ABSTRACT

Objective: β-galactosidase is an important enzyme which hydrolysis the lactose into monosaccharides. The present study was aimed to elucidate the ability of β-galactosidase production with the different fungal strains using various agricultural waste materials. Methods: The β-galactosidase expression in fungal strains was evaluated under different optimal conditions like pH, temperature, carbon source, nitrogen source and inoculums size under submerged and solid state fermentation. Results: Aspergillus terreus showed maximum enzyme production under optimized culture conditions on 3rd day of incubation at an optimum pH 6.0, temperature 30 °C, inoculum size 3% in fermentation medium using pomegranate peel as a substrate by SmF. In solid state fermentation the optimum culture condition of β-galactosidase under maximum in pH 5.0, 40°C as temperature, 4% inoculum size. Enhanced production occurred in submerged on addition of 4% pomegranate peel and 3% ammonium nitrate as nutritional factors, as well as in solid state fermentation 3% pomegranate and 4% of ammonium nitrate as a nitrogen source. Conclusion: It can be concluded that the pomegranate peel can be more effectively used as a substrate for the maximum enzyme production in both fermentation which may find good industrial application due to low cost and easy availability of substrate.

KEYWORDS: Agro industrial residues, Solid state fermentation, Submerged fermentation, Aspergillus terreus, β-galactosidase.
INTRODUCTION

Enzymes have played an important role in many aspects of life since the dawn of time. They are organic biocatalysts, which govern, initiate and control biological reactions important for life processes. β-galactosidase (EC.3.2.1.23) is an important enzyme in the food and pharmaceutical industry. It is a specific example of a glycosidase enzyme. Glycosidases catalyze hydrolysis of a glycosyl linkage. The enzyme hydrolyzes the lactose into monosaccharide, such as glucose and galactose. This enzyme is industrially important because it can be used to avoid lactose crystallization in sweetened, condensed and frozen dairy products such as ice cream and condensed milk and solve problems associated with whey utilization and disposal. In addition, it used to avoid the problems of lactose intolerance by individuals who are deficient in lactase. β-galactosidase with its transgalactosylation property finds prominent medical applications such as treatment of disorders and development of digestive supplements. It also has lots of potential applications in food processing, bioremediation, biosensor, diagnosis and treatment of disorders.

The β-galactosidase widely distributed in nature or rather, in plants, particularly almonds, peaches, apricots, apples, animal organs such as intestine, brain, placenta and testis. β-galactosidase occurs widely in nature and is produced by a number of microorganisms, such as Kluyveromyces lactis and Kluyveromyces marxianus (formerly known as Kluyveromyces fragilis and Saccharomyces fragilis) and moulds such as Aspergillus niger and Aspergillus oryzae, Mucor pusillus and also from bacterial cultures like Bacillus coagulans, Bacillus stearco-thermophilus, Bacillus circulans, Escherichia coli, Lactobacillus bulgaricus, Lactobacillus thermopila.

Several enzymes of industrial importance have been extracted from the fungi belonging to the genus Aspergillus. The importance of this genus is a model organism for fungal enzyme production. The selection of an inexpensive and easily available substrate together with a suitable producer microorganism, optimization of culture conditions and effective processing are essential to reduce the cost of enzyme preparation. Fermentation is a method of generating enzymes for industrial purposes. Fungal biomass can be produced by solid state fermentation (SSF) and submerged state fermentation (SmF). More that 75% of the industrial enzymes are produced using SmF, one of the major reasons being that SmF supports the utilization of organisms to a greater extent.
The activity of the enzyme is influenced by the type of strain, cultivation conditions (temperature, pH, aeration, agitation, incubation time) and the growth medium composition (particularly carbon and nitrogen sources) [13, 14, 15].

The objective of this study to screening and optimizing the various fungal strains to produce β-galactosidase by using agricultural wastes as a media under submerged and solid state fermentations. Natural isolates produced very low concentration of β-galactosidase, this attempts were made to increase the productivity by optimizing parameters with emphasis on carbon, nitrogen source, pH and temperature by using pomegranate as substrate constant.

MATERIALS AND METHODS

Organism and inoculum preparation

Fungal strains were isolated from garden soil, Coimbatore, Tamil Nadu by dilution plate method. The strains were morphologically identified by Agharkar Research Institute, Pune. Fungal strains were maintained on PDA at 30°C for subsequent use.

Submerged fermentation

The medium for maintenance of the culture was composed of the following (in g/L): Lactose - 20.0, NaNO₃ - 2.0, K₂HPO₄ - 1.0, KCl - 0.5, MgSO₄. 7H₂O - 0.5, FeSO₄. 5H₂O - 0.01, with pH adjusted to 5.0 (Citrate phosphate buffer, 100m mol L⁻¹).

Solid state fermentation:

In case of solid state fermentation 250 ml Erlenmeyer flasks were separately charged with 5.0 g of agro residues were optimized basal solution (OBS) minus carbon substrate and containing (g/L): Casein,-5.2; K₂HPO₄-1.0; KCl- 0.5; MgSO₄ .7H₂O- 0.5; FeSO₄ .5H₂O - 0.1; with pH adjusted to 5.0 (Citrate phosphate buffer, 100m mol L⁻¹).

Effect of various agricultural residues on β-galactosidase production

Screening of several agro-industrial residues (lemon peel, pine apple peel, musk melon peel, banana peel, musambi peel, pomegranate peel, orange peel) were evaluated as carbon source to produce β-galactosidase by using fungal strains under submerged and solid state fermentation.

Screening of microorganisms

The isolated fungal strains were screened with X-gal and oNPG disc method. Lactose medium (0.5% lactose, 0.5% peptone, 0.3% beef extract, and 1.5% agar) was used in a plate-
based screening of microorganisms aiming at the identification of strains producing β-galactosidase. The ability of the different isolates to produce β-galactosidase was examined on lactose medium plates containing 50µg mL⁻¹ of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a chromogenic substrate and 1mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) as an additional inducer for the synthesis of β-galactosidase.

**oNPG disc method**

Lactose medium containing (g/L) Lactose - 20.0, NaNO₃ - 2.0, K₂HPO₄ - 1.0, KCl - 0.5, MgSO₄. 7H₂O - 0.5, FeSO₄. 5H₂O - 0.01, with pH adjusted to 5.0) was used in a plate-based screening of microorganisms by using oNPG disc. Fungal strains were spread on agar plates and placed the oNPG disc on Petri plate and incubated for 35 °C up to 3 days [16].

**Optimization of culture conditions for maximum enzyme production**

Optimization of cultural parameters by using both solid state and submerged fermentation medium for β-galactosidase production was studied at different pH (2-8), temperature (20-70°C), incubation time (1-7 days), Substrate concentration (1-5%) and inoculum size (1-5%). Effect of various carbon source (lemon peel, pine apple peel, musk melon peel, banana peel, musambni peel, pomegranate peel, orange peel) nitrogen sources (ammonium nitrate, potassium nitrate, ammonium sulphate, casein, and urea) and its various concentrations were also studied.

**β-Galactosidase assay**

β-Galactosidase activity was determined by using o-nitrophenyl- β -d-galactopyranoside (oNPG) as substrate. 50µl of crude enzyme sample were added to 950µl of 2.5mM oNPG solution in 100mM citrate-phosphate buffer. Following 10 min incubation in a water bath at 60°C, 1ml of 10% (w/v) Na₂CO₃ solution was added and the absorbance of the final mixture was measured at 410 nm in order to determine the amount of released o-nitro-phenol (oNP). One unit of β-galactosidase activity was defined as the amount of the enzyme required to liberate 1 µmole of oNP per minute under assay conditions [17].

**Statistical analysis**

The data were expressed as Mean ± SD from triplicate determination (n=3).
RESULTS
To screening the agro industrial residues for enzyme production both SmF and SSF process were done. β-galactosidase has catalytic property to hydrolyze disaccharides into monosaccharide. Hence the present study was aimed to utilize the agro waste material for the production of β-galactosidase which may boost up high economic returns in many industries. Screening was done by the X- gal and oNPG disc method. The nutrient agar plates containing fermentation medium was adjusted to pH 5.0. The cultures were spread on a plate and placed the disc on plates.

Different fungal species (Aspergillus niger, Aspergillus terreus, Aspergillus flavus, Penicillium brevicompactum and Fusarium oxysporum) and influence of supplementation of different agro industrial residues including orange peel, pomegranate peel, musk melon peel, lemon peel, musambi peel, pine apple peel, banana peel to fermentation medium for β-galactosidase production were used in this study. Among the tested agro industrial residues pomegranate peel served as best substrate for the β-galactosidase production by Aspergillus terreus (Fig. 1 and 2).

![Screening of β-galactosidase producing fungal strains by using various agro industrial residues under submerged fermentation.](image)

Fig. 1. Screening of β-galactosidase producing fungal strains by using various agro industrial residues under submerged fermentation.
The different concentration of pomegranate peel for β-galactosidase production were also studied which showed a good production at a concentration of 4% in SmF and 3% in SSF (Fig. 3).

Time course of enzyme production plays a very critical role in enzyme synthesis. The *Aspergillus terreus* was incubated for 1-7 days (Fig. 4).
The production of β-galactosidase was maximum on 3rd day of incubation in SmF. But in SSF fermentation its showed the maximum activity on 5th day of incubation. To study the effect of pH on enzyme production, different pH levels like 2 to 8 were studied. The maximum enzyme production was occurred at a pH 6 in SmF and it was gradually reduced up to pH 8, in SSF it found maximum production in pH 5 (Fig. 5).

The effect of different incubation temperature (20-70°C) on the β-galactosidase production was investigated. The production of enzyme was maximal in flasks incubated at 30°C in submerged fermentation, while in solid state fermentation it show the maximal enzyme activity at 40°C (Fig. 6).
In the present study five different nitrogen sources (ammonium nitrate, potassium nitrate, ammonium sulphate, casein and urea) were taken and assessed for β-galactosidase production. In submerged fermentation the ammonium nitrate showed greatest enzyme production at a concentration of 3% when compared to other nitrogen sources and solid state fermentation the nitrogen concentration is found at 4 % in ammonium nitrate (Fig. 7 and 8).
In the present study, the effects of different sizes of inoculums were also explored (Fig. 9). The maximum enzyme production was found in submerged fermentation to be optimal when flasks were inoculated with 3% of inoculums size and in solid state fermentation 4% inoculums size by *Aspergillus terrus*.

**DISCUSSIONS**

The selection of an inexpensive agro residues and easily available substrate together with suitable producer microorganism, optimization of culture conditions and effective downstream processing are essential to reduce the cost of enzyme preparation \[18\]. To screening the fungal strains the oNPG disc becomes yellow, the organisms were capable of producing β-galactosidase. There is no colour changes on disc it indicates absence of β-galactosidase. In X-gal medium colonies producing β-galactosidase were shown in blue colour \[19\]. Pavani et al., (2011) reported that the maximum β-galactosidase production was observed during the 5th day of incubation by *Aspergillus flavus* on submerged fermentation.
Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme production. The pH changes observed during the growth of the organism affects product stability in the medium \[^{[21]}\]. In pH the present study were supported by Wang et al. (2004) who reported that the β-galactosidase induced by a mutant *Penicillium sp.* in SSF is optimum at pH 5.5 - 6.5 \[^{[22]}\] and Rajoke et al. (2003) also reported that the highest β-galactosidase production was high at pH 5.5 by *Klumeromyces marxians* \[^{[23]}\]. Incubation temperature of the fermentation medium is an important factor has insightful influence on metabolic activities of microorganisms. Furlan et al. (2001) indicated an optimum temperature of 35°C for the maximum production of β-galactosidase by *K. marxianus* \[^{[24]}\]. Nizamuddin et al., (2008) reported the optimum enzyme production was occurred at 28°C for β-galactosidase production from *Aspergillus* species \[^{[18]}\]. Nitrogen source is an important amendment that effects enzyme production. Basil (1981) observed good enzyme production when nitrate, phosphate, ammonium, potassium, and sodium ions were added to the growth medium \[^{[25]}\]. Inoculum size certainly has an effect on the rate of production \[^{[26]}\]. In contrast Pavani et al., (2011) reported that the maximum β-galactosidase production was observed with an inoculums size of 7.5% from *Aspergillus flavus* \[^{[20]}\].

**CONCLUSION**

In this study, screening of β-galactosidase from different fungal strains, the highest enzyme production was found at *Aspergillus terrus* by using pomegranate peel as a substrate. The optimal pH, temperature, inoculum size and incubation time for enzyme production was found to be 6, 30 °C, 3% and 3\(^{rd}\) day of incubation respectively in SmF. In solid state fermentation the optimum culture condition of β-galactosidase under maximum in pH 5.0, 40°C as temperature, 4% inoculum size, 3% pomegranate and 4% of ammonium nitrate as a nitrogen source. To promote enzyme production, the evaluation of carbon and nitrogen sources was studied and it was found that pomegranate peel and ammonium nitrate when the medium was supplemented with 4% and 3% respectively. From the present study it can be concluded that pomegranate peel has the potential producer of β-galactosidase enzyme from *Aspergillus terrus* which could have potential application for wide range of industries.

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