In this study 94.83% genetic similarity (2 × numbers of common bands/total bands) was calculated between parental and mutant strain of *A. niger*. It is therefore suggested that RAPD assay is sufficient to differentiate the wild and the mutant strains which are low and high enzyme producing strains, respectively. In future, these polymorphic DNA fragments can be utilized for converting into sequence characterized amplified regions (SCARs), which would help to overcome the concern of lack of reproducibility of the RAPD assay. RAPD analysis can be utilized efficiently [20] to differentiate wild or mutant cells, intra-specific and inter-specific strains of organisms [22] in developing countries where complex facilities are not available.

We studied both strains of *A. niger* for their ability to synthesize both α- and β-galactosidase in the presence of mono-, di- and homo-polymeric polysaccharides and mixed polysaccharides in time course study. Enzyme production was found to be dependent on the nature of the carbon source applied in the culture media. Among all carbon sources wheat bran was the most effective supporter of enzymes. The carbohydrate contents of wheat bran and rice bran were the most effective inducers of α/β-Gal production. Good results were also obtained with rice polishing, rice bran and sugarcane bagasse (Online supplementary data Table 2). Mutant culture on different carbon sources supported larger levels of α/β-Gal synthesis. These studies indicated that monosaccharides supported low level of α/β-Gal biosynthesis in the wild cells while mutant cells were (twofold) improved for product formation over the parental culture. Similar trend was also reported by Rincon et al. [11]. Among disaccharides, lactose was the best source followed by sucrose. Wheat bran was a good source for production of α-galactosidase [1] and other enzymes [2–4].

It is important that for production of α/β-Gal, wheat bran was superior to all other substrates and can be easily and cheaply obtained from local flour mills and would be more economical for large scale enzyme production. Among bagasse, rice husk, rice bran, rice polishing and corncobs, the rice polishing was the best stimulator of α/β-Gal producer followed by rice bran. The induction on corn cobs, rice husk, rice polishing, rice bran, bagasse and wheat bran media yielded 1.67–2.86-fold α/β-Gal when compared to glucose and galactose. It was noteworthy that α/β-Gal

was also produced by cultures during growth on pure cellulosic substrates. This might have occurred due to slow release of glucose and thus inducing the cells to synthesize both enzymes as reported earlier [23]. Alternatively, a common mechanism of induction for cellulases [10] and α/β-Gal might have induced α/β-Gal but its production from arabinose, galactose, sucrose and lactose indicated that it was produced constitutively as well.

The effect of nitrogen sources on production of both enzymes was variable and depended on the test fungi and the nitrogen compound used in the studies. Organic nitrogen sources, namely, CSL, urea and fish meal, favoured more production of these enzymes and supported the work of other authors [24–26] who also used various nitrogen sources in their studies and concluded that organic nitrogen sources were the promising sources for enzyme production. The product yield of α/β-Gal supported by *A. niger* and its mutant is several fold higher than the reported values by other workers on *Aspergillus* spp., yeast cultures and their mutants or some recombinants harbouring heterologous genes for α/β-Gal [1–6, 25–27].

Increase in inoculum level from 1 to 3 ml/flask caused substantial increase in enzyme synthesis but after 4 ml inoculum/flask, a decline in synthesis of both enzymes was noticed. This might be due to enhanced competition of cells for carbon, nitrogen and other nutrients, which could lead to scarcity in nutrient availability. This could lead to cessation of growth and reduced enzyme synthesis. Other authors also reported similar trend in their published work [1, 3, 4].

Initial pH of the medium did not strongly effect enzyme production. SSF provided good buffering capacity. The enzyme production remained unaffected in a wide range of pH (3–7). But maximum activity was recorded at pH 5.5 like other strains of fungi [1–5]. This characteristic suggested that both organisms were suitable for enzyme production from wheat bran-CSL medium in SSF. Temperature optimization studies confirmed that both strains of *A. niger* produced maximum activity in mesophilic range (28–32°C) and confirmed the work of other researchers [1–6]. These studies also revealed that with increase in temperature, the enzyme production system possessed by the mutant was more resistant to higher temperature.

Conclusions

The potential of the newly developed mutant derivative for production of both α- and β-galactosidases in the SSF with wheat bran-CSL medium was quite promising. The mutant supported the formation of 196.8 and 631.0 IU/gds of α-and β-galactosidase respectively and was twofold improved over the wild culture. RAPD studies confirmed that mutant strain acquired significant change in its genetic makeup and was related with genotypic variability. These results suggest that more promising differences exist between two strains producing both enzymes. Mutant strain could be easily differentiated based on forming unique amplification products and were the result of acquired genetic changes by mutation. Enhanced production of these enzymes from the mutant derivative in SSF might provide a significant boost to harness the economic feasibility and commercial scale viability of these useful enzymes for preparation of lactose-free milk, nutritional improvement of bean -based foods, and recovery of sugar from beet molasses.

References

1. Wang CL, Li DF, Lu WQ, Wang YH, Lai CH (2004) Influence of cultivating conditions on the α-galactosidase biosynthesis from a novel strain of *Penicillium* sp. in solid-state fermentation. Lett Appl Microbiol 39:369–375
2. Liu CQ, Chen QH, Tang B, Ruan H, He GQ (2007) Response surface methodology for optimizing the fermentation medium of alpha-galactosidase in solid state fermentation. Lett Appl Microbiol 45:206–212
3. Anisha GS, Sukumaran RK, Prema P (2008) Evaluation of alpha-galactosidase biosynthesis by *Streptomyces griseoalbus* in solid-state fermentation. Lett Appl Microbiol 46:338–345
4. Shankar SK, Mulimani VH (2007) Alpha-galactosidase production by *Aspergillus oryzae* in solid-state fermentation. Bioresour Technol 98:958–961
5. Oliveira O, Teixeira JA, Lima N (2007) Development of stable flocculent *Saccharomyces cerevisiae* strain for continuous *Aspergillus niger* β-galactosidase production. J Biosci Bioeng 103:212–218
6. Yanai K, Nakane A, Hirayama M (2001) Molecular cloning and characterization of the fructo-oligosaccharide producing β-galactosidase gene from *Aspergillus niger* ATCC 20611. Biosci Biotechnol Biochem 4:466–473
7. Rajoka MI, Latif F, Khan S, Shahid R (2004) Kinetics of improved productivity of β-galactosidase by a cycloheximide-resistant mutant of *Kluyveromyces marxianu*. Biotechnol Lett 26:741–746
8. Viniegra-Gonzalez G, Favela-Torres E (2006) Why solid-state fermentation seems to be resistant to catabolite repression? Food Technol Biotechnol 44:397–406
9. Haq I, Khurshaid S, Ali S, Ashraf H, Qadeer MA, Rajoka MI (2001) Mutation of *Aspergillus niger* for hyper-production of citric acid from black strap molasses. World J Microbial Biotechnol 17:35–37
10. Rajoka MI, Bashir A, Hussain MRA, Ghauri MT, Malik KA (1998) Mutagenesis of *Cellulomonas biazotea* for improved production of cellulases. Folia Microbiol 43:15–22
11. Rincon AM, Codon AC, Castrejon F, Benitez T (2001) Improved properties of baker's yeast mutant resistant to 2-deoxy-D-glucose. Appl Environ Microbiol 67:4279–4285
12. Liu Z, Yang X, Sun D, Song J, Chen G, Juba O, Yang Q (2010) Expressed sequence tags-based identification of genes in a bio-control strain *Trichoderma asperellum*. Mol Biol Rep. doi: 10.1007/s11033-010-0019-0 (available online)

13. Ghally M, Ramos AM, Dokmetzian D, Lopez SE (2007) Genetic variability of *Phytophthora sojae* isolates from Argentina. Mycologia 99:877–883
14. Ashokkumar B, Gunasekaran P (2002) β-Fructofuranosidase production by 2-deoxyglucose resistant mutants of *Aspergillus niger* in submerged and solid-state fermentation. Indian J Exp Biol 40:1032–1037
15. Smith DC, Wood TM (1991) Isolation of mutants of *Aspergillus awamori* with enhanced production of extracellular xylanase and β-xylosidase. World J Microbiol Biotechnol 7:343–354
16. 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
17. Miller GL (1959) Use of dinitrosalicylic reagent for determination of reducing sugars. Anal Chem 31:426–428
18. Shirlaw DWG (1969) A practical course in agriculture chemistry. Pergamon Press, New York
19. Pirt SJ (1975) Principles of cell cultivation. Blackwell Scientific, London. ISBN 0-63208150-3
20. Zhang AL, Zhang TY, Luo JX, Fu CY, Qu Z, Yi GH, Su DX, Tu FZ, Pan YW (2009) Inducible expression of human angiostatin by AOXI promoter in *Pichia pastoris* using high-density cell culture. Mol Biol Rep 36(8):2265–2270
21. Afzal S, Saleem M, Yasmin R, Naz M, Imran M (2010) Pre and post cloning characterization of a beta-1,4-endoglucanase from *Bacillus* sp. Mol Biol Rep 37(4):1717–1723
22. Yoshimitsu M, Higuchi K, Fan X, Takao S, Medin JA, Tei C, Takenaka T (2010) Sequencing and characterization of the porcine alpha-galactosidase A gene: towards the generation of a porcine model for Fabry disease. Mol Biol Rep. doi:10.1007/s11033-010-9985-S (available online)
23. Hrmova M, Petrakova E, Biely P (1991) Induction of cellulose- and xylan-degrading enzyme systems in *Aspergillus terreus* by homo- and hetero-disaccharides composed of glucose and xylose. J Gen Microbiol 137:541–547
24. Gao J, Weng H, Zhee D, Yuan M, Guan F, Xi Y (2008) Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn Stover. Bioresour Technol 99: 7623–7629
25. Ademark P, Larsson M, Tjerneld F, Stålbrand H (2003) Multiple α-galactosidases from *Aspergillus niger*: purification, characterization and substrate specificity. Enzym Microb Technol 29: 441–448
26. Kuhad RC, Kapoor M, Rustagi RE (2004) Enhanced production of an alkaline pectinase from S*treptomyces* sp. RCK-SC by whole cell immobilization and solid-state cultivation. World J Microbiol Biotechnol 20:35–37
27. Kondoh K, Morisaki K, Kim WO, Park GG, Kaneko S, Kobayashi H (2005) Cloning and expression of the gene encoding *Streptomyces coelicolor* A3 (2) α-galactosidase belonging to family 36. Biotechnol Lett 27:641–647