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ORIGINAL ARTICLE

Inulinase production by *Saccharomyces* sp. in solid state fermentation using wheat bran as substrate

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Abstract A newly isolated inulinase-producing *Saccharomyces* sp. from spontaneously fermented sugar cane was employed for inulinase production in solid state fermentation. All the substrates used—wheat bran, rice bran, banana peel, orange peel and bagasse—supported inulinase production. However, the highest inulinase production (78.29±0.13 U/g ds, units per gram of dry substrate) was recorded with wheat bran, while orange bagasse gave the lowest inulinase production (22.47±0.01 U/g ds). A time course of inulinase production revealed that maximum inulinase production of 90.15 U/g ds was reached at 72 h of fermentation. A 24-h-old inoculum and an inoculum density of 4% was also found to be optimal, yielding inulinase production of 88.5±0.02 U/g ds and 80.9±0.25 U/g ds, respectively, by the *Saccharomyces* sp. Furthermore, a moisture content of 65% was found to support maximum inulinase production of 81.5±0.02 U/g ds. pH and temperature studies showed pH 5.5 and 35°C to be optimum for inulinase production of 91.0±0.60 U/g ds and 89.5±0.06 U/g ds, respectively, by this *Saccharomyces* sp.

Keywords Inulinase · Solid state fermentation · *Saccharomyces* sp.

Introduction

Inulinases (E.C. 3.2.1.80; β-fructanohydrolase)—are enzymes belonging to the glycoside hydrolase family 32 (GH32)—have recently received much attention due to their wide applications in the production of high fructose syrup for various industries such as the food and beverage industries, medicines, and also the production of ethanol from fermentation of the fructose produced. Inulinases are capable of catalyzing the hydrolysis of inulin, a polysaccharide composed of fructose unit chains linked by β-(2,1)-D-fructosyl-fructose bonds) of various length, terminated generally by a single glucose unit (linked by an α-D-glucopyransoyl bond), to produce inulo-oligosaccharides and fructo-oligosaccharides (Kim et al. 2008). Inulin is a storage carbohydrate in many members of the Liliaceae, Amaryllidaceae, Graminaeae, Asteraceae and is accumulated in the underground roots and tubers of several plants including Jerusalem artichoke (*Helianthus tuberosus*), chicory (*Cichorium intibus*), dahlia (*Dahlia pinnata*), dandelion (*Taraxacum officinale*) (Gupta and Kaur 1997; Trojanova et al. 2004), and globe artichokes (Gupta and Kaur 1997).

Fructose is of great importance in food industries as it is commonly used as an alternative to sucrose as a sweetener. Sucrose has been found to cause problems related to corpulence, cariogenecity, and artherosclerosis (Vandamme and Derycke 1983). Also, fructose has been reported to exhibit a sweetness synergy effect when used in combination with other sweeteners (Hanover and White 1993). The relative sweetness of fructose blended with sucrose, aspartame, or saccharin is perceived to be greater than the sweetness calculated from the individual components. Medically, fructose has also been found to increase iron absorption in children, stimulate calcium absorption in postmenopausal women (Heuvel et al. 2000), stimulate growth of Bifidobacteria in the large and small intestine (Durieux et al. 2001), and prevent colon cancer (Rowland et al. 1998). The industrial production of fructose, however, involves the use of α-amylase, amyloglucosidase and glucose isomerase acting on starch, yielding a syrup consisting of 55% fructose and 45% glucose (Gill et al. 2004). Although various separation

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techniques, such as chromatography, have been developed to separate fructose produced by this method, thereby enhancing fructose production, these add to the cost of production and hence increase the price of fructose. Furthermore, the quantity produced is insufficient to meet the high demand for fructose by various industries, hence the need for a more economical and higher yielding procedure for the production of fructose from alternative sources.

Inulinases, which are capable of producing 95% fructose in a single enzymatic catalysis on inulin, have being proposed as an alternative source of fructose production. The ability to screen for and isolate new inulinase-producing microorganisms that can meet the conditions favorable for industrial applications could go a long way towards satisfying the high demand for fructose syrup by various industries in Nigeria. This paper therefore reports inulinase production by a *Saccharomyces* sp. isolated from spontaneously fermented sugar-cane (*Saccharum officinarum*) in solid state fermentation (SSF) using wheat bran as substrate.

Materials and methods

Sample procurement

Sugar-cane was purchased from a retailer at Sabo market in Ibadan metropolis and transferred to the laboratory, where it was washed thoroughly with distilled water to remove sand particles. Commercial wheat bran and rice bran were obtained from a milling site at Ogbomosho, while banana peel, orange peel and bagasse were obtained from De'Moris Food Canteen, University of Ibadan, Nigeria.

Isolation procedure

The yeast strain was isolated from spontaneously fermented sugar-cane obtained from a retailer at Sabo market in Ibadan metropolis. Based on conventional yeast identification procedures, the yeasts strain was identified as belonging to *Saccharomyces* species. This yeast isolate was maintained on yeast extract-peptone-sucrose (YPS) agar medium containing (g/l) yeast extract 2.5, peptone 5.0, sucrose 15.0 and agar 20.0 at 4°C in the refrigerator.

Inoculum preparation

Inocula were prepared by transferring 1 ml cell suspension of each of the isolates into a liquid medium (100 ml) containing (g/l) sucrose (20.0), yeast extract (5.0), K_2HPO_4 (5.0), NH_4Cl (1.5), KCl (1.15), and $MgSO_4{\cdot}7H_2O$ (0.65), in an Erlenmeyer flask (Mazutti et al. 2006). Flasks were incubated at 30°C and 150 rpm for 24 h.

Solid state fermentation

Substrate and pre-treatment

Commercial wheat bran (2 kg), rice bran (2 kg) and 1 kg each of banana peel, orange peel and bagasse were sun-dried to constant weight. The banana peel, orange peel and bagasse were milled using a blender (QBL-15 L40, Qlinnk Shang-Hai, China) into coarse particles. The milled banana peel, orange peel and bagasse, wheat bran and rice bran were passed through a sieve of 0.5 mm size, and the coarse particles were used for further studies

Substrates used for SSF were prepared by the modified method of Xiong et al. (2007). Briefly, 2 g of each of the dried (dried to constant weight by sun drying) substrates was mixed with 0.5 ml acidified mineral solution containing mg/l 3.0 mg $MnSO_4{\cdot}H_2O$, 9.0 mg $FeSO_4{\cdot}6H_2O$, 2.5 mg $ZnSO_4{\cdot}7H_2O$ and 3.5 mg $CaCl_2$, and the pH adjusted to 6.0 in 100 ml Erlenmeyer flasks plugged with non-absorbent cotton wool. The moisture content of the substrate was also adjusted to 65% by the addition of appropriate quantity of sterile distilled water. The contents of the flasks were sterilized at 121°C for 15 min, after which they were allowed to cool to ambient temperature. The cooled substrates were then inoculated with the yeast isolates at 4% inoculum level and incubated at 30°C for 72 h in static mode.

Extraction of inulinase

The enzyme produced was extracted according to the method of Mazutti et al. (2006). Sodium acetate buffer (0.1 M; pH 4.8) was added to the fermented medium at ten times (1:10) (v/w) and incubated at 30°C with agitation at 150 rpm for 30 min. This whole content was then filtered through Whatman filter paper No. 1 and the supernatant used as the crude enzyme preparation.

Measurement of inulinase activity

Inulinase activity was determined according to Burkert et al. (2006). Crude enzyme extract (0.1 ml) was incubated at 50°C for 15 min with 0.9 ml sodium acetate buffer (0.1 M, pH 5.5) containing 2% inulin. Thereafter, the enzyme was inactivated by keeping the reaction mixture at 90°C for 10 min. The reaction mixture was then assayed for glucose as a reducing sugar using the DNSA method (Miller 1959). Absorbance of the reaction mixture was measured using a Jenway Spectrophotometer at 540 nm. One unit of inulinase activity was defined as the amount of inulinase enzyme that produced 1 μmol fructose per minute under standard assay conditions.

Time course of inulinase production by *Saccharomyces* sp.

The time course of inulinase production was monitored so as to investigate at what phase during the growth of the organism the inulinase was produced. Wheat bran medium was inoculated with a 24-h-old inoculum and incubated at 30°C for 120 h. Inulinase production was monitored at 24-h intervals by assaying for inulinase during the period of fermentation (120 h).

Effect of inoculum age

The effect of different ages of inoculum was carried out by testing 12, 18, 24 and 36-h-old inocula in wheat bran substrate with 60% moisture content. Incubation was at 30°C for 72 h, after which inulinase activity was determined as described above.

Effect of inoculum size

Five different inoculum densities of 1, 2, 4, 6 and 8% of 24-h-old culture were tested at 60% moisture content in wheat bran. Cultures were incubated separately at 30°C for 72 h and inulinase activity determined thereafter.

Effect of initial moisture content

Effect of initial moisture content was assayed by employing the method of Selvakumar and Pandey (1999). Five different initial moisture levels (50, 55, 60, 65, and 70%) were created separately in the wheat bran substrate. These were inoculated separately with 24-h-old inoculum. Fermentation was carried out for 72 h at 30°C, after which inulinase activity was determined as described above.

Effect of pH of the fermentation substrate

The effect of initial pH of the fermentation substrate (wheat bran) was studied by adjusting the wheat bran differently to pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. A 24-h-old inoculum was used, and fermentation was allowed to proceed for 72 h at 30°C, after which inulinase activity was determined as described above.

Effect of incubation temperature

The effect of temperature of incubation on inulinase production by the *Saccharomyces* sp. in wheat bran was carried out by employing the method of Selvakumar and Pandey (1999) using wheat bran. Inulinase activity was determined after incubation for 72 h. The temperatures tested were 25°C, 30°C, 35°C and 40°C.

Statistical analysis

Results obtained in this study were subjected to analysis of variance using ANOVA, and separation of means was carried out by Duncan's multiple range test (Duncan 1955).

Results and discussion

Choice of substrate

All the substrates used in this study—wheat bran, rice bran, banana peel, orange peel and bagasse—supported microbial growth and inulinase production by *Saccharomyces* sp.; however, the highest inulinase production was found on wheat bran followed by banana peel, and the lowest enzyme production was found on orange bagasse (Table 1). Wheat bran has also been reported by Selvakumar and Pandey (1999) as supporting highest inulinase production by *Kluveromyces* sp. and *Staphyloccocus* sp. in SSF. Variation in the degree of product formation on different substrates could result from the fact that, besides the substrate acting as the carbon source, other components of the medium, such as yeast extract, peptone, K_2HPO_4, also affect cellular growth and product formation, as reported by Kalil et al. (1999)

Time course of inulinase production

The results of the time course of inulinase production (Fig. 1) show that inulinase production increased with time of fermentation. Maximum accumulation of 90.15 U/g ds was observed at 72 h. After 72 h, the enzyme titer showed a decrease with increased time of fermentation. It has been reported that inulinase production by most yeasts is growth-associated, and reaches a maximum near stationary phase (Al-Dagal and Bazaraa 1998). The decline in inulinase production after a certain time could be as a result of exhaustion of carbon source in the medium or catabolic

Table 1 Inulinase production by *Saccharomyces* sp. on different solid substrates. Data are means of three replicates±SE of means. Values followed by the same letters are not significantly different by Duncan's multiple range test ($P \leq 0.05$)

Substrate	Inulinase activity (U/g ds)
Wheat bran	78.29±0.13 a
Banan peel	68.77±0.38 e
Rice bran	60.11±0.11 c
Orange bagasse	30.08±0.62 b
Orange peel	22.47±0.01 b

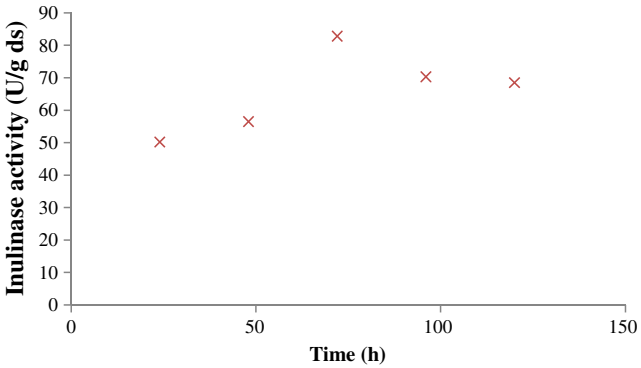

Fig. 1 Time course of inulinase production by *Saccharomyces* sp. Data are given as means±SEM, n=3

repression (Vandamme and Derycke 1983). It could also be due to the secretion of proteolytic proteins that are known to cause protein denaturation (Gupta et al. 1994).

Effect of inoculum age on inulinase production

Figure 2 shows that maximum inulinase production of 88.5 ±0.02 U/g ds was achieved with a 24-h-old inoculum, while the minimum amount of inulinase produced (60.4± 0.29 U/g ds) was with 12-h-old inoculum. Al-Dagal and Bazaraa (1998) reported that inulinase production by microorganisms is growth associated; hence, the low inulinase production with inoculum age below 24 h could be attributed to the fact that yeast culture might not have yet entered the exponential phase of growth. At 24 h old, the cultures might have entered the exponential phase of growth and are metabolically active (Singh et al. 2007). A decrease in enzyme titer after 24 h might be due to the fact that the organisms could have entered the stationary phase of growth and are not metabolically active. This study shows that it is important to use inoculum of appropriate age for optimal enzyme yield.

Effect of inoculum size on inulinase production

Figure 3 shows the effect of inoculum density on inulinase production by *Saccharomyces* sp. Maximum inulinase production of 80.9±0.25 U/g ds was obtained at 4% inoculum level, while minimum inulinase production of 50.8±0.12 U/g ds was obtained at 1% inoculum level. A 4% inoculum level was also reported by Selvakumar and Pandey (1999) to be appropriate for inulinase production by *Kluveromyces marxianus* in SSF. Low enzyme concentration at an inoculum level below 4% could be as a result of the fact that there is an inadequate yeast biomass to utilize the required amount of substrate necessary for optimal enzyme yield as suggested by Shafiq et al. (2002), while low enzyme yield after 4% inoculum level could be because high concentrations of inoculum depleted the substrate nutrients concentrations necessary for optimum product formation.

Effect of moisture content on inulinase production

The effect of different percent moisture content on inulinase production is illustrated in Fig. 4. Maximum inulinase production (81.5+0.02 U/g ds) was recorded at a 65% moisture level while the lowest was recorded at 50% (50.6± 0.10 U/g ds). A similar trend was also reported by (Selvakumar and Pandey 1999). Low enzyme concentration at low moisture content could be as a result of reduction in solubility of nutrients of the substrate, a low degree of swelling, and high water tension (Ellaiah et al. 2002). The low inulinase production at high moisture content might be due to a reduction in the porosity of the system, which can

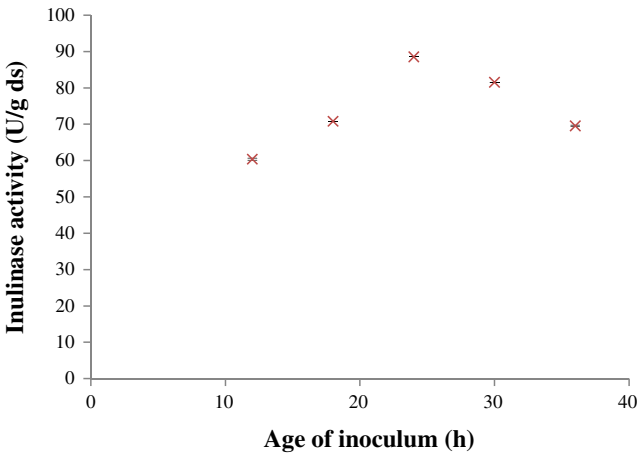

Fig. 2 Effect of inoculum age on inulinase production by *Saccharomyces* sp. Data are given as means±SEM, n=3

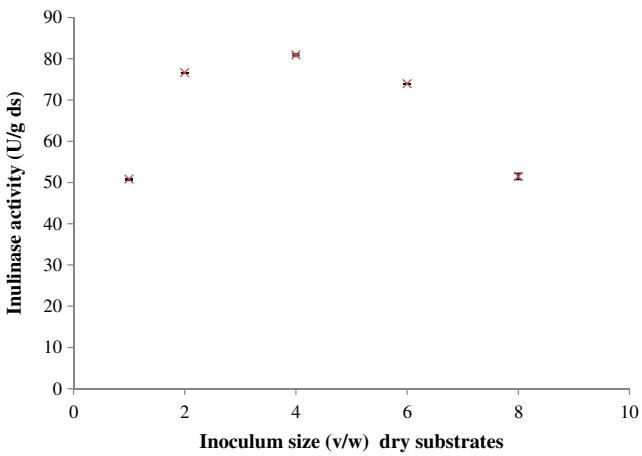

Fig. 3 Effect of inoculum density on inulinase production by *Saccharomyces* sp. Data are given as means±SEM, n=3

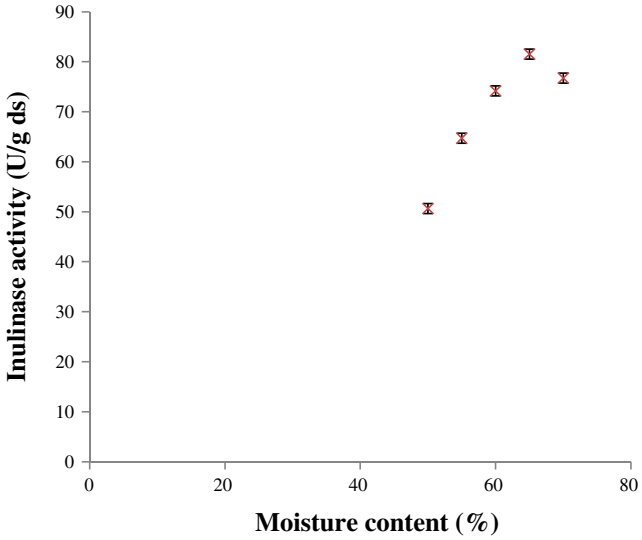

Fig. 4 Effect of different percentage moisture content of substrates on inulinase production by *Saccharomyces* sp. Data are given as means± SEM, $n=3$

lead to oxygen transfer limitation. High metabolite production at relatively low moisture level is said to be advantageous since the chance of contamination of fermentation medium by other microorganisms having high water activity is reduced (Ngadi and Correia 1992).

Effect of initial pH of fermentation

The results of the effect of pH on inulinase production by *Saccharomyces* sp. in wheat bran at 65% moisture content are shown Fig. 5. Highest inulinase titer of 91.0±0.60 U/g ds was recorded at pH 5.5 while lowest enzyme titer of 60.7± 0.14 U/g ds was recorded at pH 3.5. This optimum pH of 5.5 is within the range limits described for yeasts: 3.5–5.5 (Manzoni and Cavazzoni 1988). The pH of the medium in SSF is an important factor for microbial growth and metabolite production as its affects the metabolic activities of the organisms and hence product synthesis (Pandey 1992; Silva-Santisteban and Filho 2005). Kalil et al. (1999) also reported that the pH was most significant factor as regards enzyme production by *K. marxianus* var. *bulgaricus*, with an optimum pH of 3.5 in sucrose medium, while an optimum pH 6.0 was also recorded for the same strain in inulin medium by Selvakumar and Pandey (1999). In general, it is noted that the pH varies among the different microbial species according to the type of substrate. Complex media contain substances such as salts, which can inhibit cellular growth and even some microbial enzymes (Singh and Bhermi 2008).

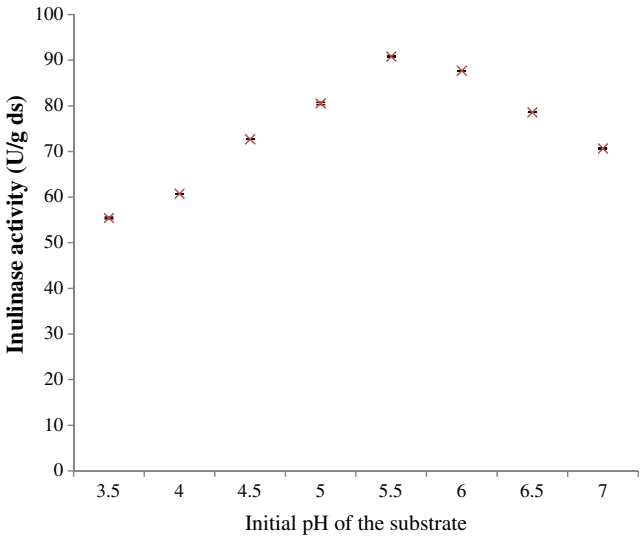

Fig. 5 Effect of different pH values on inulinase production by *Saccharomyces* sp. Data are given as means±SEM, $n=3$

Effect of incubation temperature

This study showed that maximum inulinase production (89.5±0.06 U/g ds) was at 35°C, while minimum inulinase production (64.5±0.03 U/g ds) was at 25°C (Fig. 6). Although a number of studies have reported maximum production of inulinase by yeasts at 30°C in SmF (Cazetta et al. 2005; Silva-Santisteban et al. 2006), maximum production was found to be at 35°C in this study. Selvakumar and Pandey (1999) reported that maximum inulinase production in SSF for *Kluyveromyces marxianus* occur at 37°C. The shift in optimum temperature by this organism could be a function of the type of microorganism, the porosity, the particle diameter and the depth of the substrate (Pandey et al. 1999).

From the present results, the newly isolated *Saccharomyces* sp. is a promising organism for inulinase production in SSF. Also, the fact that the inulinase activity in this study was

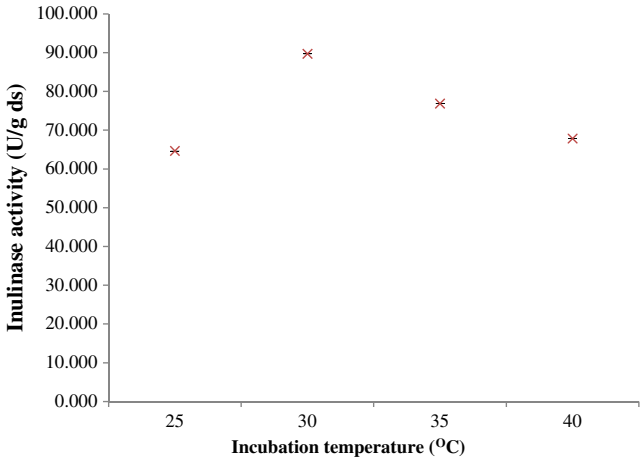

Fig. 6 Effect of incubation temperature on inulinase production by *Saccharomyces* sp. Data are given as means±SEM, $n=3$

determined using inulin as the carbon source gives further credit to the potential industrial importance of this organism. Various reports in the literature have determined inulinase activity using sucrose as the carbon source; however, the hydrolysis of inulin is slower than that of sucrose. Nevertheless, further work is required to characterize the inulinase produced by this organism so as to determine its suitability for industrial application.

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