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Isolation and Screening of Fungal Strains for β-Galactosidase Production

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Abstract**—**Enzymes are the biocatalysts which catalyze the biochemical processes and thus have a wide variety of applications in the industrial sector. β-Galactosidase (E.C. 3.2.1.23) also known as lactase, is one of the prime enzymes, which has significant potential in the dairy and food processing industries. It has the capability to catalyze both the hydrolytic reaction for the production of lactose hydrolyzed milk and transgalactosylation reaction for the synthesis of prebiotics such as lactulose and galactooligosaccharides. These prebiotics have various nutritional and technological benefits. Although, the enzyme is naturally present in almonds, peaches, apricots and other variety of fruits and animals, the extraction of enzyme from these sources increases the cost of enzyme. Therefore, focus has been shifted towards the production of low cost enzyme from the microorganisms such as bacteria, yeast and fungi. As compared to yeast and bacteria, fungal β-galactosidase is generally preferred as being extracellular and thermostable in nature. Keeping the above in view, the present study was carried out for the isolation of the β-galactosidase producing fungal strain from the food as well as the agricultural wastes. A total of more than 100 fungal cultures were examined for their potential in enzyme production. All the fungal strains were screened using X-gal and IPTG as inducers in the modified Czapek Dox Agar medium. Among the various isolated fungal strains, the strain exhibiting the highest enzyme activity was chosen for further phenotypic and genotypic characterization. The strain was identified as *Rhizomucor pusillus* on the basis of 5.8s RNA gene sequencing data.

Keywords**—**β-galactosidase, enzyme, fungus, isolation.

I. Introduction

THERE is an ever increasing demand for the utilization of the biocatalyst; especially those synthesized by microbial fermentation in the industrial sector. One such enzyme is β-galactosidase, which has a great demand in the food as well as in the dairy industry [1]. β-galactosidase or lactase (E.C. 3.2.1.23) is the member of the glycoside hydrolase family of enzymes that are responsible for cleaving the glycosidic bond between two or more carbohydrate molecules or between a carbohydrate and the another molecule [2]. This enzyme has two main vital applications in the dairy industry, such as lactase is capable of catalyzing the hydrolysis reaction. In this reaction, the enzyme catalyzes the conversion of lactose into its respective monosaccharide units; thereby providing various health benefits such as offering lactose hydrolyzed milk as suitable alternatives for lactose intolerant people. Moreover, utilization of lactose hydrolyzed milk prevents the lactose crystallization in frozen, sweetened dairy products such as ice-creams, condensed milk [3], [4]. Besides the hydrolysis reaction, β-galactosidases are also able to catalyze the transgalactosylation reaction resulting in the synthesis of lactulose and galactooligosaccharide, potential prebiotics having various nutritional benefits [5]. Owing to these properties and to fulfill the demands of the consumers, there is a need for the low cost production of the enzyme.

Lactases are naturally present in plants such as almonds, peaches, apricots and in animal organs such as brain and placenta [6]. Apart from this, microorganisms are also major sources of this enzyme [7]. Among all the natural sources, microorganisms are the preferred source for the enzyme production in terms their ability to grow on low cost agro-industrial wastes; which can minimize the cost of production. Moreover, the higher yields can be obtained from microbial sources [8]. A variety of microbes such as bacteria especially lactic acid bacteria, yeast of the genus *Kluyveromyces* and fungi (*Aspergillus oryzae, A. niger, etc.*) have been widely used for the enzyme production.

Although various microbial species have been exploited for β-galactosidase production, still β-galactosidase from fungus is of special interest as the enzyme synthesized is extracellular and thermostable by nature [9]. Extracellular enzymes are of economic significance, since the production cost arising from the additional techniques to extract the enzymes is low as compared to intracellular enzymes. The selection of suitable substrate along with the microorganism giving the higher yield of enzyme, efficient processing techniques are the prime factors determining the cost of the enzyme production.

Keeping the above in view, the present study was carried to isolate the novel fungal strain from the various food and agricultural wastes having high enzyme producing capability.

II. Materials and Methods

A. Isolation of Fungal Cultures

1. Collection of Samples

Different food and agro-industrial wastes, such as whey from the Verka Milk Plant, Sangrur, Punjab (India), peels of the different fruits and vegetables (kitchen waste), and other sources such as compost, manure and garden soil had been taken from different areas of Punjab and nearby states of India.

2. Isolation of the Fungal Cultures

The isolation of the fungal cultures was carried following the method of [10], along with some modifications. All the isolates were grown on modified czapek dox supplemented with chloramphenicol (100 mg L^{-1}) to avoid the growth of

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bacteria and yeasts. The plates were incubated at 28 ºC and 45-50 ºC. Morphological identification of the isolates was observed and the colonies were further sub-cultured to obtain pure cultures which were stored at 4ºC until further use.

3. Screening of the Fungal Cultures

The lactase producing fungal strains were initially screened by adding 50 µL of X-gal (5-bromo-4-chloro-3 indole-β-D galactopyranoside (20 mg/mL in DMSO) as inducer in the agar plates. The plates were further incubated in the respective temperatures (28 ºC and 45-50 ºC) for 3-5 days. The fungal cultures capable of producing *β*-galactosidase were chosen for further studies.

B. Procurement of Fungal Cultures

The fungal cultures, *Aureobasidium pullulans* NCIM 1050, *Aspergillus oryzae* NCIM 1212, *A. niger* NCIM 616, and *Aspergillus flavus* MTCC 9349 were procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune and Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India respectively.

C. Production of β-Galactosidase by Submerged Fermentation

The growth and the fermentation media were prepared according to the method described by [11], to determine the potential of the fungal isolates in the production of the enzyme. The medium constituted of the following components (g/L): lactose (10.0), peptone (1.5), yeast extract (1.0), $(NH_4)H_2PO_4$ (7.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (1.0) and $CaCl_2$ (0.3). The fermentation media was inoculated with 2% spore suspension and incubation was carried for 7-8 days at 45-50 ºC. The fungal strain exhibiting higher enzyme activity as compared to the other isolates as well as from the standard culture was chosen for further studies.

D. Determination of Enzyme Activity

The enzyme assay was carried out by following the method of [12]. The culture was centrifuged at 5000 rpm for 10 min. The supernatant was used for enzymatic assay. The enzyme solution (0.2 mL) was mixed with the ONPG (1 mL) in sodium acetate buffer (0.1 M, pH 4.5). The enzyme solution along with the substrate was incubated at 50 ºC for 5 min. The reaction was stopped by using 1 mL of sodium carbonate (10%) and the absorbance was read at 420 nm (DR 5000, HACH, Germany).

One unit of enzyme activity is equivalent to 1 micromole of ortho-nitrophenol liberated per min. under standard assay conditions.

E. Identification and Characterization of the Isolated Culture

The identification of the fungal strain was carried by means of both phenotypic and genotypic characterization. The identification studies were carried from Institute of Microbial Technology (IMTECH), Chandigarh. The phenotypic characterization was carried using maximum likelihood model based on Tamura-3 parameter model [13] and the genotypic characterization was done using 5.8s rRNA gene sequence data [14].

F. Determination of the Cell Biomass and Enzyme Activity of the Fungal Isolate

The fungal isolate was grown for 14 days and the sample was withdrawn at every 24 h and the weight of the mycelium was checked to determine the growth pattern of the fungal isolate. Moreover, the enzyme activity of the fungal isolate was also checked at 24 h time interval.

III. Results and Discussion

The study was carried to isolate the fungal strains capable of producing elevated levels of the enzyme *β*-galactosidase. The fungal strains were grown on cheap agro-industrial wastes and further tested for their potential in *β*-galactosidase production.

A. Isolation of the Fungal Isolates

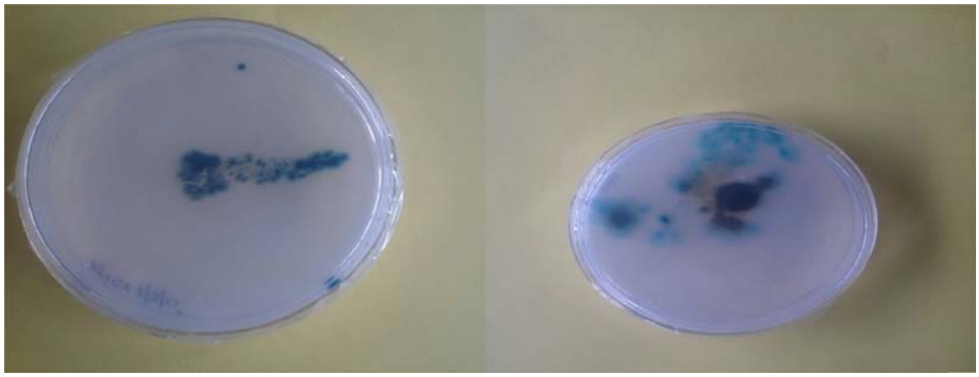
The fungal cultures were isolated from different food as well as agro-industrial wastes collected from the different parts of Punjab and other parts of India. A more than 100 fungal colonies were isolated when grown on the different agricultural as well as industrial wastes as well as from the other natural sources. These colonies were further grown on the modified czapek dox agar to obtain the pure culture.

B. Screening of the Fungal Isolates

The fungal colonies obtained were further screened by adding X-gal in the agar medium. The fungal colonies having the potential to synthesize *β*-galactosidase enzyme appeared blue in colour (Figs. 1 (a), (b)); whereas those fungal colonies that were not able to synthesize the enzyme did not show any blue colour. Among the 100 strains isolates, 45-50 fungal cultures showed blue colour when grown on media containing X-gal. The strains exhibiting the blue colour were chosen for further studies and the enzyme activity of the fungal cultures was determined. During the screening of the *β*-galactosidase producing strains, among the 72 colonies, 13 strains formed blue coloured colonies [15].

C. Production of β-Galactosidase by Submerged Fermentation

The fungal isolates were grown in the fermentation medium and the enzyme activity of the isolates were determined and compared with the standard fungal culture that showed the maximum enzyme activity. The enzyme activity of the different fungal isolates has been depicted in Table I. Among all the isolated fungal strains, BPTT showed the maximum enzyme activity (2.14 IU/mL) in comparison to the standard strain, *Aureobasidium pullulans*, which had an enzyme activity of 1.7 IU/mL as shown in Fig. 2. The highest enzyme producer strain was isolated from the rotten banana peel.



(a) (b)

Fig. 1 Fungal colonies showing blue colour when grown in X-gal media

TABLE I
ENZYME ACTIVITY OF THE FUNGAL ISOLATES

S.No.	Fungal Isolates	Enzyme Activity (IU/ml)
1	CD	0.112
2	CDT	0.0419
3	W1	0.646
4	W2	0.217
5	W3	0.065
6	W4	0.531
7	W5	0.066
8	WT	0.177
9	WTT	0.148
10	S1	0.171
11	S2	0.075
12	ST	0.147
13	CML1	0.578
14	CML2	0.373
15	CML3	0.039
16	VK	0.474
17	CLL1	1.26
18	S	0.029
19	ST	0.045
20	CLK1	0.064
21	CLK2	0.02
22	CLH	0.047
23	MK	0.500
24	PIIT	0.053
25	CLLT	0.043
26	CWL	0.530
27	ML	0.039
28	CLKT	0.090
29	WLKT	0.064
30	W6	0.193
31	CVT	0.303
32	MLT	0.58
33	VKT	0.434
34	MLHT	0.0405
35	SE	0.092
36	SET	0.215
37	PII	0.249
38	SM1T	1.52
39	SM3_1T	0.41
40	CLLT	0.13
41	P_2T	0.44
42	FSI_1T	0.189
43	FSI_2T	0.089
44	FSI_3T	0.044
45	BPTT	2.14

D. Identification and Characterization of the Isolated Culture

The phenotypic as well as genotypic characterization of the strain identified the strain as *Rhizomucor pusillus.* The analysis involved 13 nucleotide sequences (Fig. 3). The gene sequence data obtained from 5.8s rRNA sequence analysis revealed the presence of 558 base pairs as shown in Fig. 4. Moreover, it was seen that at the initial stages, the colonies were white in colour, which later became deep grey. The sporangiospores were umbellately branched, spherical in shape and brown to grey in colour. The spores had a smooth cell wall, about 5 µm in diameter. Moreover, the culture had an optimum temperature at 45ºC.

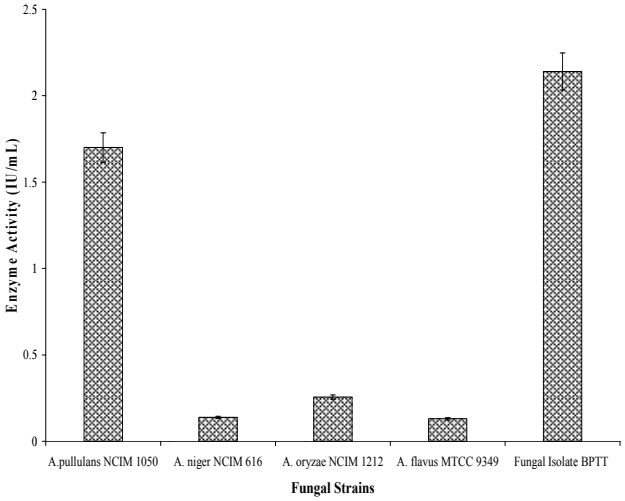


Fig. 2 Comparison of the fungal isolate with the standard cultures

E. Determination of the Cell Biomass and Enzyme Activity of the Fungal Isolate

The weight of the mycelium was determined at every 24 h to determine the growth pattern of the fungal isolate. From the results as shown in Fig. 5, it was observed that the fungal isolate showed maximum growth till the 9th day and after that a decrease in the mycelium weight was observed with further

increase in the incubation time. Furthermore, the enzyme activity of the isolate was found to increase till the 8th day of incubation (2.04 IU/mL), after which a decrease in activity was observed with further increase in incubation time up to 14 days.

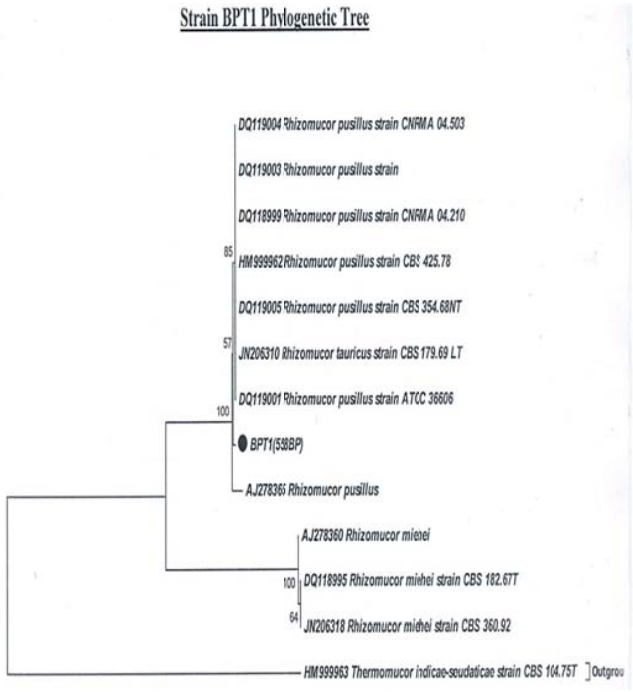


Fig. 3 Phylogenetic tree construction of the fungal isolate [13]

>BPT1 (558BP) *Rhizomucor pusillus* (99.8%); ITS/5.8S rRNA gene sequence data

ACATTAAAGTTGTGGAATCGTGGTGACCTCTATTGGTGAGCCGCGA
TTCTCTCCTTTTTGTGAAATGTTCTGAGGGATTGCTCCAGATCTCT
CGACCTTTTATTTTACATATTTGATTGACTGTTGTTTAACAAATTG
AAAGTTTTGGATCAGAAATGATTCAAGACGATAAAATTTCAAAACA
ACTTTAAGCAATGGATCACTTGGTTCTCGCATCGATGAAGAGCGTA
GCAAATTGCGAAAAGTAATGCGATCTGCAGCCTTTGCGAATCATCG
AATTCTCGAACGCACCTTGCACCCTTTGGTTCATCCATTGGGTACG
TCTAGTTCAGTATCTTTATTAACCCCTAAAGGTTTATTTTTTGATA
AATCTTTGGATTTGCGGTGCTGATGGATTTTCATCCGTTCAAGCTA
CCCGAACAATTTGTATGTTGTTGACCCTTGATATTTCCTTGAGGGT
TTGCATTGGTATCTAATTTTTTACCAGTGTGCTTCGAGATGATCAA
GTATAAAGGTTCATCACCACAATAATTTCACTATGGTCTGACTAGT
GGATAC

Fig. 4 Gene sequence data of the fungal isolate BPTT [14]

IV. Conclusions

The potential *β*-galactosidase producer fungal strain BPTT had been isolated from the rotten banana peel and from the genotypic as well as phenotypic studies; the strain was identified as *Rhizomucor pusillus*. The strain was capable of producing higher levels of *β*-galactosidase showing an activity of about 2.14 IU/mL as compared to the standard fungal strains. Furthermore, the strain showed maximum growth till the 9th day of incubation, after which a stationary phase was observed. The fungal isolate showed a maximum activity of 2.04 IU/mL during the 8th day of incubation, beyond which with further increase in the incubation time up to 14 days, a decrease in the activity was observed. The fungal isolate proved to be an efficient *β*-galactosidase producer at high temperatures as compared to the standard strains available. Thus, this culture had the potential to be used for the production of *β*-galactosidase, which could be further used in various industrial processes for the production of prebiotics.

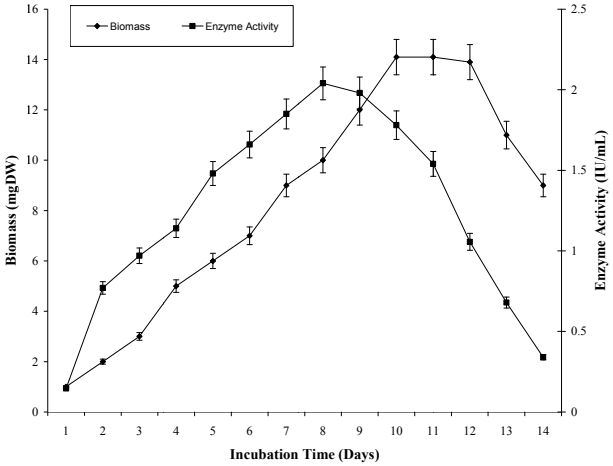


Fig. 5 Cell Biomass and enzyme production of the fungal isolate *Rhizomucor pusillus* with respect to incubation time

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