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BIOSYNTHESIS OF GLUCOAMYLASE FROM *CANDIDA LIPOLYTICA* USING SOLID STATE FERMENTATION

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Abstract

The quest for enzymes of industrial importance from novel sources is continuous. Glucoamylase is a commercially important enzyme that has extensive industrial applications. In this project, glucoamylase production by *Candida* sp. using solid state fermentation (SSF) is reconnoitered. For enzyme production, four different species of *Candida,* such as *C. lipolytica*, *C. famata*, *C. tropicalis* and *C. utilis* were explored. Among these, *C. lipolytica* produced the maximum glucoamylase after 72 h fermentation. Among the eight diluents tested for the glucoamylase biosynthesis by *C. lipolytica*, the best diluent was found to be D5 containing (%, w/v) soluble starch 0.5, $MgSO_4$ 0.05, $(NH_4)2SO4$ 0.1, KH_2PO_4 0.3, yeast extract and 0.4 wheat bran. The optimum glucoamylase production was obtained with inoculum size 1.5 ml, the substrate to the diluent ratio 1:1, and the initial pH of the diluent pH 5. These results showed that *C. lipolytica* can produce glucoamylase using relatively inexpensive agricultural waste.

Key words: Glucoamylase, Amyloglucosidase, γ-amylase, *Candida*, Solid state fermentation.

Introduction

Hydrolase glucan 1,4-alpha-glucosidase, alternatively known as amyloglucosidase, glucoamylase or γ-amylase (EC 3.2.1.3), is a glycoprotein that hydrolyzes terminal (1→4)-linked and (1→6) linked α-D-glucose of starch and related polysaccharides (Marin-Navarro and Polaina, 2011; Kumar *et al*., 2012; Diler *et al*., 2015; Karim *et al*., 2016; Wu *et al*., 2023). Glucoamylases belong to glycosyl hydrolase (GH) families 15 and 97 in the carbohydrate-active enzyme (CAZy) database (Wu *et al*., 2023). Glucoamylases are used in the commercial saccharification process of starchy feedstock, along with amylases, to produce syrup such as glucose, fructose, dextrose and bioethanol. Therefore, these are extensively employed in a large number of industries, especially paper, textile, food, fine chemical, brewing and distilling (Pandey *et al*., 2000; Gomes *et al*., 2005; Goh *et al*., 2012; Dura *et al*., 2014; Karim *et al*., 2016; Tong *et al*., 2021; Zong *et al*., 2022, Zong *et al*. 2023). Glucoamylase is also plied in a coupled assay for the quantification of starch and glycogen and for diagnosing pancreatic disease with α-D-glucosidase (Kilonzo *et al*., 2010).

Among different fermentation techniques, submerged fermentation (SmF) is commonly employed for microbial enzyme biosynthesis. But lately, solid state fermentation (SSF) is gaining interest because of several benefits such as higher productivity, ease of use of microbial consortia, better product stability, cost-effectiveness and provision of growth conditions that mimic the natural environment. Furthermore, the availability of a variety of agricultural waste products, i.e. wheat straw, rice bran, rice husk, peanut husk, cotton husk, soy husk, sugarcane bagasse, corn gluten bran, brewing residues (barley), citrus pulp, soy, corn and cotton straw etc. as substrate for the industrial enzymes production, make SSF highly attractive (Graminha *et al*., 2008; Haq *et al*., 2012; El-Shishtawy *et al*., 2014; Cheirsilp & Kitcha, 2015; Carvalho *et al*., 2023). However, starchy substrates are more promising for glucoamylase production (Li *et al*., 2007). Furthermore, in addition to suitable microorganisms and substrate for the SSF, other process parameters such as medium composition, initial pH, growth temperature, diluent ratio and inoculum size are needed to be optimized to achieve high production of enzymes by the fermentation process.

A variety of plants and microbes, including fungi and bacteria, are reported for glucoamylase production. However, fungi are the primary producers of glucoamylase for commercial processes (Alva *et al*., 2007; Zambare, 2010; Tong *et al*., 2021). Nevertheless, in the recent years, many yeast strains such as *Cryptococcus* sp., *Aureobasidium pullulans, Saccharomycopsis fibuligera*, *Lipomyces starkeyi*, *Saccharomyces cerevisiae*, *Candida Antarctica, C. pelliculosa, C. famata, C. boidinii* and *C. guilliermendii* are being explored for the amyloglucosidase production (Horvathova *et al*., 2004; Mohamed *et al*., 2007a; Mohamed *et al*., 2007b; Hostinova & Gasperik, 2010; Rani *et al*., 2010).

Significantly less literature is available on the biosynthesis of glucoamylase by *Candida* using SSF. Hence, this project is designed to explore *Candida* sp. for glucoamylase production using SSF. Moreover, to our knowledge, it is the first report on the biosynthesis of glucoamylase by *C. lipolytica* using SSF.

Methodology

Microbial strains: The yeast strains *C. liplytica* (NRRL Y-1095), *C. famata* (NRRL Y1449), *C. tropicalis* (NRRL Y-1552), and *C. utilis* (NRRL Y-900) were used in the present work. Yeast extract-Peptone-Dextrose (YPD) agar slants were used to maintain the individual cultures.

Fermentation: 250 ml individual conical flasks containing sterilized 10 g substrate with 10 ml diluent were inoculated with 1 ml (1.6×10^5 cells) vegetative inoculum followed by 72 h incubation period at 30°C in a static incubator. For the enzyme extraction from fermented bran, 100 ml phosphate buffer (0.02 M) pH 7.0±0.1 was used (Haq *et al*., 2012).

Glucoamylase assay: Glucoamylase assay was performed by the method Mohamed *et al*., (2007a).

Enzyme unit: Under the standard assay conditions, one enzyme unit is the glucoamylase protein required to liberate reducing sugar (equivalent to 1.0 mg of glucose) per min.

Diluents: To moisten the solid substrate, eight different diluents were tested (%, w/v) for solid state fermentation (Table 1) in the present work.

Statistical analysis

Computer software Costat to determine significant differences at $p \leq 0.05$ and to compare treatment effects between parallel replicates.

Results and Discussion

Strain selection: *C. lipolytica*, *C. famata*, *C. tropicalis* and *C. utilis* were tested for the biosynthesis of glucoamylase (Table 2). The highest production of the enzyme (34.7±1.2 U/min) was obtained using *C. lipolytica.* At the same time, *C. tropicalis* gave the minimum output of glucoamylase. Therefore, *C. lipolytica* was selected as the best glucoamylase producer in the present work. Mohamed *et al*., (2007a) isolated and screened different yeast isolates for glucoamylase production and reported maximum production by *C. famata*.

Selection of diluent: Moisture contents and nutrient supplements strongly influence the glucoamylase production (Bertolin *et al*., 2003). To explore the influence of diluent on the biosynthesis of glucoamylase, eight different diluents were tested (Fig. 1). Among these, D5 gave maximum glucoamylase production (54.6±1.43 U/min). It could be because D5 contained carbon and nitrogen sources in adequate amounts and ratios that are required not only for the enzyme production but also for the microbial growth (Haq *et al*., 2002; Bertolin *et al*., 2003).

Substrate selection: Different substrates (banana peel, potato peel, corn flour, wheat bran and rice husk) were tested for the enzyme biosynthesis by *C. lipolytica* (Fig. 2). The suitability order of substrate was wheat bran > rice husk > potato peel > banana peel > corn flour. The best amyloglucosidase biosynthesis was obtained using wheat bran. Many other workers have reported this agro-industrial waste as the best substrate for SSF because it contains sufficient carbohydrates, proteins, ash, fibers, fats and amino acids to support microbial growth and subsequent enzyme production. Moreover, wheat bran provides a large surface area and appropriate porosity for better air circulation and consequently, supports better microbial growth (Babu & Satyanarayana, 1995; Raza *et al*., 2011; El-Shishtawy *et al*., 2014).

Table 1. Composition of diluents.

Diluent	Composition (%, w/v)
D1	Starch 1.0, NaCl 0.035, yeast extract 0.1, malt extract 0.1, K_2HPO_4 0.28, pH 5.0.
D2	Soluble starch 1.0, $(NH_4)_2PO_4$ 0.1, KH_2PO_4 0.1, urea 0.3, $MgSO_4$ 0.1, pH 5.0.
D3	Yeast extract 1.0, Glucose 1.0, KH_2PO_4 0.025, $(NH_4)_2SO_4$ 0.1, pH 5.0.
D4	Soluble starch 0.5, KH_2PO_4 0.2, NaCl 0.1, $(NH_4)_2SO_4$ 0.5, $MgSO_4$ 0.1, peptone 0.1, pH 5.0.
D5	Soluble starch 0.5, KH_2PO_4 0.3, $(NH_4)_2SO_4$ 0.1, $MgSO_4$ 0.05, and yeast extract 0.4, pH 5.0.
D6	Starch 1, yeast extract 0.3, urea 0.05, KH_2PO_4 0.3 and $MgSO_4$ 0.05, pH 5.
D7	Yeast extract 0.25, $MgSO_4$ 0.05, $(NH_4)_2SO_4$ 0.2, $CaCO_3$ 0.3, KH_2PO_4 0.1, pH 5.0.
D8	Soluble starch 2.0, Peptone 0.5, $MgSO_4$ 0.344, yeast extract 0.25, NaCl 0.5, $(NH_4)_2HPO_4$ 0.25, KH_2PO_4 1.0, pH 5.0.

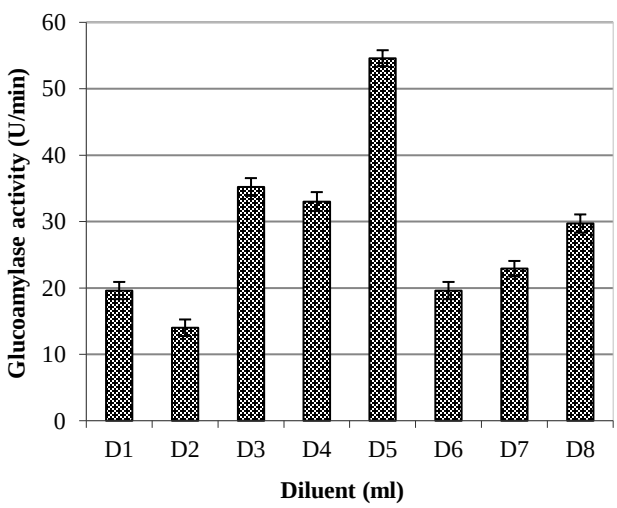

Fig. 1. Selection of diluent for the production of glucoamylase by *C. lipolytica* under SSF*
*Incubation period 72 h, Incubation temp 30°C
Y-error bars indicate the standard deviation (±SD) among the parallel duplicates, which differ significantly at $p \leq 0.05$. LSD= 0.478.

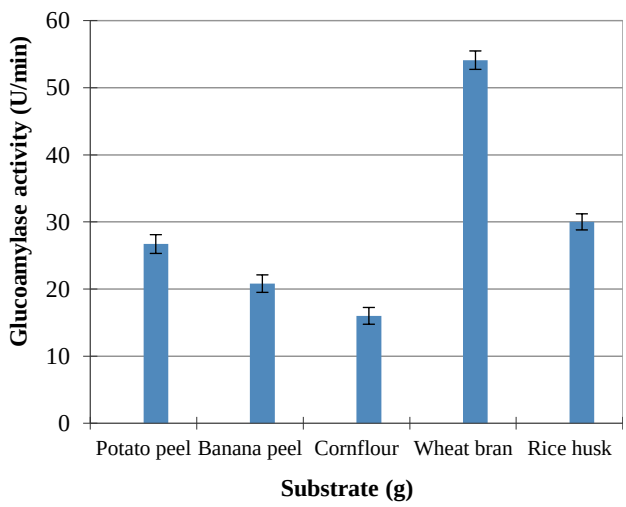

Fig. 2. Substrate selection for the production of glucoamylase by *C. lypolitica* under SSF*
*Incubation period 72 h, Incubation temp 30°C, initial pH 5.0
Y-error bars indicate the standard deviation (±SD) among the parallel duplicates, which differ significantly at $p \leq 0.05$. LSD= 0.78.

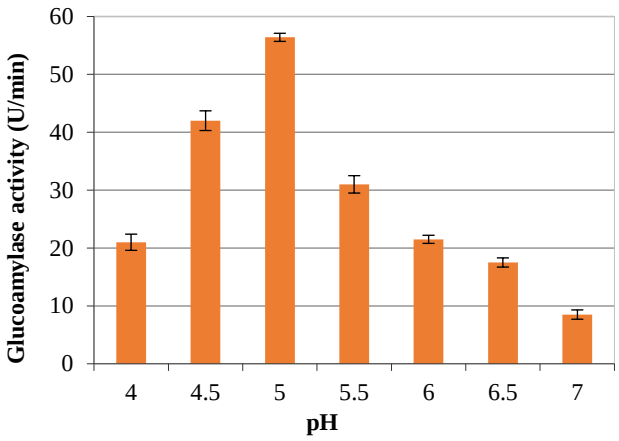

Fig. 3. Effect of initial pH on the production of glucoamylase by *C. lipolytica* using SSF
*Incubation period 72 h, Incubation temp 30°C, initial pH 5.0
Y-error bars indicate the standard deviation (±SD) among the parallel duplicates, which differ significantly at $p \leq 0.05$. LSD= 0.28.

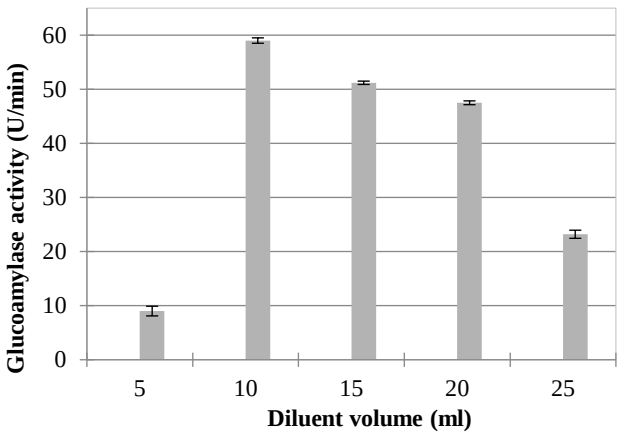

Fig. 4. Effect of diluent volume on the production of glucoamylase by C. *lipolytica* using SSF*
*Incubation period 72 h, Incubation temp 30°C, initial pH 5.0
Y-error bars indicate the standard deviation (±SD) among the parallel duplicates, which differ significantly at $p \leq 0.05$. LSD= 0.39

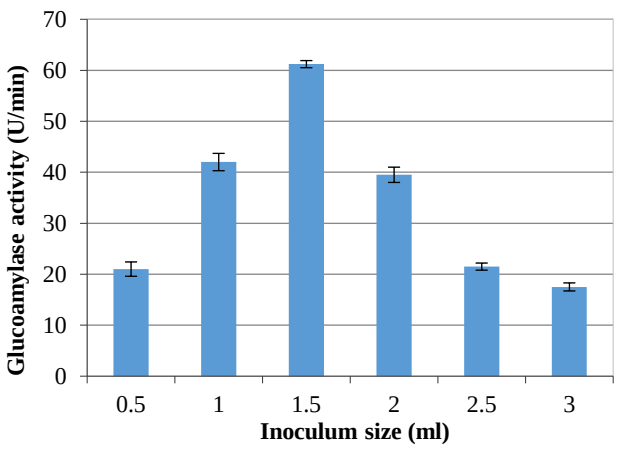

Fig. 5. Effect of inoculum size on the production of glucoamylase production by *C. lipolytica* using SSF
*Incubation period 72 h, Incubation temp 30°C, initial pH 5.0
Y-error bars indicate the standard deviation (±SD) among the parallel duplicates, which differ significantly at $p \leq 0.05$. LSD= 1.93S.

Table 2. Selection of *Candida species* for the production of glucoamylase using SSF*.

Microorganism	Glucoamylase activity (U/min)
Candida lipolytica	34.7 ± 1.2
Candida utilis	30.1 ± 1.8
Candida famata	20.7 ± 2.5
Candida tropicalis	10.3 ± 3.7

*± Indicates the standard deviation (±SD) among the parallel duplicates, which differ significantly at $p \leq 0.05$. LSD= 0.97

Effect of initial pH of diluents: pH has a very profound effect on the microbial metabolic activities (Ellaiah *et al*., 2002) and enzyme production. To assess the initial pH effect of diluent on the biosynthesis of glucoamylase by *C. lipolytica*, pH ranged from 4.0-7.0 was tested for the individual fermentation batches. The production of the enzyme was maximum at pH 5 (Fig. 3). Further increase or decrease from this optimum value reduced the enzyme activity. Mohamed *et al*., (2007a) reported similar findings for the production *C. famata* biomass as well as subsequent glucoamylase production.

Effect of diluent volume: The effect of different volumes of (5-25 ml) of optimized diluent on the enzyme biosynthesis by C. *lipolytica* was also studied. Glucoamylase production was maximized (59±0.5 U/min) when the fermentation flask containing 10g of the substrate was supplemented with 10 ml of diluent (Fig. 4). At higher diluent volumes, less enzyme production was recorded. It is because higher moisture contents in the SSF are responsible for increasing the stickiness of the substrate that, in turn, decreases substrate porosity and oxygen transfer rate (Haq *et al*., 2012). These findings are in accordance with the other workers (Sun *et al*., 2009; Lonsane *et al*., 1985).

Optimization of inoculum size: The size of inoculum has a direct effect on both the microbial growth and the production of enzyme (Kaur & Satyanarayana, 2004). Thus, the effect of inoculum size ranged from 0.5-3.0 ml on the biosynthesis of glucoamylase by *C. lipolytica* was also investigated (Fig. 5). The maximum enzyme units (61.2±0.75 U/min) were achieved using 1.5 ml inoculum. The decreased glucoamylase production at higher inoculum is attributed to the competition between rapidly growing microbial cells for the availability of nutrients (Kaur & Satyanarayana, 2004) and the rapid accumulation of toxic metabolites. Moreover, the high inoculum level leads to a rapid but unfavourable fermentation (Laluce *et al*., 2009). In contrast, low enzyme production at a lower inoculum level is associated with more time requirement for the substrate utilization (Bhatti *et al*., 2007) and subsequent enzyme production.

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