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BIOSYNTHESIS OF β-GALACTOSIDASE FROM *ASPERGILLUS ORYZAE* USING MILK POWDER AS SUBSTRATE

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Abstract

The purpose of the current study was to produce highly active and stable β-galactosidase by using submerged fermentation employing indigenously isolated fungi. Different soil and food samples were collected from various waste lands and restaurants kitchens of Lahore for fungal strains isolation. X-gal based screening resulted in the isolation of 42 β-galactosidase producing fungal strains. Out of these, highest production (112.34± 0.23 U/mL/min) of β-galactosidase was shown by fungal isolate IIB-26 that was further subjected to 18S rDNA sequencing and was identified as *Aspergillus oryzae*. This work also focused on optimizing different fermentation parameters to enhance the productivity of β-galactosidase. The maximum productivity of β-galactosidase i.e. 112.34±0.23 U/mL/min was attained using soya bean meal medium of pH 4 after 120 hours of incubation, at 30ºC, using soya bean meal as carbon and urea as nitrogen source when it was inoculated with 3% inoculum of *A. oryzae* conidial suspension. The biosynthesis of enzyme was increased 3 times after optimization of fermentation parameters. The present work would help in future to decline the incidence of hypolactasia among Asian people by developing lactase supplements.

Key words: Catalysis, Submerged fermentation, Optimization, Bioproduct.

Introduction

β-galactosidase (EC 3.2.1.23) generally known as lactase is an essential biocatalyst that results in the hydrolysis of lactose into glucose and galactose, two main reducing sugars as well as energy reservoirs (Usai-Satta, 2012). Lactase enzyme can be derived and produced from a variety of sources that include food, plants and microorganisms (Saqib *et al*., 2017). Among all the known sources, microorganisms are widely used because of the fact that they excrete large quantities of enzymes and have the capacity of producing both extracellular as well as intracellular lactase (Corgneau *et al*., 2017). Moreover, microbial derived enzymes are more specific and have high stability as compared to other sources (Kazemi *et al*., 2016). Microbial sources include *Aspergillus oryzae, A. niger, Kluyveromyces lactis* and *K. fragilis* (Lukito *et al*., 2015). *Lactobacillus* and *Bifidobacterium* species are of great importance in producing β -galactosidase enzyme (Venkateswarulu *et al*., 2020).

The aim of conducting this research was to utilize β-galactosidase for medicinal purposes, the most important of which is the treatment of hypolactasia. Hypolactasia is a common genetic disorder in which the individual fails to produce adequate amount of β-galactosidase that is responsible for lactose digestion (Ianiro *et al*., 2017). This disease is affecting more than 50 percent of the world population and is the major concern now a days. Therefore, biosynthesis of β-galactosidase would help in solving this problem by the production of β-galactosidase supplements and pellets (Lember, 2012). Lactaid pills and Lactozyme are the examples of such supplements. Lactozyme is a digestive formulation that regulates the breakdown of lactose into its main constituents (Sitanggang *et al*., 2016). β-galactosidase can also be used in food industries and can be helpful in regulating milk hydrolysis (Nivetha & Mohanasrinivasan, 2017). The hydrolyzed milk is involved in improving the flavor of various dairy products such as ice-creams by reducing the sugar crystallization and promoting the process of acidification (Silanikove *et al*., 2015) Lactase also has significant importance in synthesis of galactooligosaccharides that is an important prebiotic and aids in supporting the defense mechanism of the body with the help of gut microbial flora. Galactooligosaccharides provides an advantage by elevating the levels of gut bacteria and also prevents the attachment of *Salmonella* and *Clostridium* species (Torres *et al*., 2010). Apart from treating lactose intolerance and flavor improvement of food products, β-galactosidase also has a significant role in bioremediation (Prashar *et al*., 2016). The disposal of whey permeates into the drinking water has increased the water pollution over the last few decades that can be alleviated by using thermostable β-galactosidase. It breaks down the whey into bioethanol which is another significant metabolite and can be widely used on industrial level (Kokkiligadda *et al*., 2016).

This research work was focused on isolating various β-galactosidase producing fungal strains from the soil samples of Lahore and to achieve maximum enzyme production isolate opting optimization studies.

Material and Methods

Isolation and screening: The soil, compost and food samples such as banana peel for fungal strain isolation were collected from different agricultural and industrial sites of Lahore, Pakistan. For isolation of fungal strains, Czapek Dox Agar (CDA) media supplemented with chloramphenicol antibiotic (25mg/mL) was used. After isolation, the CDA plates containing fungal colonies were loaded with 20µL of the X-gal stock solution and further incubated at 30ºC for 2 to 3 days for blue-white screening of the β-galactosidase producing fungal isolates (Kamran *et al*., 2016). The fungal isolates that showed positive results for X-gal screening were then purified on potato dextrose agar (PDA) slants.

Enzyme production: The enzyme was produced by submerged fermentation for which lactase production media (10g lactose, 1.5g peptone, 1.0g yeast extract, 1.0g potassium phosphate, 7.0g (NH_4) H_2PO_4, 1.0g $MgSO_4$. $7H_2O$ and 0.3g Calcium chloride) was inoculated with 1mL of spore suspension and incubated at 30℃ for 4 days (Sarfaraz *et al*., 2016). After incubation, the sample was centrifuged for 10 minutes at 10000 rpm and the supernatant obtained was then used as crude enzyme for enzyme assay (Natrajan *et al*., 2012).

Enzyme assay: The estimation of enzyme activity was done by using Para- nitro phenyl- para-D- galactopyranoside (PNPG) substrate. The tubes containing 0.2mL of sample and 0.8mL of substrate were incubated at 50ºC for 10 minutes. After 10 minutes, the reaction was terminated by adding 1 mL of 10 percent Na_2CO_3 solution. The absorbance was then checked at 420nm (Sarfaraz *et al*., 2016). One unit of β-galactosidase is defined as the amount of enzyme required to release 1 micromole of pNp per minute at 50ºC (Dormeyer *et al*., 2015) The unit of β-galactosidase was calculated by using the following formula:

$$\text{Enzyme units} = \frac{\text{Concentration from the graph x Dilution factor}}{\text{Incbation time}}$$

Identification: The maximum β-galactosidase producing fungal strain was identified by 18S rDNA genome sequencing (Medema *et al*., 2011). The obtained sequence was then inserted in NCBI database for obtaining the BLAST sequence that was further used for determining homology among the sequences. After homology analysis, Clustal omega was used to construct the phylogenetic tree for identification of closely related fungal specie.

Optimization of cultural conditions: Several physical and chemical factors such as media, time of incubation, temperature, pH, carbon sources, organic nitrogen sources, inorganic nitrogen sources and inoculum size were used for optimizing the β-galactosidase production. Table 1 shows the cultural conditions that were used to optimize the biosynthesis of β-galactosidase.

Statistical analysis

All the experiments were conducted in triplicates. Computer software, costat cs6204W.exe, was applied for the statistical analysis of the results.

Results

Isolation and screening: On Czapek dox agar plates, 55 samples showed positive result for the growth of fungal isolates. Among the isolated samples, 42 isolates gave positive results for β-galactosidase production by showing blue color around their colonies. Once the fungal isolates were primarily screened by X-gal screening, β-galactosidase activity was determined by SmF and highest yield of 39.13±0.21 U/mL/min was obtained by one of the isolated fungal strains (IIB-026) as depicted in table 3.

Identification: The 18S rDNA genome sequencing report was obtained from Macrogen, Korea. The fungal DNA was isolated (Fig. 1). The DNA was sequenced as evident from figure 2. The phylogenetic tree obtained after BLAST and Clustal omega illustrated that the strain (IIB-026) that gave maximum β-galactosidase biosynthesis was recognized as *Aspergillus oryzae* (Fig. 3).

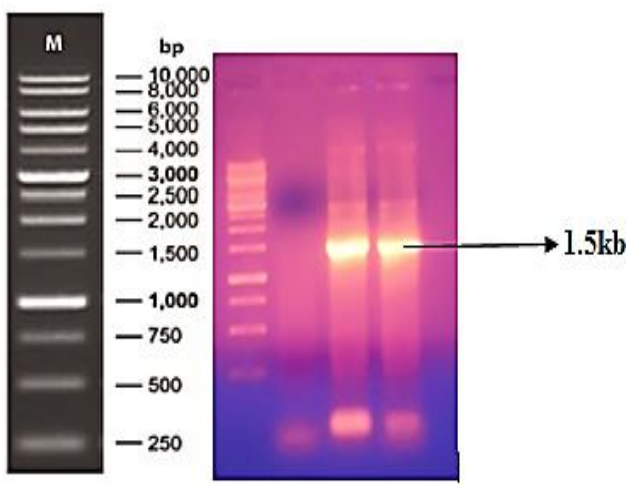

Fig. 1. Isolation of DNA from fungal isolate IIB-26.

Optimization of cultural conditions

Medium optimization: Nine different fermentation media were used for optimizing the synthesis of β-galactosidase by using the maximum producing isolate *A. oryzae* (Table 2). Among all the used media, the M7 medium (soya bean meal medium) gave the highest yield of β-galactosidase with the activity of 119.25± 0.21 U/mL/min (Fig. 4a).

Table 1. Cultural conditions used for the optimization of β-galactosidase production.

Cultural conditions	Method
Media	9 different media were used for the optimization purpose, The composition of all the media is mentioned in table 2
Incubation time	24, 48, 72, 96, 120, 144 and 168 hours
Temperature	25ºC. 30ºC, 35ºC and 40ºC
pH	3, 4, 5, 6, 7 and 8
Carbon sources	Glucose, fructose, lactose, pretreated molasses, sucrose and soya bean meal
Organic N_2 sources	Peptone, tryptone, meat extract, yeast extract
Inorganic N_2 sources	Ammonium sulfate, potassium nitrate, ammonium nitrate and urea
Inoculum size	1%, 2%, 3%, 4% and 5%

>S1_contig_1

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CCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGGCCCAGGAGGG
TTGGCAACGACCCCCCAGGGCCGGAAAGTTGGTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTC
CGTAGGTGAACCTGCGGAAGGATCATTA

Fig. 2. Genome sequence of fungal isolate IIB-26 obtained after 18s rDNA genome sequencing.

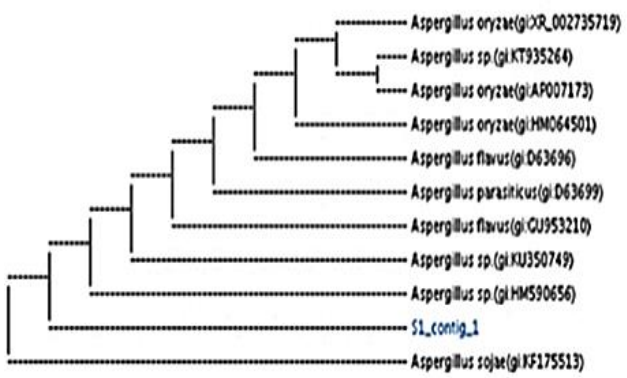

Fig. 3. Phylogenetic tree for identification of fungal strain.

Incubation time: The maximum synthesis of β-galactosidase (121.49±0.21 U/mL/min) was obtained after the incubation period of 120 hours. The activity of enzyme gradually started to decease after 120 hours (Fig. 4b).

Temperature: Different temperatures ranging from 25 to 40°C were used to optimize β-galactosidase biosynthesis. The maximum production of 118.54±0.13 U/mL/min was attained in the culture medium incubated that was incubated at 30°C (Fig. 4c). Additional increase in temperature significantly declined the enzyme activity.

pH: The pH of culture medium was maintained at 3, 4, 5, 6, 7 and 8. The maximum production was achieved at pH 4 with enzyme activity 119.74±0.07 U/mL/min (Fig. 4d).

Carbon sources: Various carbon sources such as glucose, fructose, lactose, sucrose, soya bean and pretreated molasses were investigated for the production of β-galactosidase. Maximum enzyme yield (127.11±0.17 U/mL/min) was achieved when soya bean meal was used as a carbon source. Therefore, soya bean powder was proved to be the best optimized C source (Fig. 5a).

Nitrogen sources: In the present study, different organic and inorganic nitrogen sources such as peptone, tryptone, yeast extract, urea, and ammonium sulfate were selected and optimized to enhance the biosynthesis of β-galactosidase. Among organic nitrogen sources, peptone significantly increased the synthesis of β-galactosidase with the highest activity of 122.57±0.04 U/mL/min as given in figure 5(b) whereas figure 5(c) shows that urea served as the optimized inorganic nitrogen source at which enzyme activity of 124.46 ±0.05 U/mL/min was obtained.

Inoculum size: The influence of different inoculum sizes (1%, 2%, 3%, 4% and 5%) on the synthesis of β-galactosidase was explored and it is determined that maximum activity of 112.34±0.23 U/mL/min was obtained by using 3% inoculum size (Fig. 5d).

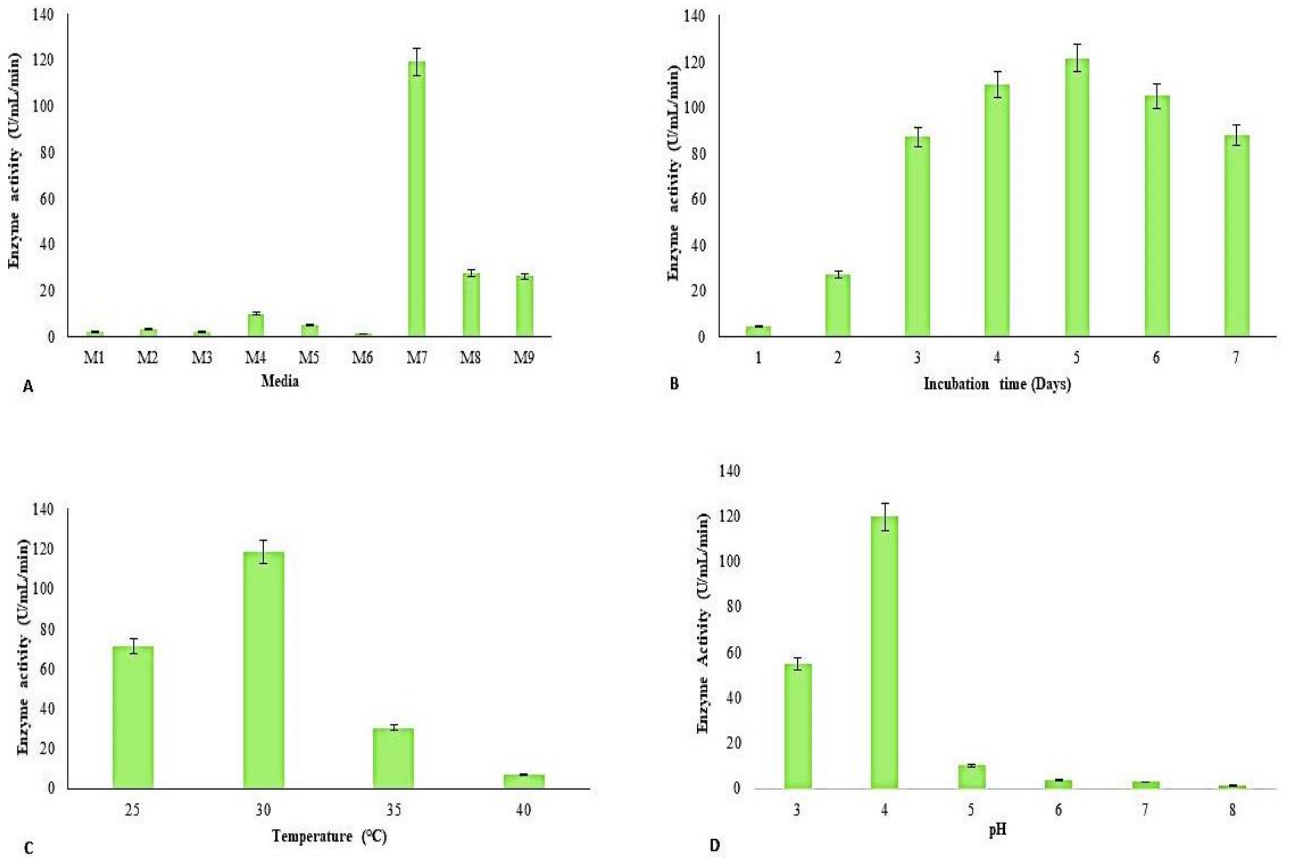

Fig. 4. Biosynthesis of β-galactosidase using *Aspergillus oryzae* by optimizing (a) Media (b) Incubation time (c) Temperature (d) pH.

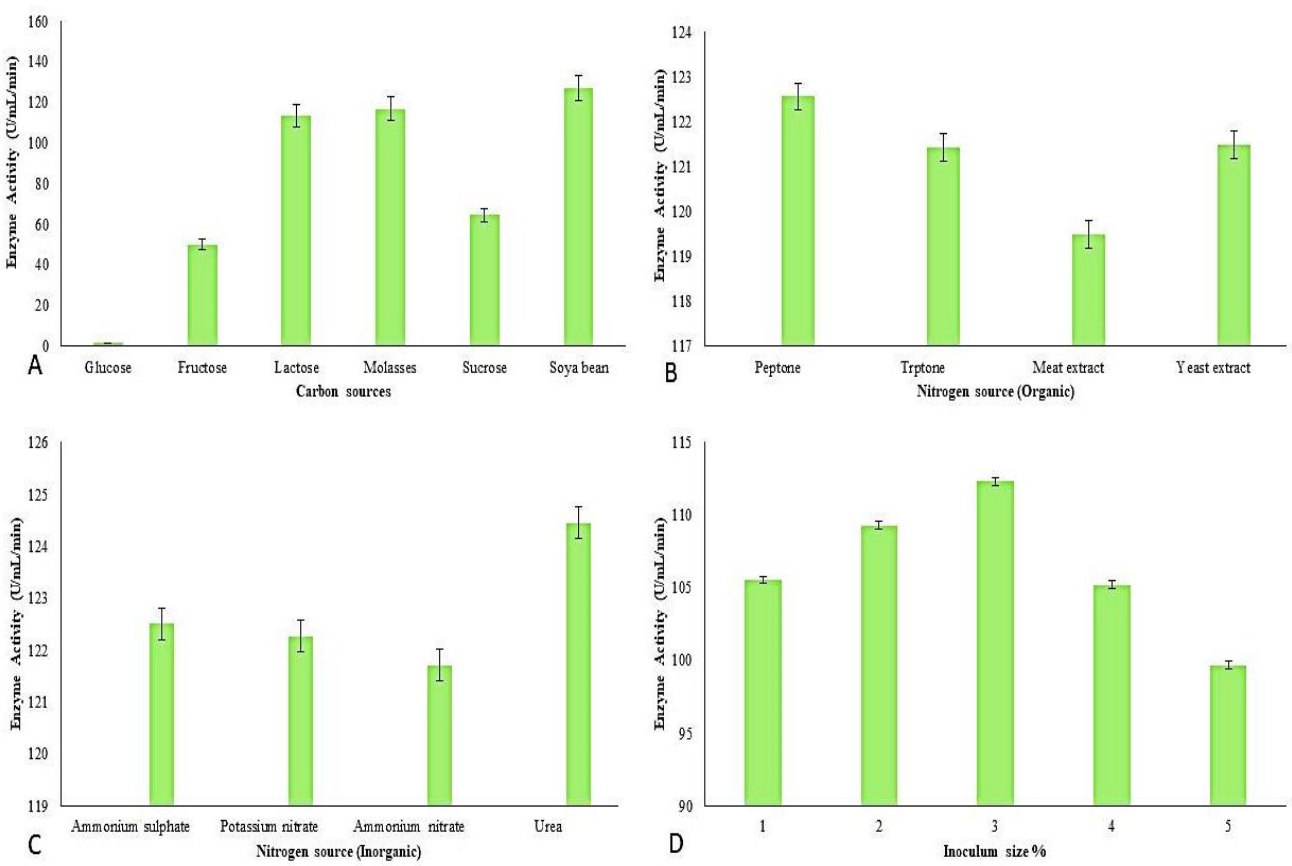

Fig. 5. Biosynthesis of β-galactosidase using *Aspergillus oryzae* by optimizing (a) Carbon sources (b) Organic nitrogen sources (c) Inorganic nitrogen sources (d) Inoculum size.

Table 2. Composition of optimization media.

Media	Composition g/L
M1	Lactose (20g), $NaNO_3$ (2g), K_2HPO_4 (1g), KCl (0.5g), $MgSO_4.7H_2O$ (0.5g), $FeSO_4.5H_2O$ (0.01g) (Shindia *et al*., 2006)
M2	Lactose (2g), peptone (0.4g), yeast extract (0.4g), KH_2PO_4 (0.2g), $Na_2HPO_4.12H_20$ (0.8g), $MgSO_4.7H_2O$ (0.025g) (Silverio *et al*., 2016)
M3	Lactose (2g), $(NH_4)_2SO_4$ (0.2g), K_2HPO_4 (0.1g), whey (2g), peptone (0.5g), yeast extract (0.3g) (Alikkunju *et al*., 2016)
M4	Lactose (4g), yeast extract (4g), $MgSO_4.7H_2O$ (1g), tryptophan (0.1g) (Sarfaraz *et al*., 2016)
M5	Lactose (9g), asparagine (2g), KH_2PO_4 (1g), $MgSO_4.7H_2O$ (0.5g), $FeSO_4$ (0.2mg), $ZnSO_4$ (0.2mg), $MnSO_4$ (0.1mg), biotin (5µg), thiamine (100µg) (Sun *et al*., 2018)
M6	Lactose (10g), asparagine (3g), KH_2PO_4 (1g), $MgSO_4.7H_2O$ (0.5g), KCl (0.5g), $FeSO_4.7H_2O$ (0.1g) (Sun *et al*., 2018)
M7	Soya bean meal (30g), milk powder (10g), KH_2PO_4 (1.8g), yeast extract (1.8g), $MgSO_4.7H_2O$(0.1g), $FeSO_4.7H_2O$ (5mg), $MnSO_4$ (5mg) (Domingues *et al*., 2004)
M8	Lactose (10g), peptone (1.5g), yeast extract (1.0g), KH_2PO_4 (1.0g), $(NH_4)H_2PO_4$ (7.0g), $MgSO_4.7H_2O$(1.0g), $CaCl_2$ (0.3g) (Vinderola and Reinheimer, 2003)
M9	Lactose (10g), peptone (10g), yeast extract (10g), dextrose (20g), $(NH_4)_2SO_4$ (5g), KH_2PO_4 (1g), K_2HPO_4 (3g), $MgSO_4.7H_2O$ (0.5g) (Cardoso *et al*., 2017)

Table 3. Estimation of β-galactosidase activity using submerged fermentation.

Fungal isolates	β-galactosidase activity (U/ml/min)	Fungal isolates	β-galactosidase activity (U/ml/min)
IIB 01	32.49 ± 0.10	IIB 022	6.90 ± 0.22
IIB 02	7.86 ± 0.07	IIB 023	0.24 ± 0.19
IIB 03	7.91 ± 0.21	IIB 024	36.18 ± 0.25
IIB 04	13.63 ± 0.13	IIB 025	34.80 ± 0.25
IIB 05	1.5 ± 0.17	IIB 026	39.13 ± 0.21
IIB 06	0.189 ± 0.20	IIB 027	18.13 ± 0.12
IIB 07	9.84 ± 0.04	IIB 028	1.44 ± 0.24
IIB 08	2.01 ± 0.07	IIB 029	13.46 ± 0.17
IIB 09	12.01 ± 0.22	IIB 030	11.54 ± 0.11
IIB 010	10.44 ± 0.15	IIB 031	3.94 ± 0.06
IIB 011	7.8 ± 0.21	IIB 032	10.02 ± 0.23
IIB 012	25.0 ± 0.04	IIB 033	4.84 ± 0.04
IIB 013	2.1 ± 0.13	IIB 034	12.30 ± 0.21
IIB 014	1.54 ± 0.07	IIB 035	11.14 ± 0.22
IIB 015	1.40 ± 0.16	IIB 036	6.74 ± 0.10
IIB 016	2.57 ± 0.10	IIB 037	4.85 ± 0.16
IIB 017	35.01 ± 0.21	IIB 038	6.13 ± 0.07
IIB 018	1.07 ± 0.05	IIB 039	4.06 ± 0.25
IIB 019	23.0 ± 0.01	IIB 040	1.44 ± 0.13
IIB 020	9.64 ± 0.04	IIB 041	7.22 ± 0.19
IIB 021	16.44 ± 0.17	IIB 042	19.59 ± 0.04

Discussion

The selection of cheap agricultural and industrial wastes for the isolation of β-galactosidase producing fungi and the optimization of fermentation parameters are important to minimize the cost of enzyme production with respect to industrial point of view. CDA medium was selected in the present study for the isolation purpose due to the fact that it is preferred medium for the isolation of fungal species such as *Aspergillus* and *Trichoderma*. The same medium was employed by Panesar *et al*., (2016) for isolating *Aspergillus flavus* for synthesis of β-galactosidase. The best reported fungi for production of lactase enzyme in the current study was *Aspergillus oryzae*. Soya bean meal media gave the maximum enzyme production of 119.25± 0.21 U/mL/min after 120 hours of incubation time as it contains the proper blend of all the essential nutrients required for the survival of fungal spores. Similar medium was optimized in the study of Martarello *et al*., (2019) and gave highest yield of the enzyme. Anumukonda & Tadimalla (2015) used clarified whey media but obtained inhibitory impact on β-galactosidase biosynthesis that

might be due to negative effect of amino acid (tryptophan and biotin) on fungal growth. The reason for long incubation time is due to the slow growth of *A. oryzae* in the fermentation medium (Pavani *et al*., 2011). In this study, the highest enzyme production (118.54±0.13 U/mL/min) was attained at 30ºC which shows that the maximum producing fungal isolate belongs to the mesophilic range and carries out its metabolic reactions best at mesophilic temperature at which maximum production of β-galactosidase was obtained (Vidya *et al*., 2014). In the present work, enzyme activity was found to be inhibited at 40ºC that can be due to extreme heat creation in the medium (Subramaniyam & Vimala, 2012). Carbon source is an essential component in the culture medium as it is the source of energy for the growth and survival of fungal spores. The current study shows that soya bean served as the optimized source of carbon as it contains high percentage of carbohydrates in its composition (Hu *et al*., 2010). Peptone and urea were determined as the optimized N_2 sources that enhanced the β-galactosidase synthesis by releasing mineral components in the medium that helps in promoting the fungal growth (Pant *et al*., 2015). Soya bean meal, urea and peptone have also been reported as the optimized C and N_2 sources in the finding of Hu *et al*., (2010) and Hsu *et al*., (2007). Maximum enzyme production was achieved at 3% that might be due to rapid consumption of nutrients by *A. oryzae* (Irfan *et al*., 2016). Some studies reported maximum production at 7% inoculum size that might be due to the slow rate of microbial growth (Chen *et al*., 2015).

Conclusion

Aspergillus oryzae was identified as a potent source of β-galactosidase production in the present work. Moreover, optimization of cultural parameters significantly increased the biosynthesis of β-galactosidase. The current research work would benefit in producing cost effective and industrially feasible β-galactosidase which can be utilized to augment the enzyme biosynthesis and improving food quality.

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