OPTIMAL CULTURAL CONDITIONS FOR INDUSTRIAL ENZYME PRODUCTION BY USING SHAKEN FLASK TECHNIQUE OF SUBMERGED FERMENTATION

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Abstract

In this work optimization parameters in submerged fermentation were studied for the production of Invertase from *Aspergillus flavus* (Link 1809; IBGE 07) using agricultural based by-products (sunflower waste, cotton stalk, rice husk, date syrup and molasses) as sources of carbon. Effects of incubation time period (24-240 h), various cultural media (CM1, CM2, CM3, CM4 and CM5) sources of nitrogen (Corn steep, Casein, Potassium Nitrate Albumin Ammonium Sulphate Urea and Yeast Extract), pH (4.0-9.0), temperatures (30-70° C), inoculum size ($4x10^6$-$8x10^6$ conidia) and agitation rate (50-300 rev/min) were also investigated for maximum invertase production. Optimal conditions for the production of invertase (7.41 U/mL) by *Aspergillus flavus* were observed when the strain was grown on culture medium CM1 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 40° C, inoculum size of $6x10^6$ conidia in 50 mL of culture medium and agitation rate of 200 rev/min.

Introduction

The modern biotechnological setup due to increasing demand of enzymes has motivated the need for enlarged survey of microorganisms surviving and producing enzyme in extreme conditions (Mamma *et al.,* 2008). For the production of large quantities of enzymes filamentous fungi have biotechnological importance (Ahmed *et al.,* 2011; 2014).

Invertase (E. C. 3.2.1.26), splits sucrose into glucose and fructose. It is one of the most widely used enzymes by food industry in making chocolate covered cherries. This enzyme is also used in paper industry and to make artificial honey in which it contributes to anti-bacterial properties (Phadtare *et al.*, 2004; Kotwal & Shankar, 2009; Safarik *et al.*, 2009; Kulshrestha, 2013).

In the present work specific interest has been focused on agricultural based by-products like sunflower cotton stalk rice husk, date syrup, and molasses because they are usually related with pollution. Being the cost effective sources of carbon agricultural wastes have a potential for conversion into useful products (Mamma *et al.,* 2008). In this work the secretion of invertase by *Aspergillus flavus* in submerged fermentation was carried out.

Materials and Methods

Strains: Strain of *Aspergillus flavus* (Link 1809; IBGE 07) was obtained from the Institute of Biotechnology and Genetic Engineering, University of Sindh Jamshoro and culture were maintained as followed by Dahot (1986). In the present study 4 days old slants were used for inoculation.

Conidia count: Number of conidia of each fungus was counted by haemocytometer. Spore suspension was maintained around $4x10^6$ conidia/mL and they were added to 50 mL of fermentation media in 250 mL of flask.

Hydrolysis of agriculture waste: Each agricultural waste (cotton stalk, sunflower waste and rice husk) were hydrolyzed as reported earlier (Ahmed *et al*., 2011; 2014).

Determination of invertase activity: Invertase activity was determined by following Akgol *et al.*, (2001). One unit of invertase activity is the amount of enzyme, which releases 1 mg of inverted sugar in 5 min at 35º C and pH 5.5.

Optimization of enzyme production parameters: All experiments were done in such a way that the parameter optimized in one experiment was fixed in the next experiments for the production of enzyme.

Culture medium: First of all the most suitable culture medium was determined. For invertase production 50 mL of following culture media were used in 250 mL flask having composition (in g/L)

CM1: Dextrose 10, peptone 5, epsom salt 5, KH_2PO_4 5, common salt 2.5, ferrous sulphatehepta hydrate 0.01, $ZnSO_4.7H_2O$ 0.002, $MnSO_4.H_2O$ 0.001 and thiamine hydrochloride 0.001 (Burrel *et al.*,1966).
CM2: Yeast extract 10, peptone 20 and sucrose 20. (Dworschock & Wickerham, 1961).
CM3: Yeast extract 20, peptone 40, sucrose 20, $KH_2(PO_4)_2$ and epsom salt 1 (Souza *et al*., 2007).
CM4: $NaNO_3$ 3, KCl 0.5, epsom salt 0.5, ferrous sulphate hepta hydrate 0.01, K_2HPO_4 1, Sucrose 30 (Almeida *et al*., 2005).
CM5: Sucrose 40, corn steep liquor 30, $NaNO_3$ 3, KH_2PO_4 0.5, epsom salt 0.05, $CaCO_3$ 2.5 (Poonawalla *et al*., 1965).

Incubation time period: After the determination of the most suitable culture medium, optimum incubation time period was determined. It was done by growing the strain on CM1 (Culture medium) at various time periods from 24-240 h.

Carbon sources: After the optimization of incubation time the most suitable carbon source was determined. It was done by replacing the glucose (control) of culture medium (CM1) by various wastes including sunflower wastes, cotton stalk, rice husk, which were hydrolyzed by 0.3*N* $H_2SO_4$and 0.6*N* H_2SO_4. Date syrup and molasses were used 0.5 % and 1 % in place of glucose (control).

Nitrogen sources: After the determination of the most suitable carbon source various nitrogen sources were checked for optimum production of enzymes. It was done by replacing peptone of culture medium (CM1) by corn steep, casein, potassium nitrate, albumin, ammonium sulphate, urea and yeast extract.

Incubation temperature: The most suitable culture medium CM1 (with the most suitable carbon and nitrogen source) was tested on varying temperature from 20-70^o C to determine the most suitable incubation temperature for the production of enzymes.

Initial pH of medium: The initial pH of a medium has an effect on growth and productivity of microorganism. A range of pH from 4.0-9.0 was checked for optimum enzymes production.

Inoculum size: Productivity was also checked in terms ofnumber of conidia in 50 mL of optimized culture medium in order to obtain the optimized inoculum size of culture medium. The number of conidia was counted by haemocytometer.

Agitation rate: Effect of agitation rate was also checked for optimization at 50, 100, 150, 200, 250 and 300 rev/min in orbital shaking incubator.

Results and Discussions

Effect of culture media: Effects of various culture media on invertase production by *Aspergillus flavus* after 24 h, at temperature 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Fig. 1). The strain was grown on five different culture media *i.e.* CM1, CM2, CM3, CM4 and CM5. It was capable of growing well on all types of culture media but production of invertase was maximum (2.62 U/mL) on culture medium CM1, which was selected for the next study.

Effect of incubation time period: The effects of incubation time periods on invertase production by *Aspergillus flavus* in CM1 at temperature 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are given (Fig. 2). Invertase activity was measured at regular interval of 24 h and it was found that the maximum activity (3.54 U/mL) was observed after 48 h of incubation. On prolonged incubation enzyme activity was decreased, which might be due to denaturing of enzyme or synthesis of inhibiting metabolite (Mamma *et al.*, 2008). Incubation time period of 48 h was reported for invertase production by *Aspergillus fumigatus* and *Aspergillus flavus* (Kulshrestha. *et al*., 2013) .

Effect of carbon sources: The effects of various carbon sources on invertase production by *Aspergillus flavus* after 48 h in CM1 at temperature 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are exhibited (Fig.3). It was observed that invertase activities were lower in case of 0.3*N*sulphuric acid hydrolysed agriculture waste (2.49, 2.27 and 2.13 U/mL for cotton stalk, sunflower waste and rice husk, respectively) and 0.5 % of molasses and date syrup (2.87 and 2.69 U/mL respectively). Invertase activities were closed to control,

glucose (3.54 U/mL) when 0.6*N* sulphuric acidhydrolysed agriculture waste (3.65, 3.39 and 3.51 U/mL for cotton stalk, sunflower waste and rice husk, respectively) and enzyme activities were higher when 1 % of molasses (4.47 U/mL) and date syrup (4.76 U/mL) were used. Uma *et al*. (2012) have reported pomegranate peel as the appropriate carbon source for invertase production by *Cladosporium cladosporioides*.

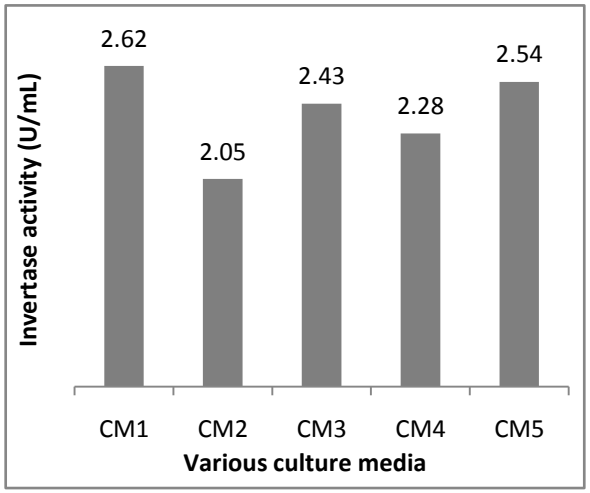

Fig. 1 Effects of various culture media on invertase production by *Aspergillus flavus* after 24 h, at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

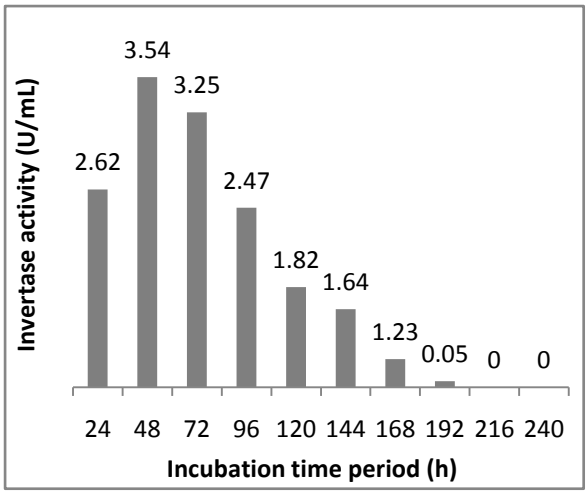

Fig. 2 Effects of incubation time periods on invertase production by *Aspergillus flavus* in CM1 at 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

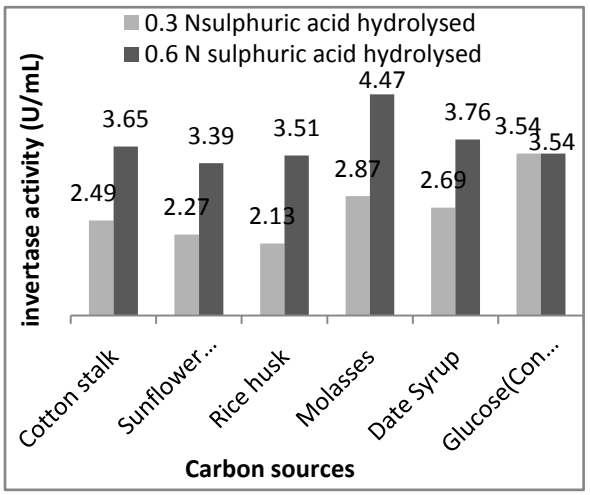

Fig. 3 Effects of various carbon sources on invertase production by *Aspergillus flavus* after 48 h in CM1 at 30° C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

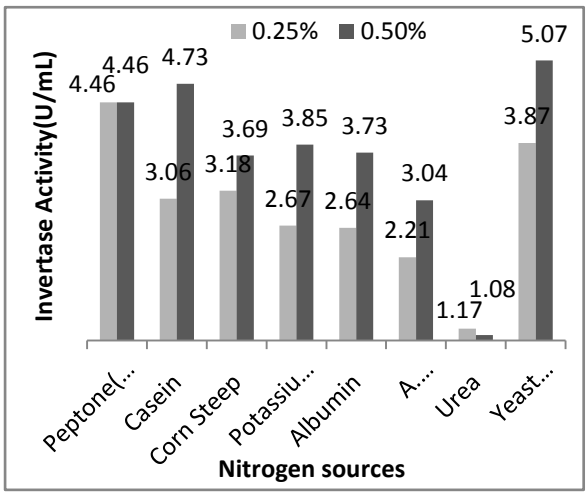

Fig. 4 Effects of various nitrogen sources on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source at 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

Effect of nitrogen sources: The effects of various nitrogen sources on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source at temperature 30°C, initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are presented (Fig. 4). Various nitrogen sources (corn steep liquor, casein, potassium nitrate, albumin, ammonium sulphate, urea and yeast extract) were used in 0.25 and 0.50 % in place of peptone (control having enzyme activity 4.46 U/mL). The strain showed the capability of utilizing well all types (except urea) of nitrogen sources but yeast extract was found to be the best (3.87 U/mL in 0.25 % and 5.07 U/mL in 0.50 %). Yeast extract was also reported as the best nitrogen source for *Candida utilis*, *Saccharomyces cerevisiae* (Dworschack & Wickerham, 1961) and *Aspergillus ochraceus* (Guimarães *et al*., 2007).Very low values (0.17 and 0.08 U/mL) of invertase activities were observed when urea was used as nitrogen source. It might be due to denaturing effect of urea on invertase (Hussain *et al*., 2010).

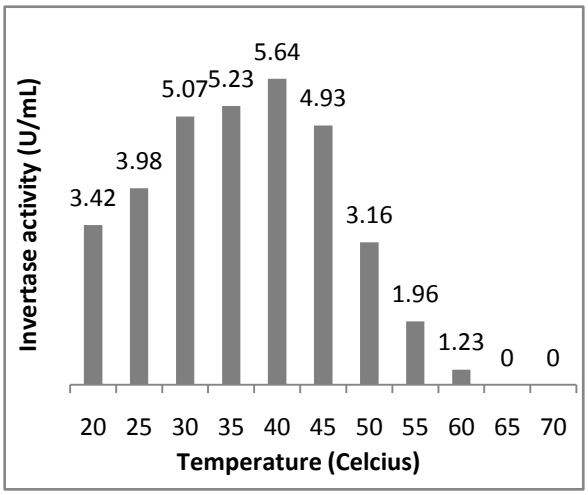

Fig. 5 Effects of incubation temperatures on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

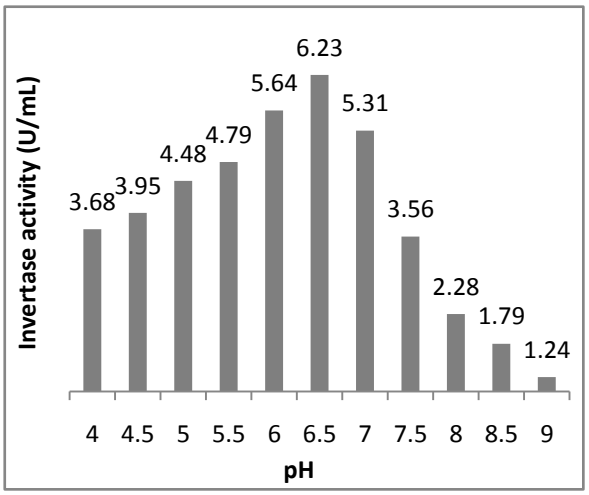

Fig. 6 Effects of initial pH of fermentation medium on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source at 40°C, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min.

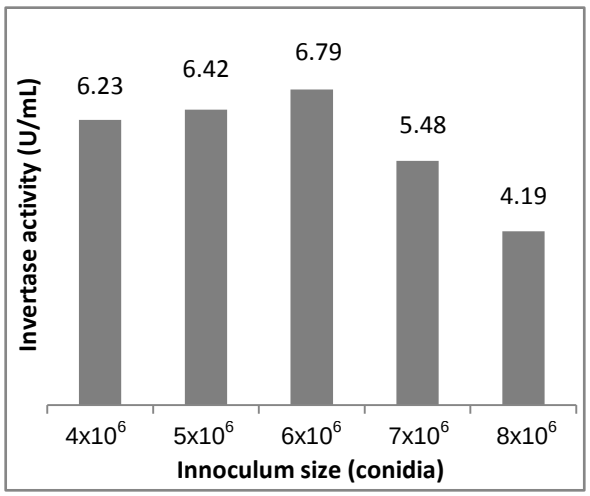

Fig. 7 Effects of inoculum sizes on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 40°C, initial pH 6.5 and agitation rate 50 rev/min.

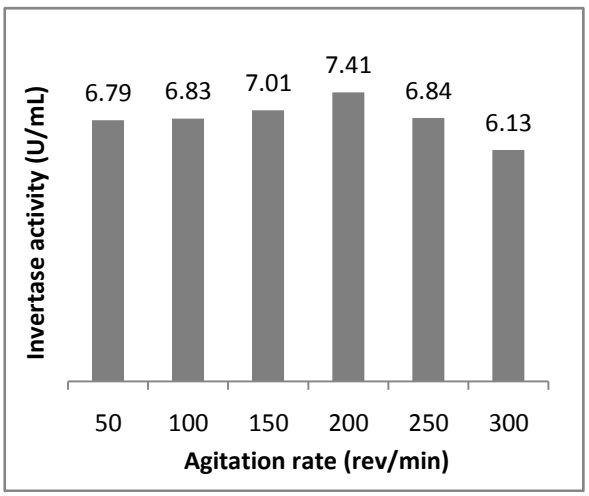

Fig. 8 Effects of agitation rates on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at 40° C, initial pH 6.5 and inoculum size $6x10^6$ conidia.

Effect of temperature: The effects of incubation temperatures on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, at initial pH 6.0, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are exhibited (Fig. 5).The fermentation medium was incubated at a range of temperatures 20-70° C. Invertase activity was the highest (5.64 U/mL) about 40° C. Similar optimum temperature was reported for *Penecillium lilacinum* by Ahmed *et al*. (2011). The strain showed thermo stability up to 60° C (0.23 U/mL), which is a requirement for industrial use of a microorganism (Mamma *et al*., 2008).

Effect of initial pH: The effects of initial pH of fermentation medium invertase on production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, temperature 40°C, inoculum size $4x10^6$ conidia and agitation rate 50 rev/min are plotted (Fig. 6). A range of pH (4.0 to 9.0) was studied and found that initial pH of 6.5 would be the best for maximum enzyme production (6.23 U/mL). After and before the pH enzyme activities were decreased. Similar optimum pH for invertase production was reported by Dworschack & Wickerham (1961) form *Candida utilis* and *Saccharomyces crevisiae*.

Effect of inoculum Size: The effects of inoculum sizes on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, temperature 40° C, at initial pH 6.5

and agitation rate 50 rev/min are presented (Fig. 7).Flasks were added with $4x10^6$-$8x10^6$ conidia and maximum invertase activity (6.79 U/mL) was observed when $6x10^6$ conidia were added to the medium. Researchers had used inoculum size in varying percentages (Dahot, 1986; Guimarães *et al*., 2007; Mamma *et al*., 2008; Ahmed *et al*., 2011). Large inoculum size causes overgrowth and nutritional imbalanced resulting less production of enzyme (Dahot, 1986; Guimarães *et al*., 2007; Mamma *et al*., 2008).

Effect of agitation rate: The effects of agitation rates on invertase production by *Aspergillus flavus* after 48 h in CM1 containing molasses as carbon source, yeast extract nitrogen source, temperature 40° C, at initial pH 6.5 and inoculum size $6x10^6$ conidia are shown (Fig. 8). The fermentation medium was agitated at 50, 100, 150, 200, 250 and 300 rev/min. Invertase activity was maximum (7.41 U/mL) at 200 rev/min. Literature survey exposed that researchers have reported various agitation rates for different enzymes production by different microorganisms (Dahot, 1986; Quiroga *et al*., 1995; L'Hocine *et al*., 2000; Rubio *et al*., 2002; Bhatti *et al*., 2006).

Conclusion

Optimal conditions for the production of invertase (7.41 U/mL) by *Aspergillus flavus* were observed when the strain was grown on culture medium CM1 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 40°C, inoculum size of $6x10^6$ conidia in 50 mL of culture medium and agitation rate of 200 rev/min.

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