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CULTURAL CONDITIONS FOR THE INCREASED PRODUCTION OF INDUSTRIAL ENZYME BY *MUCOR GEOPHILLUS* OUDEM. ALONGWITH PARTIAL PURIFICATION AND CHARACTERIZATION

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ABSTRACT

Ever increasing growth of food and biotechnological industries has built up a stress over researchers for the large investigation of microorganisms, which could be used in industries. In the present work cultural conditions for the production of Invertase from *Mucor geophillus* using agricultural wastes in submerged fermentation along with partial purification and characterization were studied. Maximum quantity of invertase (5.25 U/mL) was produced when the strain was grown on growth conditions (molasses, carbon source; yeast extract, nitrogen source; incubation time, 48 h; pH, 6.5; temperature, 35° C; inoculum size, $5x10^6$ conidia; agitation rate, 150 rpm). The enzyme was also partially purified by salt precipitation and column chromatography. Temperature and pH optima were 55° C (106.8 U/mL) and 5.5 (121.7 U/mL), respectively. The strain was producing enzyme even up to pH 9.0 and 60° C, so it might be a useful strain for industrial utilization.

Key words: *Mucor geophillus*, Invertase, submerged fermentation.

INTRODUCTION

Invertase (*β*-D-fructofuranosidase, Enzyme Commission No. is 3.2.1.26) splits sucrose by hydrolysis and releases glucose and fructose (Ahmed *et al.* 2015). It is widely used enzyme by various industries especially food industry in making chocolate covered candies, in paper industry, powder milk for infants, in the preparation of jams and to make artificial honey (Phadtare *et al.*, 2004; Safarik *et al.*, 2009; Kotwal and Shankar, 2009; Kulshrestha *et al.*,2013; Ahmed *et al*., 2015a)

Ever increasing growth of food and biotechnological industries has built up a stress over researchers for the large investigation of microorganisms, which could be used in industries (Mamma *et al.*, 2008). A large variety of plant, animal and microbial sources are used for the production of commercial enzymes but the best choice is always microbial sources due to their rapid growth and easier manipulation (Luxhoi *et al.*, 2002; Kulshrestha *et al.*, 2013). Various fungi like *Saccharomyces cerevisiae* (Bokosa *et al.*, 1992), *Aspergillus ochraceus* (Guimarães *et al.*, 2007), *Aspergillus flavus* (Uma *et al*., 2010), *Candida utilis* (Zafar and Aslam, 2013), *Aspergillus niveus* (Guimarães *et al.*, 2009), *Aspergillus niger* (Ashokumar *et al.*,, 2001) and *Cladosporium cladosporioides* (Uma *et al.*, 2012) have been reported for the production of invertase.

The elevated enzyme production depends upon various factors like the strain, culture medium, incubation time period, sources of carbon and nitrogen, temperature, pH, agitation rate and conidia count or inoculum size (Uma *et al*., 2010; Kulshrestha *et al.*, 2013; Ashokumar, 2001; Aslam *et al.*, 2013; Guimarães *et al.*, 2007; Zafar and Aslam, 2013; Ahmed *et al.* 2011; Ahmed *et al.*, 2014; Ahmed *et al.*, 2015; Ahmed *et al.*, 2015a).

In the present work local fungus (*Mucor geophillus* Oudem.) was used and optimization parameters were determined for increased enzyme (invertase) production by using various agricultural based cellulosic wastes as sources of carbon. Utilization of cellulosic waste as a carbon source has two benefits first is the reduction in atmospheric pollution as these wastes are usually disposed of by environment non-friendly manner and second is the production of useful products for humans.

MATERIALS AND METHODS

Materials

Majority of chemicals used in this study were of lab grade [BDH (UK), Sigma-Aldrich (USA), E-Merck (Germany), Fluka (Switzerland) and Acros (Belgium)]. All other chemicals were of the highest possible purity. Strains of *Mucor geophillus* [Oudem., Archives Néerlandaises 7: 278 (1902)], were obtained from the fertile soil of NED University Karachi and culture of strain were maintained (Ahmed *et al.*, 2015).

Methods

Present effort was carried out with shaken flask technique of submerged fermentation in which conical flask (250 mL) containing 50 mL of culture medium was incubated in shaking incubator at temperature 30° C, pH 6.0, inoculums size $4x10^6$ conidia, agitation rate 50 rpm. Conidia count was performed by haemocytometer. Each agricultural based by-product (such as cotton stalk and sunflower) was grinded to convert into powdered form and then hydrolysed (Ahmed *et al*., 2011). The total protein (Lowry *et al*., 1951), total carbohydrate (Dubois *et al.*, 1956) and reducing sugar (Miller, 1959) in the samples were determined. Akgol *et al*. (2001) method was used to determine invertase activity. Enzyme sample of 0.1 mL was added in 2.5 mL acetate buffer (50 mM, pH 5.5) containing 0.1 mL sucrose (300 mM) and then incubated for 5 minutes at 35° C then added 1.0 mL of DNS, boiled for five minutes and cooled at room temperature and absorbance was measured at 540 nm. One unit of invertase activity (U) is the quantity of enzyme, which releases 1 mg of inverted sugar in 5 min at 35° C and pH 5.5.

Optimization of parameters

In this study all parameters were optimized step by step (Ahmed *et al*., 2015). First of all the most suitable culture medium was determined [M_1 (Burrel *et al.*, 1966), M_2 (Dworschack and Wickerham, 1961), M_3 (Souza *et al*., 2007), M_4 (Almeida *et al*., 2005) and M_5 (Poonawalla *et al*., 1965)]. Then the most suitable incubation time period, carbon source, nitrogen source, temperature, pH, conidia count (inoculum size) and agitation rate were determined step by step (Ahmed *et al*., 2015).

Purification and characterization of enzyme

After optimization, purification was done by ammonium sulphate precipitation (20-85 %) and then by DEAE-cellulose column (Ahmed *et al*., 2015). Temperature and pH optima (*i.e*. effects of temperature and pH on invertase activity) were fixed by the method of Akgol *et al*. (2001).

RESULTS AND DISCUSSIONS

Effect of culture media

The strain was grown on five different reported culture media M_1, M_2, M_3, M_4 and M_5. Effects of various culture media on enzyme production by *M. geophillus* after 24 h, at temperature 30° C, initial pH 6.0, conidia count (inoculum size) $4x10^6$ and agitation rate 50 rpm are presented (Fig. 1). The strain was growing well on all mentioned culture media but invertase production was noted maximum (1.25 U/mL) on culture medium M_1, which was selected for the afterward study.

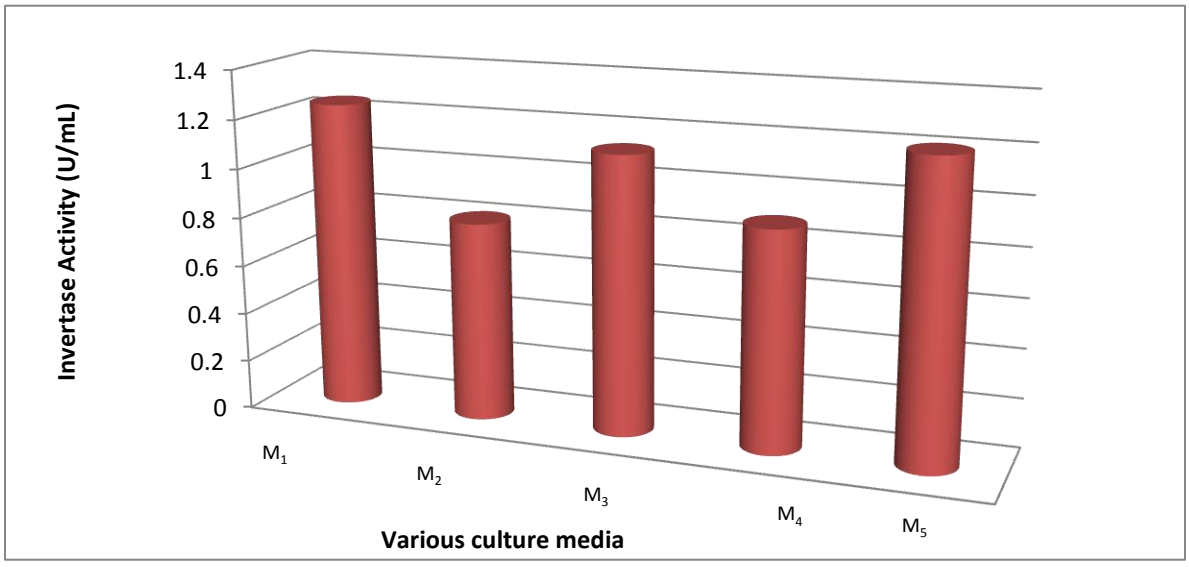

Fig. 1. Effects of various culture media on the production of invertase by *M. geophillus* Oudem. (Incubation time 24 h, temperature 30° C, pH 6.0, conidia count $4x10^6$ and agitation rate 50 rpm.

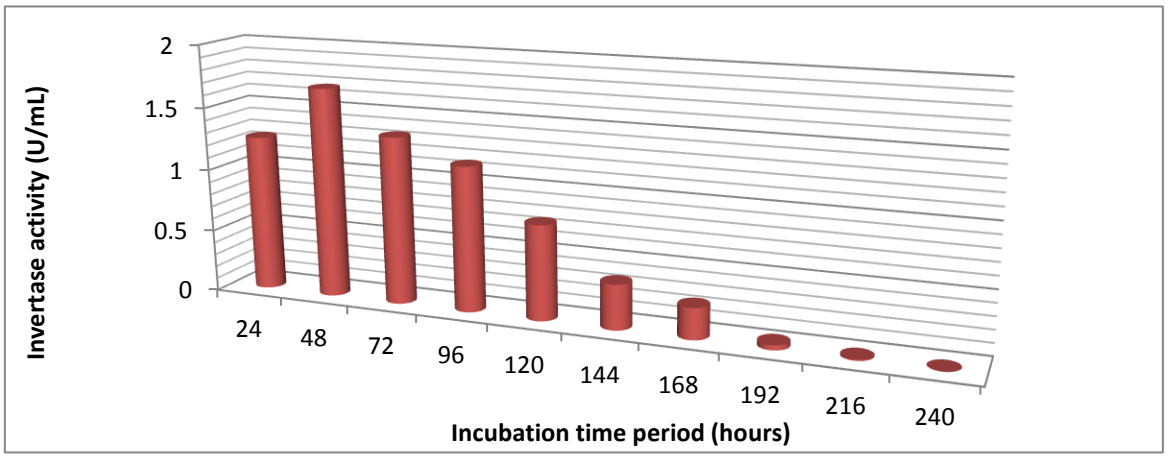

Fig. 2. Effects of various incubation time periods on the production of invertase by *M.geophillus* in M_1 (at temperature 30º C, pH 6.0, conidia count $4x10^6$ and agitation rate 50 rpm).

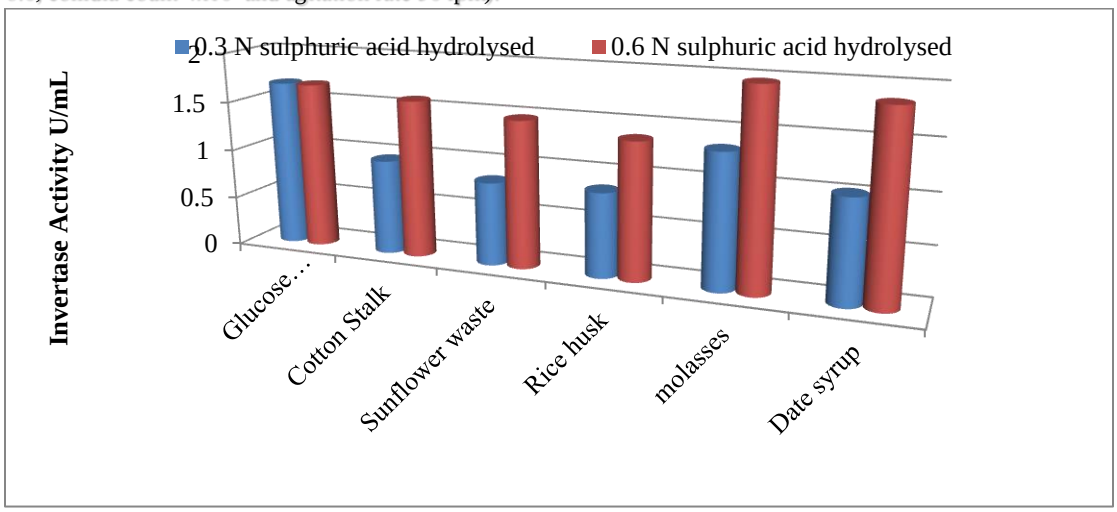

Fig. 3. Effects of various energy or carbon sources in M_1 for invertase production by *M. geophillus* (Incubation time 48 h, at 30º C, initial pH 6.0, conidia count $4x10^6$ and agitation rate 50 rpm)

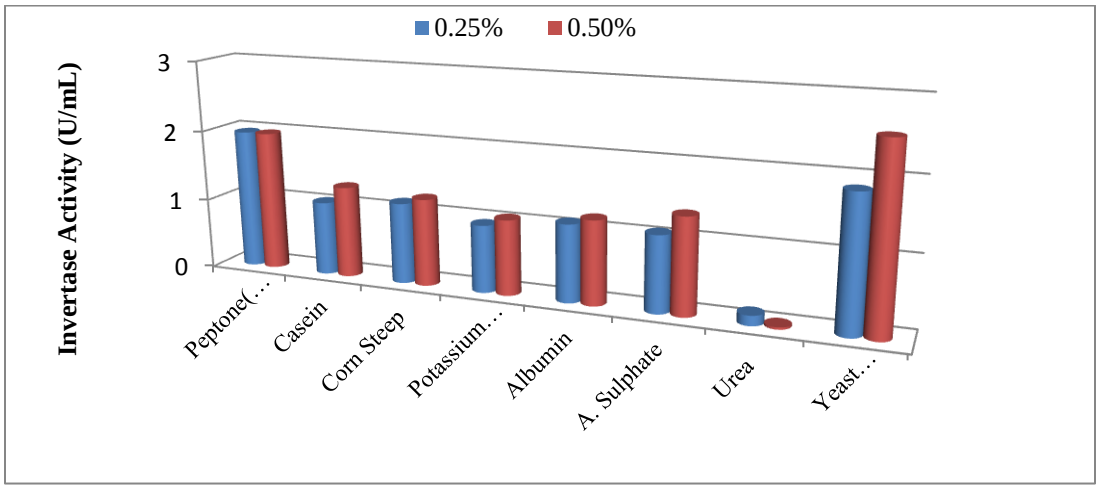

Fig. 4. Effects of various sources of nitrogen in M_1 on invertase production by *M. geophillus* (incubation time 48 h, containing molasses, temperature 30^o C, pH 6.0, conidia count $4x10^6$ and agitation rate 50 rpm)

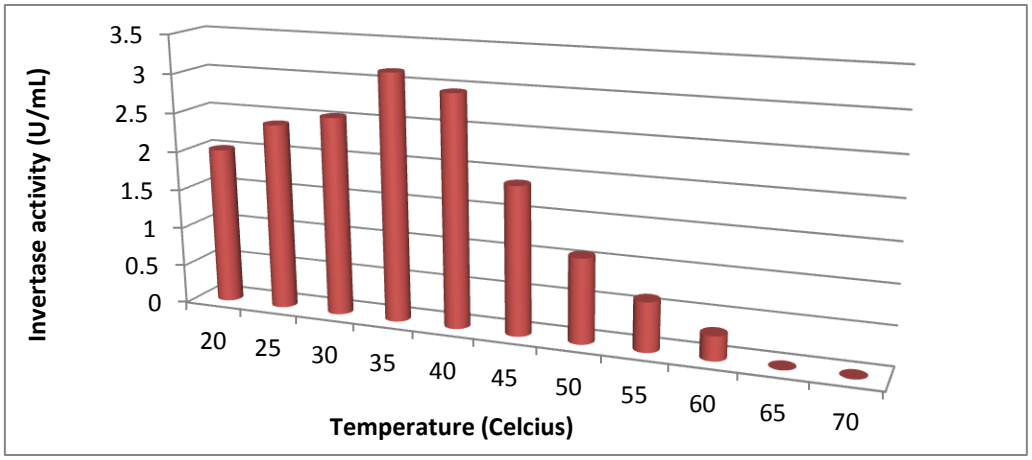

Fig.5. Effects of incubation temperatures on the production of invertase in M1 by *M. geophillus* (Incubation time 48 h, containing molasses, yeast extract, pH 6.0, conidia count $4x10^6$ and agitation rate 50 rpm)

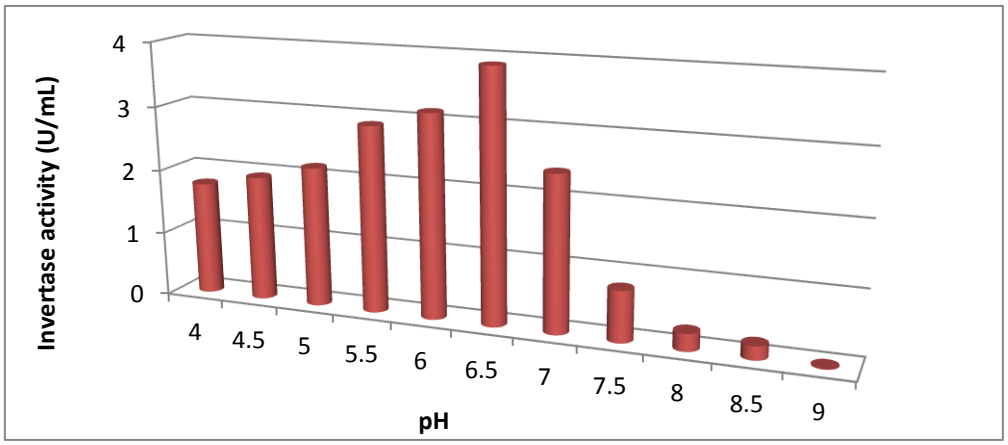

Fig.6. Effects of initial pH of growth medium in M_1 for invertase production by *M. geophillus* (Incubation time 48 h, containing molasses, yeast extract, at temperature 35º C, conidia count $4x10^6$ and agitation rate 50 rpm)

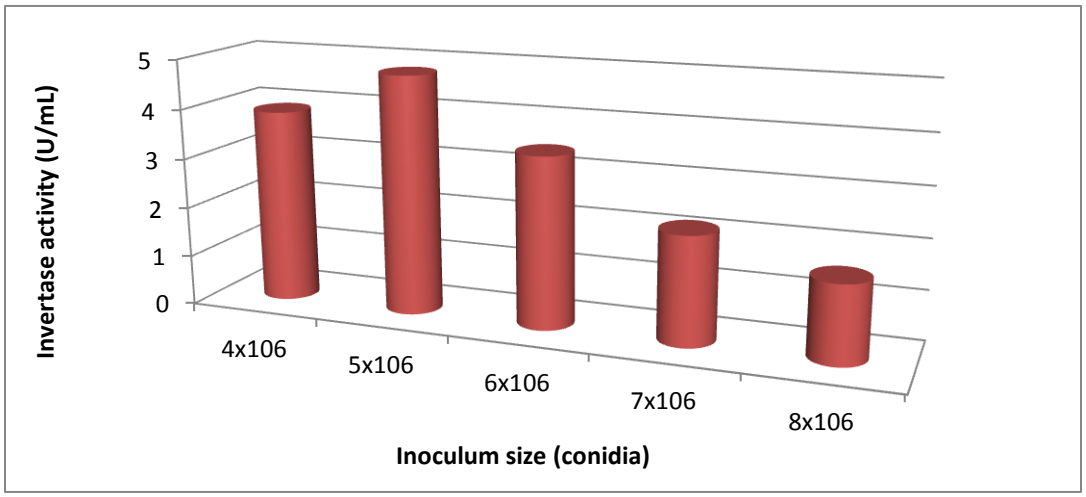

Fig.7. Effects of conidia count in M1 for invertase production by *M. geophillus* (incubation time 48 h, containing molasses, yeast extract, at temperature 35° C, pH 6.5 and agitation rate 50 rpm)

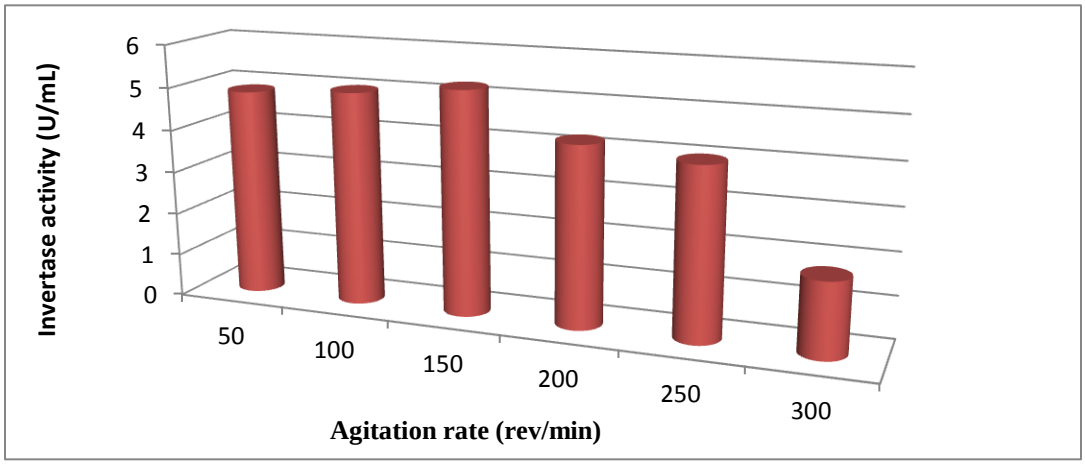

Fig.8. Effects of agitation rates (rpm) of M_1 on invertase production by *M. geophillus* (Incubation time 48 h, containing molasses, yeast extract, at temperature 35° C, pH 6.5 and conidia count $5x10^6$)

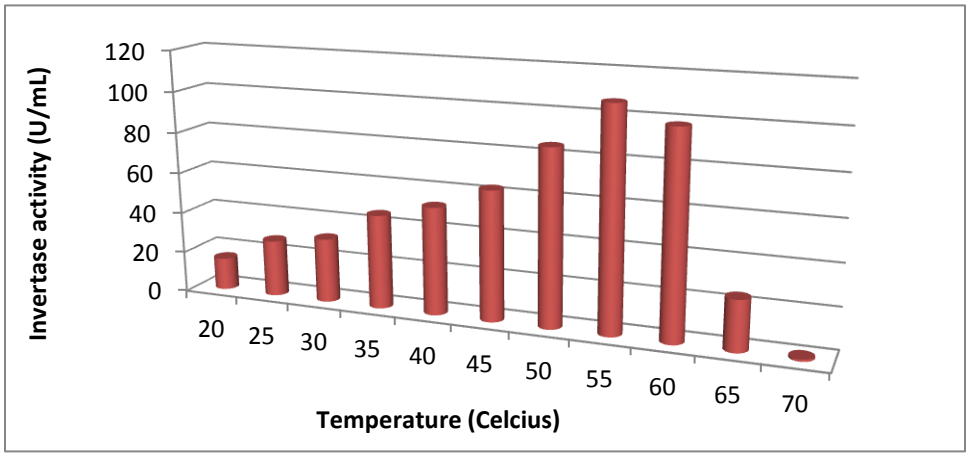

Fig.9. Temperature optima of invertase from *M. geophillus.*

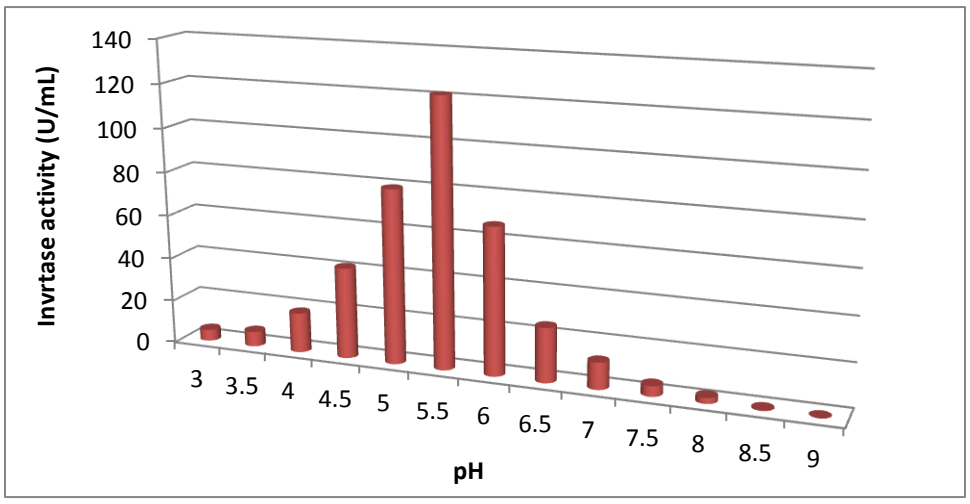

Fig.10. pH optima of invertase from *M. geophillus*.

Effect of incubation time period

Incubation time period has a profound effect on enzyme production (Ahmed *et al*., 2011; 2014; 2015). Effects of incubation time periods on invertase production by *M. geophillus* on M_1 (at incubation temperature 30^o C, initial pH 6.0, conidia count $4x10^6$ and 50 rpm agitation rate) are presented (Fig. 2). After every 24 h, enzyme activity was measured and it was observed that the maximum invertase activity (1.68 U/mL) was observed after 48 h of incubation. On sustained incubation enzyme activity was reduced which is due to synthesis of inhibiting metabolite or denaturing of enzyme (Mamma *et al*., 2008). Mizunaga *et al*. (1981) also reported 48 h as the most suitable incubation time period for invertase production by *Saccharomyces cerevisiae*.

Effect of carbon sources

The most appropriate energy or carbon source also has a deep effect on enzyme production (Ahmed *et al*., 2011; 2014; 2015). The effects of various carbon sources on invertase production by *M. geophillus* after 48 h on M_1 (temperature 30° C, initial pH 6.0, conidia count $4x10^6$ and agitation rate 50 rpm) are presented (Fig. 3). From the results it can be seen that that Invertase activities were closed to control [glucose (1.68 U/mL)] when 0.6 *N* sulphuric acid hydrolysed agriculture waste (1.59, 1.48 and 1.37 U/mL for cotton stalk, sunflower waste and rice husk respectively) while activities were lower in case of 0.3 *N* sulphuric acid hydrolysed agriculture waste (0.96, 0.84 and 0.85 U/mL for cotton stalk, sunflower waste and rice husk respectively) and 0.5 % of molasses and date syrup (1.34 and 1.03 U/mL respectively). Invertase activities were higher when 1 % of date syrup (1.97 U/mL) and molasses (1.88 U/mL) were used. In literature various carbon sources have been reported as the most appropriate for the production of various enzymes. Sugar cane bagasse was reported as the appropriate carbon source for invertase production by *Aspergillus niveus* (Guimarães *et al*., 2009).

Effect of nitrogen sources

The presence of appropriate nitrogen source in culture medium is essential because it is related with quality and quantity of enzyme (Ahmed *et al*., 2015). The effects of various nitrogen sources on invertase production by *M. geophillus* after 48 h in M_1 containing molasses as carbon source (at temperature 30° C, initial pH 6.0, conidia count $4x10^6$ and 50 rpm agitation rate) are presented (Fig. 4). It is evident from results that the strain had the capability of consuming all (except urea) of nitrogen sources but yeast extract was found to be the most appropriate (1.86 U/mL in 0.25 % and 2.54 U/mL in 0.50 %). Zafar and Aslam (2013) and Guimarães *et al*. (2007) also observed Yeast extract as the best nitrogen source for *Candida utilis* and *Aspergillus ochraceus* respectively. When urea was used as nitrogen source then very low values (0.14 and 0.03 U/mL) of invertase activities were observed which is due to denaturing effect of urea on invertase (Hussain *et al*., 2010).

Effect of temperature

Incubation temperature is another important parameter in order to obtain maximum enzyme production (Ahmed *et al*., 2011; 2014; 2015). The effects of incubation temperatures on invertase production after 48 h in M_1 containing molasses and yeast extract (at initial pH 6.0, conidia count $4x10^6$ and agitation rate 50 rpm) are presented (Fig. 5). The growth medium was incubated at a range of temperatures between 20-70° C. It was observed that enzyme activity was the highest (3.15 U/mL) around 35° C beyond that it was decreased which is due to denaturing of enzyme and formation of inhibiting metabolite (Ahmed *et al*., 2015). Similar type of temperature for invertase production was also reported by Dahot (1986) for *Penicillium expansum.*

Effect of initial pH

Initial pH of fermentation medium has direct relationship for elevated enzyme production (Ahmed *et al*., 2011; 2014; 2015). The effects of initial pH on invertase production by *M. geophillus* after 48 h in M_1 (containing molasses, yeast extract, temperature 35° C, conidia count $4x10^6$ and agitation rate 50 rpm) are plotted (Fig. 6). The range of pH (4.0 to 9.0) was examined and found that initial pH of 6.5 would be the most suitable for elevated enzyme production (3.89 U/mL) before and after the pH, decrease in activity was observed. Similar results related with pH for invertase production from *Saccharomyces cerevisiae* were also reported by Dworschack and Wickerham (1961).

Effect of inoculum size

Inoculum size (conidia count) has the effect on the quantity of enzyme (Ahmed *et al*., 2011; 2014; 2015). The effects of conidia count or inoculum sizes on invertase production by the strain on M_1 (after 48 h, containing molasses, yeast extract, temperature 35° C, at initial pH 6.5 and agitation rate 50 rpm) are presented (Fig. 7). Flasks were added with $4x10^6$-$8x10^6$ conidia and maximum invertase activity (4.79 U/mL) was observed when $5x10^6$ conidia were added to the medium. Various Scientists such as Dahot (1986), Guimarães *et al*. (2007), Ahmed *et al*. (2011;

2015) used varying inoculum sizes. Large inoculum size caused overgrowth and nutritional imbalanced resulting less enzyme production (Guimarães *et al*., 2007; Mamma *et al*., 2008; Ahmed *et al*., 2011; 2014; 2015).

Effect of agitation rate

Agitation of fermentation media is important because it ensures homogenous supply of nutrients (Ahmed *et al*., 2011; 2014; 2015). The effects of agitation rates on invertase production by *M. geophillus* after 48 h on M_1 (containing molasses, yeast extract, at 35° C, initial pH 6.5 and conidia count $5x10^6$) are presented (Fig. 8). The fermentation media was agitated at a range of 50, 100, 150, 200, 250 and 300 rpm. It was observed that invertase activity was maximum (5.25 U/mL) at 150 rpm. In literature various workers have reported various agitation rates (100-200 rpm) for enzymes production by different microorganisms [Quiroga *et al*., 1995; L'Hocine *et al*., 2000; Rubio *et al*., 2002; Ahmed *et al*. (2011; 2015)].

Purification

The extracellular invertase from *Penicillium lilacinum* was purified and results are summarised in table 1 as per following results:

Table 1. Purification scheme of extracellular invertase by *Penicillium lilacinum*.

Treatment	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude enzyme	400	5629	157.2	35.8	100	1
$(NH_4)_2SO_4$ (85%) treated	32	2194	54.2	40.47	38.97	1.13
DEAE-cellulose	5.4	1360	5.68	239.4	24.2	6.69

Chan *et al.* (1991) reported 75 % recovery of yeast's invertase with nine fold purification using 0.05*M* Tris-HCl buffer containing 0.5*M* sodium chloride at pH 7. Bhatti *et al.* (2006) purified invertase from *Fusarium solani* to homogeneity by ammonium sulphate precipitation and column chromatography *i.e.* DEAE-cellulose and Sephadex G-200.

Effect of pH and temperature on purified invertase activity

The effects of pH (Fig. 9) and temperature (Fig. 10) on purified enzyme from *P. lilacinum* were examined and it was found that invertase showed maximum activity at pH 5.5 (121.7 U/mL) and 55° C (106.8 U/mL). Above and below the conditions there was a reduction in invertase activity. The results were similar to that reported for *Aspergillus niger* by L'Hocine *et al*. (2000) while Bhatti *et al*. (2006) described optimum pH and temperature were 2.6 and 50° C respectively for invertase produced by *Fusarium solani*.

CONCLUSION

Maximum quantity of invertase (5.25 U/mL) from *Mucor geophillus was* observed when the strain was grown on culture medium M1 containing yeast extract as a source of nitrogen, molasses as a source of carbon after 48 h of incubation at initial pH 6.5, temperature 35° C, inoculum size of $5x10^6$ conidia in 50 mL of culture medium and agitation rate of 150 rev/min.

Invertase from *Mucor geophillus* was also partially purified and characterized. It was purified to about 6.69 folds than crude enzyme with the recovery of 24.2 % having specific activity 239.4 U/mg. It has pH and temperature optima 5.5 and 55° C respectively.

The strain was producing very high quantity of invertase and it was also stable at pH (up to 9) and temperature (up to 60° C), therefore, could be used effectively in industries for invertase production.

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