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Full Length Research Paper

Production and partial characterization of invertase from *Mucor geophillus* EFRL 03

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In the present study, cultural conditions for invertase production from *Mucor geophillus* using *Brassica niger* (oilcake) in batch wise submerged fermentation was investigated. The effects of time period (24 to 240 h), carbon sources [*Brassica* campestris, *B. niger*, pomegranate peel, coconut, malta peel, apple pulp and glucose (control)] and nitrogen sources (peptone, yeast extract, corn steep liquor, ammonium chloride, sodium nitrates and potassium nitrate) were checked on invertase production. The highest level of invertase was achieved using mineral medium containing 1.0% *B. niger* hydrolyzed with 0.3 N HCl and 0.5% yeast extract as carbon and nitrogen sources, respectively, and after 48 h of incubation at 45°C, initial pH was adjusted to 6.5. Invertase exhibited the maximum stability in the range of 25 to 50°C temperature and 4 to 6 pH, respectively, within 10 min of incubation. The enzyme retained more than 60 and 50% activities at 8 and 70°C pH and temperature, respectively. In this study, cost effective substrate was utilized for invertase production. The pH and thermostable invertase can be utilized in industrial process.

Key words: *Mucor geophillus*, *Brassica niger*, submerged fermentation, invertase.

INTRODUCTION

Invertase (β-D-fructofuranoside fructohydrolase, β-fructofuranosidase, sucrose invertin, saccharase EC 3.2.1.26) catalyzes the hydrolysis of sucrose which yields equimolar mixture of glucose and fructose (Tanriseven and Dogan, 2001). This mixture has higher sweetening capacity than sucrose. Invertases have many potential applications in the food industry (confectionery, syrups, condensed milk, infant foods and beverages). It is worth to mention that, invertase is also used for the production of artificial honey, plasticizing agents used in cosmetics, pharmaceutical and paper industries and recently invertase has found applications in the analytical field for the construction of sucrose biosensors (Kotwal and Shankar, 2009; Bagal et al., 2007). In addition, they exhibit transfructosylating activity at high sucrose concentrations (Kotwal and Shankar, 2009; Guimaraes et al., 2007). The conventional method for the generation of invert sugar involves acid hydrolysis of sucrose, which has low conversion efficiency, high energy consumption and thus high cost of production. Whereas, invertase splits sucrose into glucose and fructose (invert syrup) with 100% conversion efficiency without the inherent disadvantages of acid hydrolysis (Kumar et al., 2008). The product obtained by invertase has the advantage of being colorless as compared to that obtained through acid hydrolysis (Kotzelski and Staude, 1996; Danisman et al., 2004). Invertases are widely distributed in bacteria, yeasts, fungi, higher plants and in some animal cells (Rubio et al., 2002; Rubio and Navarro, 2006; Ul-Haq et al., 2008; Kotwal and Shankar, 2009). The microbial sources are preferred over other sources of invertases to meet the commercial needs.

Increasing concern on pollution that occurs from agricultural and industrial wastes has stimulated interest in converting waste materials into commercially valuable products. Number of different industries produces huge quantity of waste materials, both solids and liquids.

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Beside their pollution effects, in most cases, wastes have potential for conversion into useful product of higher value (Sangeetha et al., 2004; Mamma et al., 2008; Rashad and Nooman, 2008; Guimaraes et al., 2009). Molasses, a sub-product in sugar production, is rich in sucrose and has been reported for the production of invertase by *Saccharomyces cerevisiae* (Bokossa et al., 1993; Zech and Gorisch, 1995; Echegaray et al., 2000). In the present study, oilcake and fruit waste was utilized for invertase production from *Mucor geophillus*, the invertase characterized as thermostable and acid invertase.

MATERIALS AND METHODS

M. geophillus was isolated from soil sample of Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan. The culture was maintained on glucose agar medium at 4°C and subcultured every week. The agar medium contained (per liter of distilled water) the following: glucose 20 g, peptone 10 g and agar 15 g.

Invertase production

M. geophillus was grown in a medium containing (g per L of distilled water): glucose 10, peptone 5, $MgSO_4 \cdot 7H_2O$ 5, KH_2PO_4 5, NaCl 2.5 and $Fe_2SO_4 \cdot 7H_2O$ 0.01. The initial pH was adjusted to 6.5. The culture medium (50 ml) was autoclaved at 121°C, 15 pounds pressure for 20 min in a 250 ml shake flask, cooled to the incubation temperature of 30 ± 2°C and inoculated with 0.5 ml of a culture. After 24-h of incubation, the invertase containing supernatant was recovered by filtration and used for further analyses.

Effect of carbon source

The effect of carbon source (10 g/l) on enzyme production was assessed by replacing glucose in the above specified medium with an equal concentration of *Brassica* campestris, *Brassica niger*, pomegranate peel, coconut, malta peel and apple pulp. The inoculated flasks were incubated at the above specified conditions for 48 h.

Effect of nitrogen source

Various nitrogen sources (corn steep liquor, yeast extract, sodium nitrate, potassium nitrate and ammonium chloride) at a concentration of 2.5 g and 5 g/l were used by replacing peptone during enzyme production in the earlier specified basal medium.

Influence of initial pH

Initial pH of the enzyme production medium was adjusted in the range of 4 to 12 with 0.1 M HCl or NaOH.

Effect of temperature

Fermentations with specified media were carried out at various temperatures ranging from 30 to 60°C. The other conditions were same as described earlier.

Invertase assay

The invertase activity was measured in the culture supernatant using Sumner and Howells (1935). 1 ml of the cell-free supernatant was mixed with an equal volume of an aqueous solution of sucrose (10 g/l) as the substrate dissolved in 20 mM acetate buffer (pH 5.5). The mixture was incubated at 37°C for 15 min. Dinitrosalicylic acid reagent (2 ml) was then added and the reaction mixture was boiled for 5 min. The absorbance of the cooled reaction mixture was read at 540 nm against a blank Miller method (1959). The blank was prepared exactly as the sample with the exception that pure water was added instead of the sample. One unit of invertase activity was defined as the amount of enzyme required for liberating 1 µg of reducing sugar at 37°C per minute.

Effect of pH and temperature on invertase activity

The effect of pH in the range of 4 to 12 and temperature in the range of 25 to 90°C on invertase activity was checked by measuring the enzyme activity at different pH and temperature.

pH stability and thermostability of invertase

The pH stability of invertase enzyme was noted by measuring percentage of relative activity when 0.5 ml enzyme was mixed with 0.1 ml of buffer pH ranging between 4 and 12 using universal buffer and incubated for 10 min, while thermal stability of invertase was carried out at various temperatures ranging between 25 and 90°C for 10 min.

RESULTS AND DISCUSSION

15 fungal strains were isolated from the fertile soil of Institute of Biotechnology and Genetic Engineering and screened for invertase activity in the culture medium. The primary selection criteria were: 1) maximal secretion of invertase into the culture medium, and 2) production of invertase using cost effective substrate. Three of the isolated strains showed invertase secretion at higher rate and the best one (*M. geophillus*), which produced maximum titer of invertase was selected. The strain was capable of growing in the wide pH range of 4 to 12 and 30 to 60°C temperature.

Effect of time of incubation on synthesis of invertase *by M. geophillus* is shown in Figure 1. The growth and enzyme yield increased with passage of time and the maximum enzyme secretion was noted (35.89 U/ml) after 48 h, but on prolong incubation, production decreased. The decline of enzyme production may be due to change in pH, denaturation of enzyme or synthesis of inhibiting metabolites and other compounds damaging invertase. Similar results have been reported by Matrai et al. (2000) in the case of invertase production by *Aspergillus flavus* and *Aspergillus fumigatus*. The maximum production was achieved after 48 h of incubation.

The effect of different carbon sources (*B.* campestris,

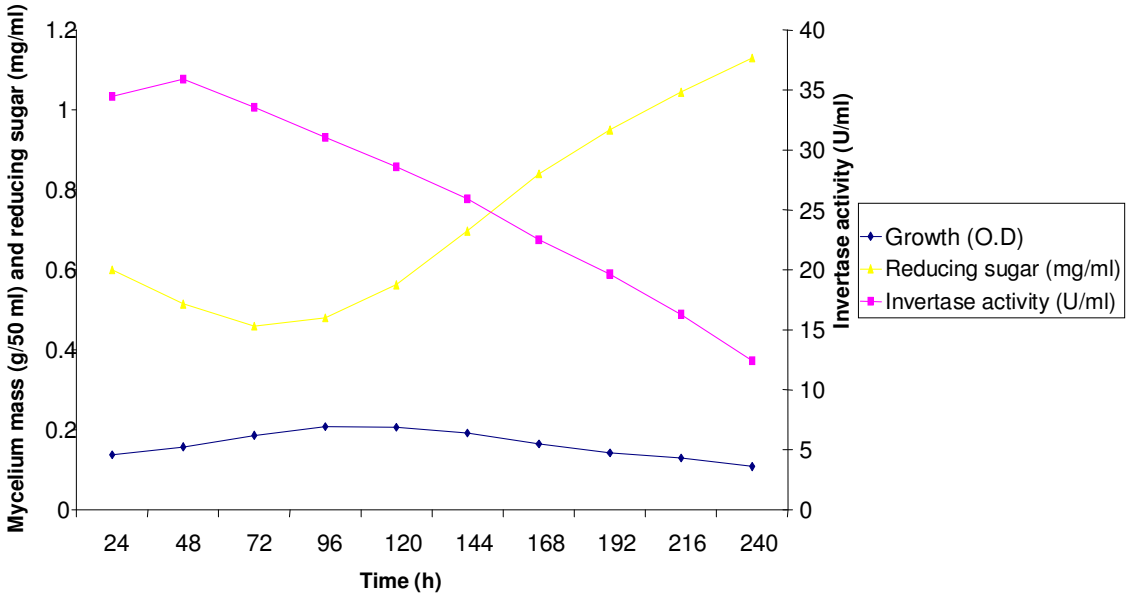

Figure 1. Batch profiles of biomass growth, invertase activity and reducing sugar concentration during enzyme production (30°C, initial pH of 6.5).

B. niger, pomegranate peel, coconut, malta peel, apple pulp by replacing glucose (control) was analyzed on the production of enzyme. It is shown in Figure 2 that *M. geophillus* grows better and secrete maximum invertase when grown on 1% *B. niger* hydrolyzed with 0.3 N HCl mineral medium at 30 ± 2°C after 48 h of incubation in comparison with other carbon sources. Agro-industrial waste causing pollution, can be utilized as raw material for the production of enzymes and many value added products. The utilization of agro-industrial residues on one hand provides useful products and on the other hand, help in solving pollution problems (Pandey et al., 2002). Among various low cost raw materials in the present study, *B. niger* produced maximum invertase. The superior effect of natural substance on enzyme synthesis may be due to presence of growth promoting substances in sufficient quantity which fulfill the requirement of microorganism along with synthesis of invertase (Figures 2 and 3).

Figures 4 and 5 show the effect of organic and inorganic nitrogen sources (0.25 and 0.5%) on invertase yield from *M. geophillus*. In this study, peptone was replaced with yeast extract, corn steep liquor, ammonium chloride, sodium nitrates and potassium nitrate. The invertase production was more pronounced with addition of yeast extract as nitrogen source in the mineral medium. Similar results have been reported by Iram et al. (2008) in the case of invertase production using yeast extract as nitrogen source from newly isolated *Fusarium* sp.

Figure 6 shows the influence of initial pH on the invertase biosynthesis by *M. geophillus* using 1.0% *B. niger* and 0.5% yeast extract in mineral medium when incubated at 30°C for 48 h. Production of invertase was observed in a wide pH range (4.0 to 12) production which shows no correlation with growth. The optimum pH 8.0 and 6.5 were noted for growth and enzyme production, respectively. The growth was extremely slow at pH 4.0 and 12.0 and reached maximum after 48 h of incubation at pH 8.0. Though, production of enzyme was maximum at pH 6.5 (39.2 U/ml), beyond this production rate, it was low. The pH dependent enzyme production might have been due to pH control over the growth of bacteria or pH dependent control of the enzyme synthesis gene expression (Young et al., 1996). The results are in the accordance with the report of Uma et al. (2010) in the case of invertase production from *A. flavus* at pH 5.0 using fruit peel waste as substrate.

Figure 7 shows the effect of incubation temperature on the invertase synthesis by *Mucor* (30 to 60°C) when grown on 1.0% *B. niger* and 0.5% yeast extract mineral medium, which was incubated for 48 h at the initial pH of 6.5. The maximum yield of invertase was noted at 45°C. Similar results were reported by Mona and Mohamed (2009) in the case of invertase production by *S. cerevisiae* NRRLY-12632 at 50°C. Temperature influence all the physiological activities in a living cell and is one of the important environmental factor to control the growth, microbial activities, normal functioning of enzyme and many enzymes control the nutritional requirement of the cell and subsequently its composition (Van Demark and Batzing, 1987).

Figure 8 shows the effect of pH and pH stability on invertase activity of *M. geophillus* using different pH of universal buffer ranging from 4.0 to 12.0. The maximum invertase activity was observed at pH 5.0. These results

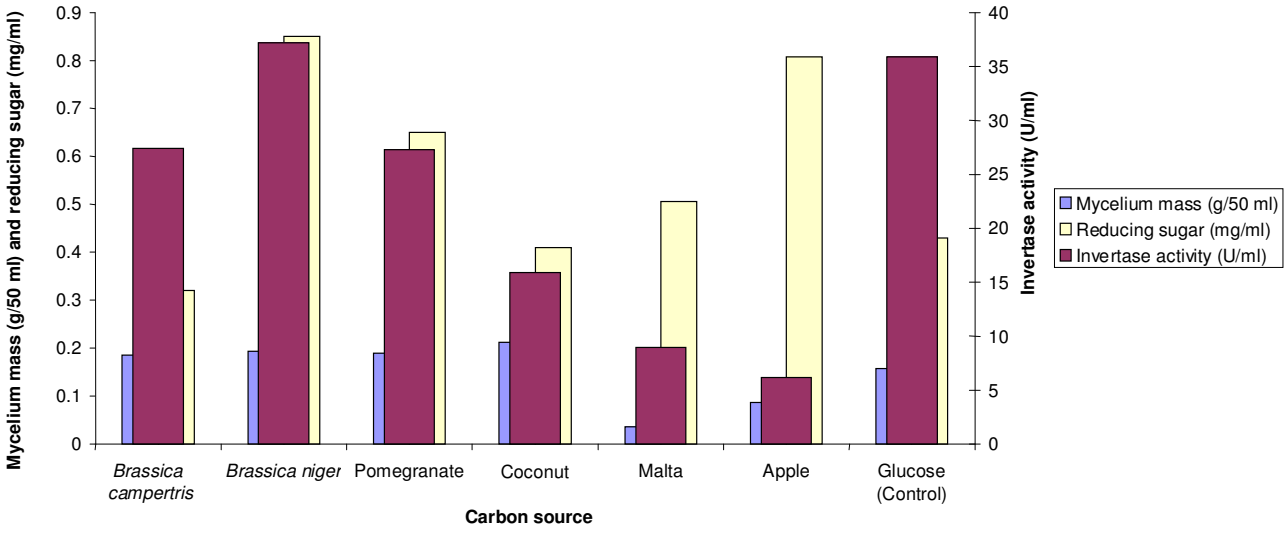

Figure 2. Effect of fruit waste and oil cake as carbon source (10 g/l initial concentration) hydrolyzed with 0.3 N HCl on biomass concentration, invertase activity and residual reducing sugar level at 48 h of fermentation (30°C, initial pH of 6.5).

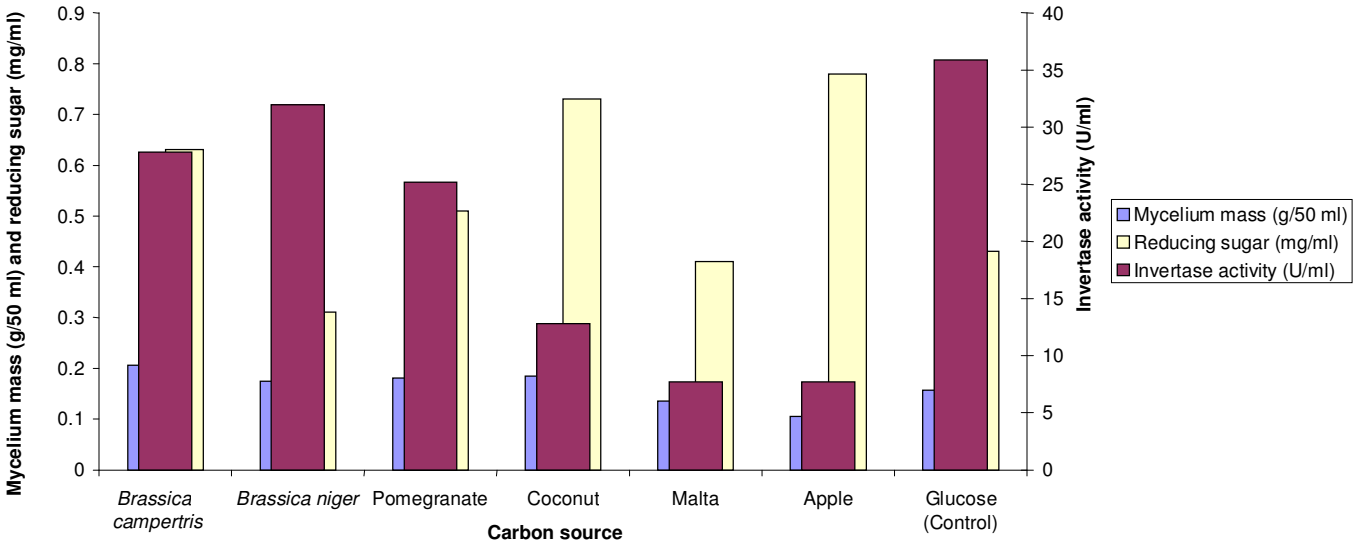

Figure 3. Effect of fruit waste and oil cake as carbon source (10 g/l initial concentration) hydrolyzed with 0.6 N HCl on biomass concentration, invertase activity and residual reducing sugar level at 48 h of fermentation (30°C, initial pH of 6.5).

are in agreement with the results of Mona and Mohamed (2009) in the case of invertase production by *S.cerevisiae* NRRLY-12632; the invertase was stable in the range of 5 to 7 pH with maximum activity at pH 6.0. The 40.1 and 2.15 U/ml invertase activities were found at pH 4.0 and 12.0 when incubated at 37°C for 15 min. The pH stability of invertase was also checked using universal buffer ranging from pH 4.0 to 12.0. *M. geophillus* invertase shows maximum stability between pH 4.0 and 6.0, and above pH 9.0 invertase activity dramatically decreased. The present result is in accordance with the results reported by Mona and Mohamed (2009) in the case of pH stability of invertase in the range of 5 to 7 pH.

Figure 9 shows the effect of temperature and thermostability ranging from 25 to 90°C on crude invertase activity of *M. geophillus*. The invertase activity increased with the increase of temperature up to 50°C and then declined. The declination of enzyme activity may be due to denaturation of invertase on exposure to higher temperatures than its optimum temperature. These findings are in accordance with several earlier reports showing optimal invertase activity at 50°C in the

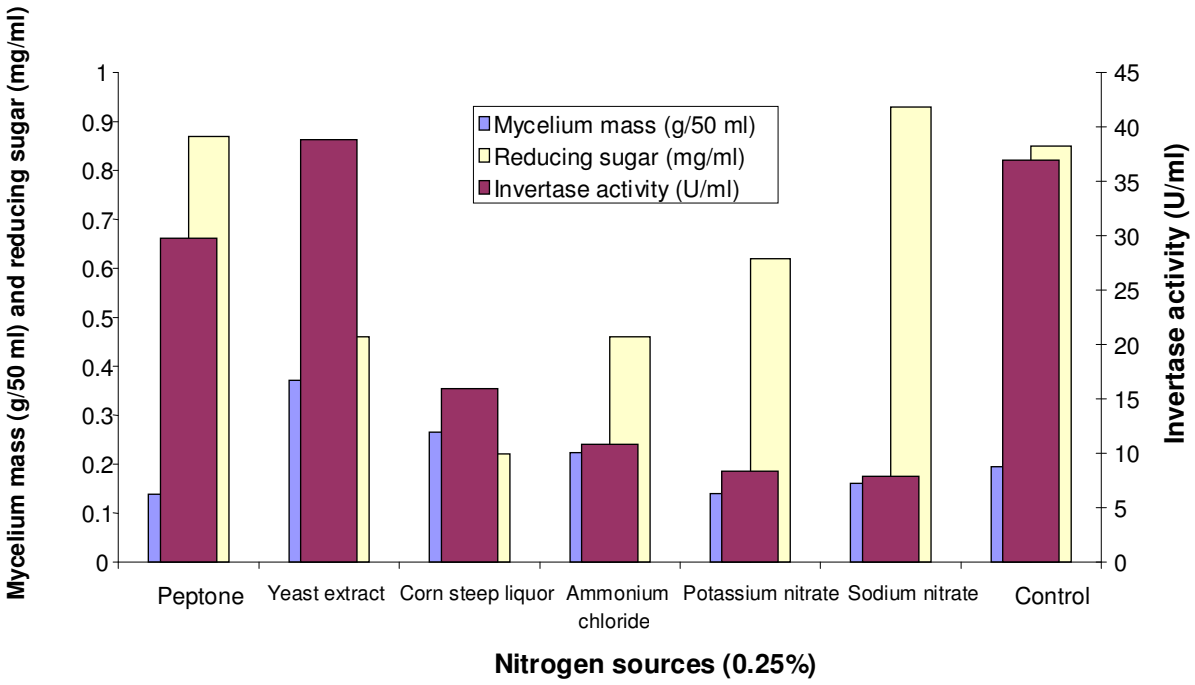

Figure 4. Effect of nitrogen source (2.5 g/l initial concentration) on invertase production, biomass concentration and residual reducing sugar level at 48 h of fermentation (30°C, initial pH of 6.5) in a *Brassica niger* (10 g/l initial concentration) mineral medium.

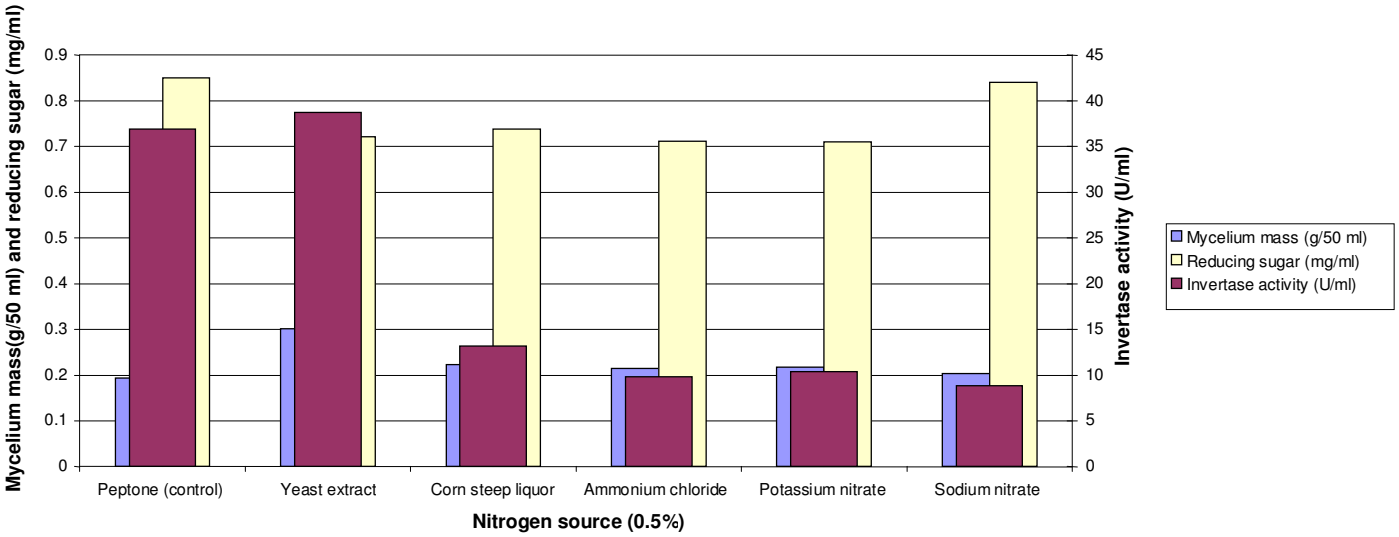

Figure 5. Effect of nitrogen source (5 g/l initial concentration) on invertase production, biomass concentration and residual reducing sugar level at 48 h of fermentation (30°C, initial pH of 6.5) in a *Brassica niger* (10 g/l initial concentration) mineral medium.

case of *S. cerevisiae* (Mona and Mohamed, 2009; Faiza et al., 2010) acid invertase from *Saccharum officinarum* at 45 and at 50°C (Iram et al., 2008). About 70 and 50% of relative invertase activities were noted after the incubation at 65 and 70°C for 10 min. The temperature stability of crude enzyme was investigated by incubating the enzyme for 10 min at different temperature ranging from 25 to 90°C and then substrate was added and processed according to Miller (1959) assay method. Crude invertase retained 98% of activity at 50°C after 10 min of incubation and then decreased with increase in temperature. About 50% activity was retained at 75°C and

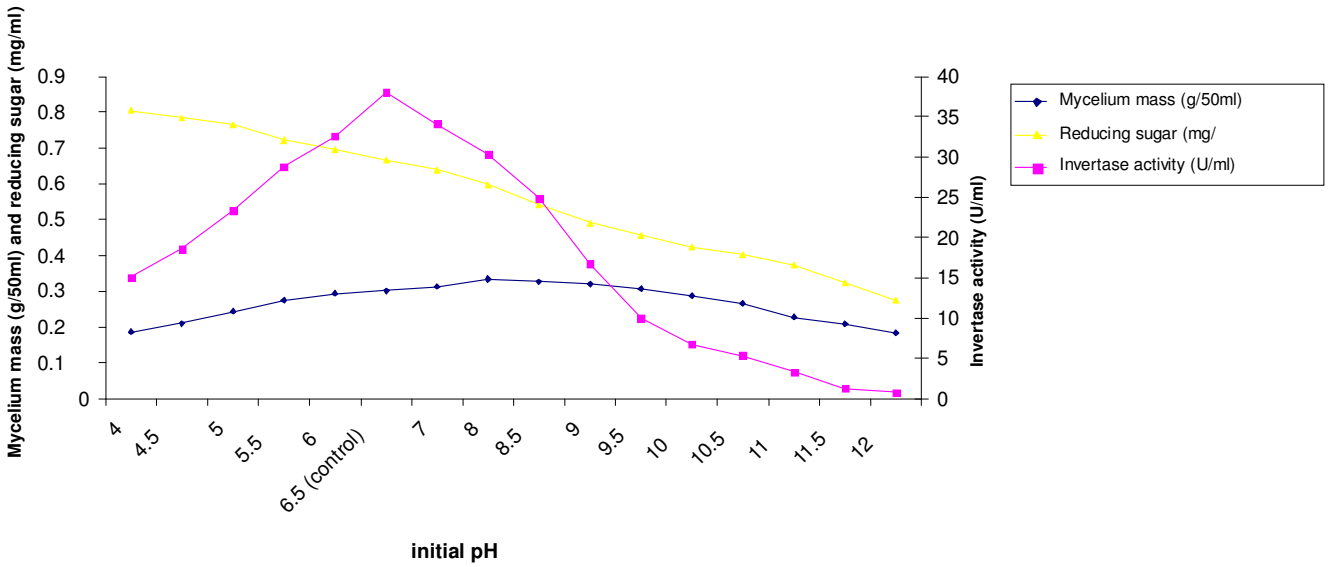

Figure 6. Effect of initial pH on invertase production, biomass growth and residual sugar concentration at 48 h of fermentation (30°C) in a mineral medium containing *Brassica niger* hydrolyzed with 0.3 N HCl and yeast extract at initial concentrations of 10 and 5 g/l, respectively.

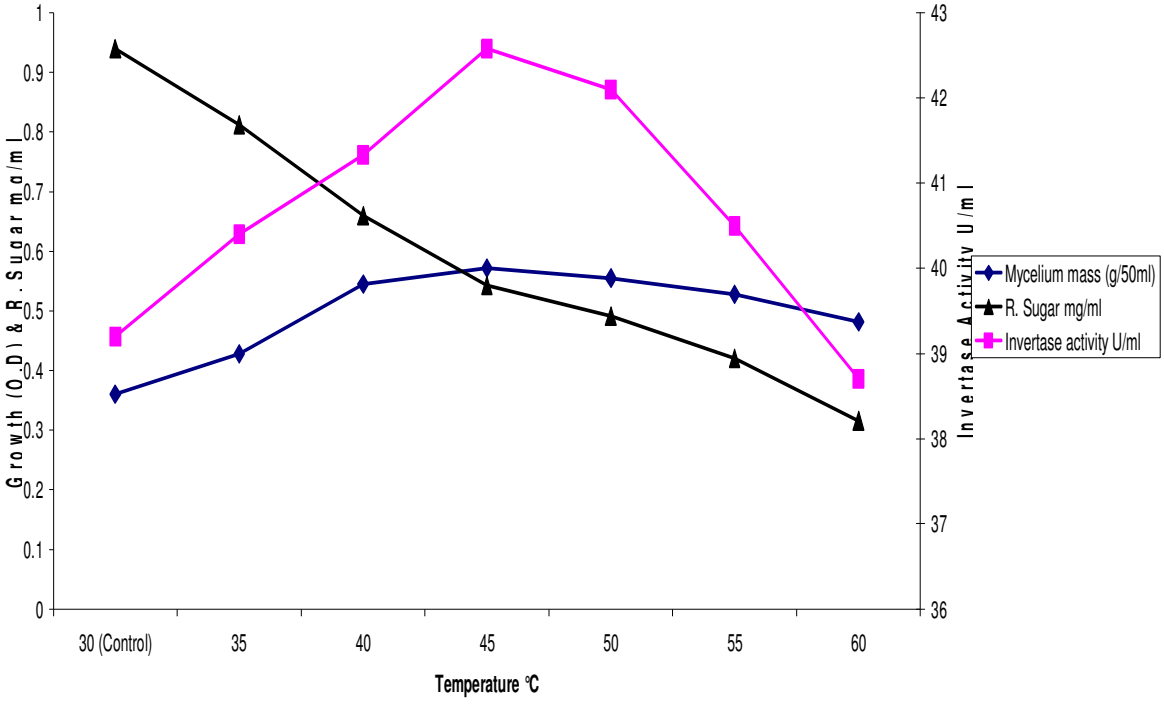

Figure 7. Effect of fermentation temperature on invertase production, biomass concentration and residual sugar concentration at 48 h. The medium initially contained *Brassica niger* hydrolyzed with 0.3 N HCl and yeast extract at initial concentrations of 10 and 5 g/l, respectively. The initial pH was 6.5.

and suggested that invertase is thermostable. These results are in accordance with results of Mona and Mohamed (2009) in the case of thermo stability of invertase activity retained by 85% at 50°C after 1 h. High temperature and low pH optima are advantageous for industrial high invert fructose syrup production, as all the conditions prevent microbial contamination and undesired color formation.

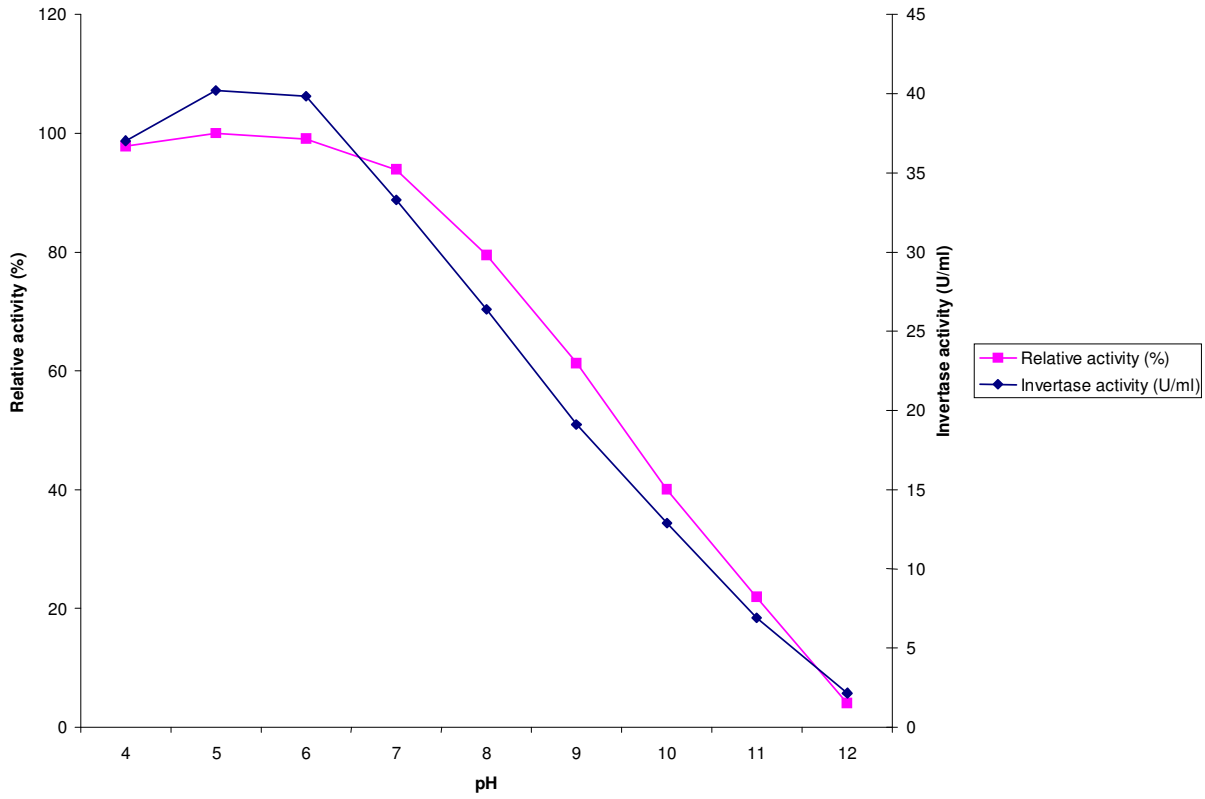

Figure 8. Effect of pH and pH stability on invertase activity produced by *M. geophillus*.

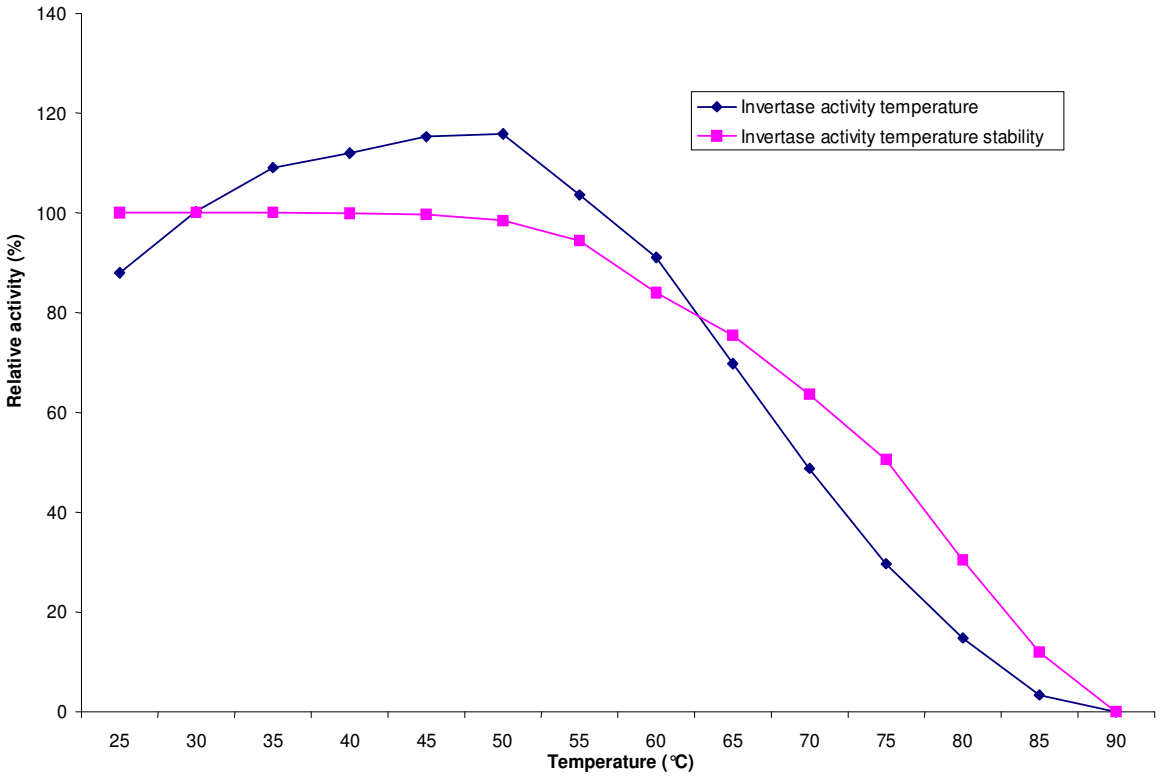

Figure 9. Effect of temperature and thermostability on invertase activity from *M. geophillus*.

Conclusion

Increasing concern on pollution that occurs from agricultural and industrial waste has stimulated interest to convert waste material into commercially valuable products. In the present study, oilcake and fruit waste have been utilized as carbon source for the production of invertase from *M. geophillus* EFRL 03. The *B. niger* is an economically cheap substrate for commercial invertase production using local isolated *M. geophillus*. The invertase produced from Mucor is pH- and thermo-stable, which satisfy the industrial needs.

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