Research Article

Isolation of β-galactosidase from a yeast sp. isolated from whey

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ABSTRACT

β-galactosidase, (E.C 3.2.1.23) is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides. Lactose utilizing yeast cells are good source of β-galactosidase. β-galactosidase has tremendous potential in various fields like food, bioremediation, biosensor, biofilm formation, diagnosis and treatment of disorders. Whey, a by-product of cheese industry possesses a challenge in terms of downstream processing consisting of large amount of unutilized lactose. Hence, the study was designed to involve isolation of yeast capable of utilizing lactose efficiently, identification of yeast, optimization of growth media for maximal production of β-galactosidase, extraction of enzyme from yeast and study its kinetic parameters. The yeast species isolated from cheese whey produced β-galactosidase enzyme which is inducible in nature. The activity of the enzyme was higher for 36 h incubation period which decreased further after 40 h onwards. The enzyme displayed maximal activity around neutral pH of 6.6, at the temperature of 37°C and substrate concentration of 2.0 mM. Km for enzyme determined was 1.6 mM. The isolated yeast strain was found to metabolize and ferment various mono and disaccharides which includes fructose, galactose, maltose, lactose, sucrose, mannitol and arabinose.

Key-words: Yeast, whey, β-galactosidase, β-galactosides, lactose.

[I] INTRODUCTION

β-galactosidase (EC 3.2.1.23) catalyzing hydrolysis of O-glycosidic linkages in β-galactosides like lactose to glucose and galactose is an important enzyme for dairy industry since disposal of whey poses a serious pollution problem for the surrounding environment.[1, 2, 3] β-galactosidase is equally important enzyme to solve the problem of nutritional disorder like lactose intolerance[4] where 70% of the world’s adult population reported to have lactose intolerance due to deficiency of lactase. Thus the hydrolytic activity of β-galactosidase is applicable in the food industry for reducing the lactose content in milk for safe consumption by lactose intolerant people. [5] The transgalactosylation activity of this enzyme has been used to synthesize galacto-oligosaccharides (GOS) for balanced gastrointestinal flora preservation. [6] The β-galactosidase enzyme is also used for the production of glucose-galactose syrup obtained by the hydrolysis of lactose contained in the whey. [7] Sweet syrup produced through lactose hydrolysis by β-gal can be used in dairy, confectionary, baking and soft drink industries. [8] β-galactosidase can be also applied for the production of biologically active galacto-oligosaccharides by causing lactose hydrolysis.[9,10] Galacto-oligosaccharides (GOS) when taken orally promote the growth of
beneficial bacteria, Bifidobacterium and Lactobacillus sp. in the large intestine which leads to the improvement of microflora and suppression of putrefaction in the gut. In addition, they have anti-adhesive and inhibitory properties to pathogens. Beta galactosidase also known as lactase is widely distributed in various microorganisms including bacteria, fungi, archaea and yeast as well as in plants and animals. The major enzymes of commercial interest are isolated mainly from the yeast Kluyveromyces lactis, K. fragilis, K. marxianus, Candida kefyr and the fungi Aspergillus niger or A. oryzae. The present work is intended to involve isolation of the yeast sp. producing beta-galactosidase enzyme, its molecular characterization, pilot scale production of enzyme using submerged fermentation, partial purification of the enzyme by salt precipitation method and optimization of enzyme activity based upon pH, temperature, incubation period and requirement of metal ions as cofactors.

II] MATERIALS & METHODS

2.1 Sample collection
The dairy effluents were collected from Katraj Dairy Industry, Pune, Maharashtra. Samples were brought to the laboratory under aseptic conditions in sterile container.

2.2 Isolation and screening of yeast for the production of beta-galactosidase
The pure culture of yeast strains was isolated from dairy effluent sample by serial dilution method using Sabouraud Dextrose Agar (pH 7.0) containing 2% lactose provided with streptomycin (20 μg/ml) to prevent the bacterial growth. The plates were then incubated at 27°C for 24 hrs. Yeast isolates were confirmed by microscopic observation after staining with cotton blue. A Morphological and biochemical study of isolated yeast strain was carried out. The purified isolates were preserved on SDA slants at ±4°C. Molecular identification of isolated yeast strain-1371 MD2 was carried out in Agharkar Research Institute, Pune, Maharashtra.

2.3 Procedure for molecular identification for isolated yeast sp
Isolated yeast sp. was grown in YPD broth at 25°C for 48 h. Genomic DNA was isolated in pure form, from the isolated yeast culture. The nearly ~350 bp rDNA fragments were successfully amplified using universal primers M13 forward (5’TGTAACGACGCGGTAG3’) and M13 reverse (5’ CAGGAAACAGCTATGACC 3’). The sequencing PCR was set up with ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit. The raw sequence obtained from ABI 3100 automated DNA sequencer was manually edited for inconsistency. The sequence data was aligned with publicly available sequences & analyzed to reach identity. The sequences of rDNA from 1371-MD2 has been assigned the GenBank accession number DQ286062.1. A database search for closely related fungal species was aligned using the Clustalw program method of the BioEdit software. After analyses of their rDNA sequences, a phylogenetic tree was generated using the program Neighbor-joining (NJ) of Mega 4.0 program by utilizing the sequence of related strains which were downloaded from GenBank.

2.4 Enzyme production
Culture media and growth conditions
2.4.1 Inoculum Preparation for the Production of beta-Galactosidase
Inoculum was prepared using SD broth inoculated with one loopful of overnight grown yeast culture grown further at ± 27º C for 24 hrs. It was then transferred to the production medium under sterile conditions.

2.4.2 Production medium
Sterile basal media containing ammonium sulphate 3.0 g/L, yeast extract 10 g/L, peptone 1 g/L and lactose 20 g/L was prepared with pH 7.0. After transfer of inoculum, fermentation with production medium was carried out at 27°C for 48 hrs when Cells when reached stationary phase. Cells were harvested from medium by centrifugation at 10,000 rpm for 10 min at ±4ºC.
The resultant cell mass was washed twice with distilled water and used further for the extraction of intracellular enzyme.

2.4.3 Extraction of enzyme

The cell mass collected was suspended in 0.1 M tris buffer (pH-6.6) and homogenized in a pre-chilled mortar and pestle using glass beads. After sufficient cell lysis the resultant extract was centrifuged at 10,000 rpm and 4°C for 20 min. The supernatant containing crude enzyme was store at 4°C used for further biochemical studies. All the enzyme activity was found associated with yeast cell lysate indicating its intracellular nature.

2.4.4 Enzyme assay

β-galactosidase assay was done with O-nitrophenol β-D-galactopyranoside (ONPG) as substrate. The assay mixture containing 0.5 ml of ONPG substrate prepared using 0.05 M tris buffer (pH-6.6) along with 0.5 ml of extracted enzyme was incubated at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃ after 10 min and liberated ONP (o-nitrophenol) was measured spectrophotometrically at 420 nm using a suitable control. One unit of enzyme activity was defined as the amount of enzyme that produced one micro-mol (µM) of o-nitrophenol per min under the assay conditions.

2.4.5 Assay of protein concentration

The protein concentration of enzyme was determined by the Lowry method by using bovine serum albumin used as a standard. [18]

2.4.6 Enzyme Purification

Purification of enzyme was carried for separation of enzyme protein from other unwanted proteins as follows.

2.4.6.1 Dialysis

Supernatant derived by centrifugation of yeast cell lysate was dialysed extensively to remove other low molecular weight components. The dialysis was carried out in Tris buffer (pH 6.6; 10 mM) at +4°C. It was used as a first step of purification process for β-galactosidase enzyme.

2.4.6.7 Ammonium sulphate fractionation

Crude enzyme with β-galactosidase activity was subjected to precipitation by ammonium sulfate with 70 % saturation under ice cooled conditions. The precipitate derived by centrifugation at 10,000 rpm for 20 min at 4°C was redissolved in a small volume of 50 mM tris buffer (pH 6.6) and dialyzed against the same buffer solution for overnight period. The dialysed enzyme was further used to check its activity and protein content.

Study of kinetic parameters of β-galactosidase

This partially purified enzyme having β-galactosidase activity was used to study the effect of pH, temperature, substrate concentration, metal ions and sugar assimilation test as follows.

Effect of pH on the enzyme activity and stability

Study regarding the effect of variable pH on β-galactosidase was carried out by measuring the enzyme activity at 37°C for 30 min. The optimum pH was determined by using ONPG as a substrate in a series of buffers of different pH values ranging from 4-10. The enzyme activity was measured using the standard assay procedure as described above.
Effect of temperature on the enzyme activity and stability
To determine the optimum temperature β-galactosidase activity was measured at different temperatures from 0°C to 90°C. Assay was carried out by incubating ONPG substrate prepared in tri buffer (pH 6.6) along with the enzyme at 37°C for 30 min.

Optimization of Substrate Concentration
The effect of variable substrate concentration on the activity of β-galactosidase was analyzed by carrying the assay procedure with 0.3-2.6 mM of ONPG in 0.1 M tris buffer at pH 6.6 along with the enzyme solution.

Effect of metal ions
The effect of various metal ions such as MgSO₄, COCl₂, MnCl₂, NaCl and HgCl₂ on β-galactosidase activity was tested by incorporating them at 10 mM concentration into a reaction mixture during normal enzyme assay.

Sugar assimilation test
A sterile basal medium containing sugars such as glucose, fructose, sucrose, maltose, mannitol, galactose, lactose, and arabinose at 2% concentration along with yeast extract (0.45%), peptone (0.75%) and phenol red as an indicator (1mg/ml) distributed in standard assay tubes. Each tube with a 1.5 mL aliquot of sterile basal medium was inoculated with 200 µL aliquot of yeast culture and incubated at 27°C for 72 hrs. Positive results for sugar fermentation were revealed by change in color from red to yellow. Control tubes were used in each set to monitor contamination.

Determination of optimum incubation period for reaction:
Effect of incubation period on β-galactosidase production was studies by cultivating yeast isolates at different incubation period (6-10 hrs). β-galactosidase activity was estimated as per the standard procedure.

[III] RESULTS
3.1 As per the results from the phylogenetic tree (Fig. 1), BLAST analysis of the sequence data (DQ286062.1); showed 99% sequence identities with Galactomyces geotrichum isolates (GQ458022.1, DQ148946.1, JN227076.1), 98 % with Galactomyces geotrichum isolate (JN227080.1) and 98% with Galactomyces sp. isolate (DQ286062.1). According to the morphological observation and molecular identification of isolated strain, 1371-MD2 belongs to Galactomyces sp.

Figure 1: Phylogenetic tree derived from of the isolated sp. of yeast

3.2 Activity of the enzyme before and after semi-purification
Ammonium sulfate precipitation results partial purification of protein by causing removal of other components in cell culture media. Protein precipitation achieves separation by the conversion of soluble proteins to an insoluble state. The semi-purified enzyme as shown in Fig. 2 was used to determine the parameters like pH,
temperature, incubation period, substrate concentration as well as effect of metal ions.

3.3 Effect of Incubation period on the activity of β-galactosidase:
Fermentation period is also an important parameter for enzyme production. Under optimized incubation period bacteria produce higher amount of enzyme.
As per Fig. 3 maximum enzyme activity was observed after 36 hr of incubation during fermentation by isolated yeast spp. With further increase in incubation period from 40-72 h decrease in enzyme activity was observed. This is due to decrease in metabolic activities of yeast cells from stationary to lag growth phase (decline phase).

3.4 Determination of Optimum pH of β-galactosidase
Production of enzyme is also influenced by pH of the production medium. pH has a profound effect on the state of ionization of acidic and basic amino acids present in the active site of enzyme. From Fig. 4 it was observed that the enzyme activity increased from pH 2-5 with maximum activity peak obtained at pH 6.0. The activity was further decreased from pH 8-10. Thus the activity of enzyme was diminished at extremes of acidic (pH-2 and below) and alkaline (pH-10 and above) pH ranges.

3.5 Determination of optimum temperature of β-galactosidase
Optimum temperature enhances the rate of enzymatic reaction by causing activation of both substrate and enzyme molecules through increase in the collisions between them. From Fig. 5 it revealed that β-galactosidase enzyme remains active at temperature varying from 20°C to 70°C. The maximal enzyme activity was shown at 40°C, thereafter showing decline in its activity. Optimal β-galactosidase production was observed between temperature range from 37-45°C.

Figure 2: Activity of the enzyme before and after semi-purification

Figure 3: Effect of Incubation period on the activity of β-galactosidase

Figure 4: Determination of Optimum pH of β-galactosidase

Figure 5: Determination of Optimum temperature of β-galactosidase
3.6 Effect of Substrate concentration on β-galactosidase activity
From Fig. 6 it indicated that the activity of enzyme goes on increasing with corresponding increasing in substrate concentration from 0.3 mM to 2.0 mM. Thereafter, the activity of enzyme remains unaffected with further increase in substrate concentration showing substrate saturation curve.

![Figure 6: Effect of Substrate concentration on β-galactosidase activity](image)

3.7 Effect of Metal ions on the activity of β-galactosidase
Metal ions are known to function as cofactor for enzyme activities and after act as ion or salt bridges among two nearby amino acid residues preserve the rigid confirmation of enzyme molecules.
Data in Fig. 8 displayed that Co²⁺ and Mg²⁺ are activators of the enzyme. Mn³⁺ ions stabilize the activity of the enzyme. The salts such as NaCl and heavy metal ions like Hg²⁺ inhibit the enzyme activity.

Table 1: Effect of inhibitors and activators on the relative activity of β-galactosidase

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Inhibitors/Activators</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>Mg²⁺ (MgCl₂)</td>
<td>107.22</td>
</tr>
<tr>
<td>3.</td>
<td>COCl₂ (CO²⁻)</td>
<td>224.09</td>
</tr>
<tr>
<td>4.</td>
<td>MnCl₂ (Mn³⁺)</td>
<td>100</td>
</tr>
<tr>
<td>5.</td>
<td>NaCl (Na⁺)</td>
<td>68.67</td>
</tr>
<tr>
<td>6.</td>
<td>HgCl₂</td>
<td>80.78</td>
</tr>
</tbody>
</table>

3.8 Sugar Assimilation Test
For all sugars positive results were observed for sugar fermentation as revealed by change in the color of dye from red to yellow (Fig. 9) From the test it indicates that the yeast spp. isolated from whey is able to ferment various monosaccharides and disaccharides.

![Sucrose](image)

![Mannitol](image)
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[IV] DISCUSSION

Isolated strain showed 98% sequence similarity with genus *Galactomyces*. Therefore as per the molecular identification reports, isolated yeast belongs to *Galactomyces sp*. The genus currently has 6 species as mentioned in the Dictionary of Fungi.

This yeast sp. isolated from cheese whey produced the beta galactosidase enzyme which was inducible in nature. The enzyme was found to be produced in the culture media of yeast within 48 hrs. of fermentation period. The enzyme exhibited maximal activity at pH 6.0 and at the temperature of 40°C. The activity of the enzyme was higher for 36 h incubation period, which was found to be decreased from 40 h onwards. Hyperbolic substrate saturation curve indicates the kinetic behaviour of enzyme as per Michaelis-Menten rule where saturation of enzyme was observed above 0.5 mg/ml concentration of ONPG substrate with corresponding value of Km observed was 1.6 mM. The isolated yeast strain was able to metabolize and ferment various sugars including various monosaccharides and disaccharides. Mg²⁺ and Co²⁺ metal ions are activators of the β-galactosidase enzyme.

REFERENCES


Fructose

Arabinose

Galactose

Maltose

Lactose

Figure 7: Sugar Assimilation Test
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