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Invertase production from *Aspergillus spp* M1 isolated from honeycomb

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A newly isolated strain *Aspergillus spp* M1 was obtained from infected honeycomb. The strain produces efficient thermostable invertase which was active in acidic pH. The partially purified invertase was optimally active at pH 6 and 60^0C. The enzyme activity was enhanced by Ca^{2+} and Cd^{2+}, while Hg^{2+} inactivates the enzyme. The maximum invertase production (238.18 U/ml) was detected by using sucrose (10 % w/v) as a carbon source while sodium nitrate (2.0 g/l) was found to be the best nitrogen source and supported maximum invertase production (233.27U/ml) in static condition.

Aspergillus spp/Microbiology

Invertase (EC: 3:2:1:26) acts on 1, 4 glycoside linkage of sucrose and splits it into D-glucose and D-fructose (Kaur and Sharma, 2005). It is intracellular as well as extracellular enzyme. Invertase is also referred as ß-fructofuranosidase as it catalyses hydrolysis of the terminal non-reducing residue of ß fructofuranoside (Gascón, 1968; Chen, 1996). Invertase is widely used in production of confectionary with liquid or soft centre, manufacturing of invert syrups, calf feed preparations and fermentation of cane molasses into ethanol (Park and Sato, 1982, Gehlawat, 2001). On industrial scale citric acid fermentation uses molasses as a feedstock, which contains principally sucrose as a carbon source. The invertase is also used in combination with glucose isomerase for sweetening the syrups. A wide range of microorganisms were reported to secrete invertase, it includes yeast *Saccharomyces cerevisiae* and *S. carlsbergensis* (Jon and Linda, 2007) and some fungi, *Penicillium spp, Neurospora spp*, *Aspergillus spp* (Poonawalla and Patel, 1965). Commercially, invertase is produced on large scale primarily by using submerged fermentation. The well-known methods for production of invertase are submerged and solid state fermentation. However, the submerged method is strongly inhibited by catabolite repression of glucose. In contrast, several studies have been reported high enzyme activity titers by solid-state fermentation (SSF) over submerged when high glucose concentrations were used (Aranda et al. 2006).

The objective of the present work was to partially characterize and determine optimum production parameter for the invertase production from newly isolated *Aspergillus* spp.

Author contributions: Mahesh Patil and Ravikiran Bhamare involved in execution of experimental work and literature survey. Dr. Ulhas Patil contributed in experiment design and scientific and statistical analysis of data.

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Materials and Methods

Microorganism

Total 14 fungal isolates were screened out as invertase producer from several locations. The fungal isolate used in present investigation designated as M1, was obtained from honeycomb; collected at R.C.P.A.S.C. college campus, Shirpur (Fig.1). It was identified as *Aspergillus spp* based on morphological and biochemical properties. It was maintained on Czapek-Dox agar slants at 4^0C.

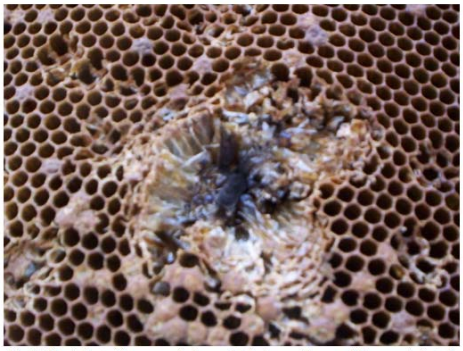

Fig.1: The infected honeycomb from which *Aspergillus spp* M1 was isolated

Screening for Invertase Producer

The fungal isolates were obtained by suspending the various samples into Czapek-Dox medium containing (g/L) sucrose 30, sodium nitrate 2.0, di-potassium phosphate 1.0, magnesium sulphate 0.5, and potassium chloride 0.5, pH 6.0. All the isolates were grown at 27^0C for 5 days with agitation (100 rpm). Efficient invertase producers were screened out by mixing 2 ml cell free broth with 2 ml Benedict's reagent; green colored precipitation indicates the positive test (Sadashivam and Manikam, 1996).

Inoculum Preparation

The inoculum consists, 4ml of spore suspension having A_{530} - 0.12 (containing 1.6×10^6 spores per ml) into 100 ml sterile basal medium.

Invertase Assay

The grown culture was harvested by filtering the broth through Whatman filter paper. The cell free extract was then taken as an enzyme source. Invertase activity was measured by method described by Bacon (1955). The suitably diluted aliquot of filtered broth was added to 1.0 ml sucrose (1 % w/v) solution prepared in acetate buffer pH (6.0). The reaction mixture was incubated at 60^0C for 20 minute; the reaction was terminated by adding 1.0 ml of 3, 5- dinitrosalicylic acid solution. The content of tubes diluted with 3.0 ml of distilled water and absorbance was measured at 540 nm. One unit of invertase activity was defined as the amount of enzyme required to produce 1M of reducing sugar per minute at pH 6.0 and 60^0C.

Fungal Growth Measurement

The culture broth was harvested by filtration and separated biomass was washed twice with cold distilled water. The washed biomass was dried in vacuum at room temperature until a constant weight was attained. Values obtained were an average of three independent determinations.

Optimization of Physical Parameters for Invertase Production

The optimization of growth and invertase production by M1 isolate was carried out under various physical conditions viz pH, temperature and rate of agitation. The effect of pH on growth and invertase production were examined by growing strain M1 in production medium containing (g/L) sucrose 100, sodium nitrate 2.0, di-potassium phosphate 1.0, magnesium sulphate 0.5, and potassium chloride 0.5 for 5 days with varying pH. The effect of temperature on growth and invertase secretion were examined by growing the strain in production medium at various temperatures (20 - 40^0C) for 5 days. The effect of agitation on growth and invertase production was studied by allowing the culture to grow 0 - 100 rpm.

Optimization of Media Ingredients for Invertase Production

Various carbon sources viz glucose, lactose, maltose, starch and molasses were examined for optimum invertase production. Each carbon source (1%) was separately added in basal medium containing (g/L), sodium nitrate 2.0, di-potassium phosphate 1.0, magnesium sulphate 0.5, and potassium chloride 0.5, pH 6.0. Similarly various nitrogen sources viz peptone, yeast extract, ammonium chloride, sodium $nitrate_3$, potassium nitrate and ammonium sulphate were examined for optimum invertase production. Each nitrogenous component (0.5 %) was separately added in basal medium containing (g/L) sucrose 100, di-potassium phosphate 1.0,

magnesium sulphate 0.5 and potassium chloride 0.5. The effect of various concentrations of sucrose from 5 to 25 % on cell growth and enzyme production was also studied.

Effect of pH on Invertase Activity

Effect of pH was assessed using partially purified and suitably diluted enzyme. Following buffers (0.1 M) were used: glycine-HCL (pH 2) acetate (pH 4.0) phosphate (pH 6.0 - 8.0), carbonate- bicarbonate (pH 9.0 - 11) KCl-NaOH (pH 12). The relative activities were based on the ratio of the activity obtained at specific pH to the maximum activity obtained and expressed as percentage. To study the effect of pH on activity of invertase, 0.08 ml of enzyme was added to respective buffer. The reaction was carried out at 60^0C for 20 min.

Effect of Temperature on Invertase Activity

The effect of temperature was determined by incubating the reaction mixture for 20 min at the different temperatures ranging from 25^0C - 65^0C. The relative activities were based on the ratio of the activity obtained at specific temperature to the maximum activity obtained and expressed as percentage.

Effect of Metal Ions on Invertase Activity

The partially purified enzyme was mixed with 5 mM concentration of various salts such as $CaCl_2$, $CdCl_2$, $HgCl_2$, $MgCl_2$, $CoCl_2$ and NaCl for 30 min and subsequently invertase activity was determined.

Result and Discussion

Among the total 14 fungal isolates, six potential strains were screened out as potential invertase producers based on their response to Benedict's reagent. The strain described here was designated as M1, gave high colored intensity among all 6 fungal isolates when cell free broth was mixed with Benedict's reagent. The fungal isolate M1 was identified as *Aspergillus spp* based on morphological and biochemical characteristics (Alexopoulos and Mims, 2002).

Aspergillus spp M1 grows in range pH 4 -7 and showed an optimum growth and maximum invertase secretion at pH 6 (Fig 2). The optimum temperature for invertase production and growth was 28^0C (Fig 3). Although the agitation promotes the growth of *Aspergillus spp* but it suppresses the invertase production. The maximum invertase production was detected when culture grown in static condition. The best carbon source for optimum growth and invertase production was sucrose and comparable results (182.25U/ml) were obtained by employing the molasses as carbon source. The subsequent increase in sucrose concentration has positive effect on both growth and invertase production. The fungi tolerate up to 15% sucrose, while it optimally grow at 10% sucrose concentration in which it shows maximum secretion of invertase (Fig 5). Recently, Zbigniew et al. (2011) studied co-production of invertase and citric acid by employing *Yarrowia lipolytica*. Among various carbon sources, the highest yield of invertase was obtained from glycerol. While, pomegranate peels waste was suggested as substrate for production of invertase from *Cladosporium cladosporioides* (Uma et al. 2012).

Table - 1: Effect of carbon sources (1%) on invertase production by *Aspergillus spp* M1 grown in the basal medium at 28^0C and pH 6.0

Carbon source	Enzyme activity (U/ml)
Control	27.26
Glucose	68.23
Fructose	75.63
Sucrose	182.25
Molasses	178.96

Table - 2: Effect of nitrogen sources (0.5 %) on invertase production by *Aspergillus spp* M1 grown in the basal medium at 28^0C, pH 6.0.

Nitrogen supplementation (1%)	Invertase activity (U/ml)
Peptone	39.48
Yeast extract	80.02
Sodium nitrate	233.27
Ammonium chloride	102.30
Ammonium sulphate	145.23
Potassium nitrate	78.25

Among the nitrogen sources studied *Aspergillus spp* M1 showed maximum invertase production in presence of sodium nitrate (233.27 U/ml), while ammonium chloride and ammonium sulphate declined the invertase production. The optimum concentration of sodium nitrate was found 0.2 %. Further increase in sodium nitrate not promotes the growth and invertase production

(Fig 6). The profile of growth and invertase production represented in Fig 7 reveals that maximum growth and highest enzyme production was achieved at fifth day while enzyme production remains constant up to sixth day. The invertase secreted by isolate M1 appeared thermostable as it exhibits an optimum activity at 60°C considerably greater than *Penicillium perperogenu* M1 (Dhake and Patil, 2005*)* and similar to *Actinomycetes* (Kaur and Sharma, 2005). Enzyme activity shows saturation after 20 minutes incubation (Data not shown). The salts like $CdCl_2$ and $CaCl_2$ were proved to enhance the enzyme activity whereas enzyme activity was reduced in presence of $HgCl_2$ and NaCl. In contrast to *Streptomycetes* (Kaur and Sharma, 2005) no detectable activity was observed in presence of $HgCl_2$ & NaCl. The invertase activity was slightly modulated by $CoCl_2$, $MgCl_2$.

Table - 3: Effect of metal ions on invertase activity upon pre-incubation at pH 6.0, 28^{0}C for 1 h

Metal ions Concentration (5mM)	% Activity
Control	100.0
Na^+	80.21
Ca^{2+}	111.22
Mg^{2+}	98.03
Cd^{2+}	105.0
Hg^{2+}	50.09
Co^{2+}	70.10

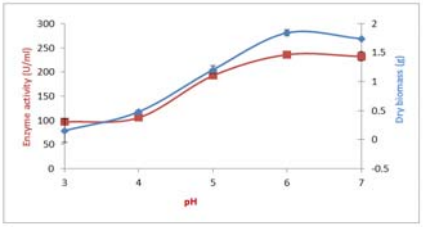

Fig. 2: Effect of pH on growth of *Aspergillus spp* M1 (——) at 28°C under static condition in nutrient medium containing (g/l): and invertase production (——).

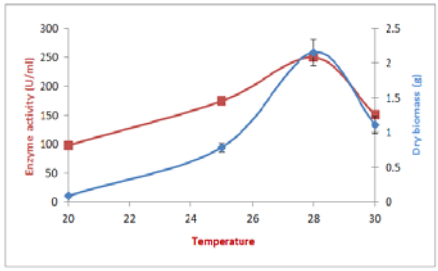

Fig. 3: Effect of temperature on growth of *Aspergillus spp* M1 (——) at pH 6.0 under static condition in nutrient medium containing (g/l): and invertase production (——).

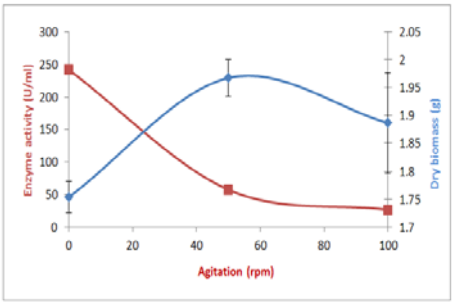

Fig. 4: Effect of agitation on growth of *Aspergillus* spp M1 (——) at pH6.0, 28°C in nutrient medium containing (g/l): and invertase production (——).

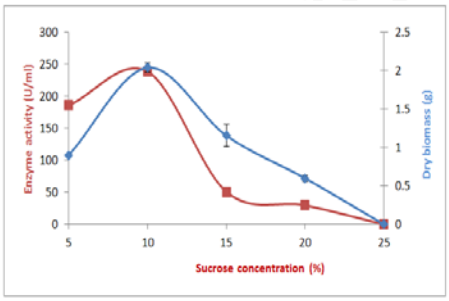

Fig.5: Effect of sucrose on growth. (——) of *Aspergillus spp* M1 at pH6.0, 28°C in nutrient medium containing (g/l): and invertase production (——).

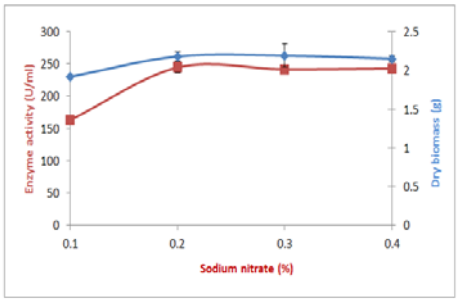

Fig.6: Effect of sodium nitrate son growth. (——) of *Aspergillus spp* M1 at pH6.0, 28°C in nutrient medium containing (g/l): and invertase production (——).

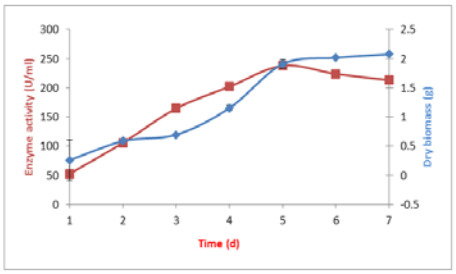

Fig.7: Profile of growth (——) of *Aspergillus spp* M1 at pH6.0, 28°C in nutrient medium containing (g/l): and invertase production (——).

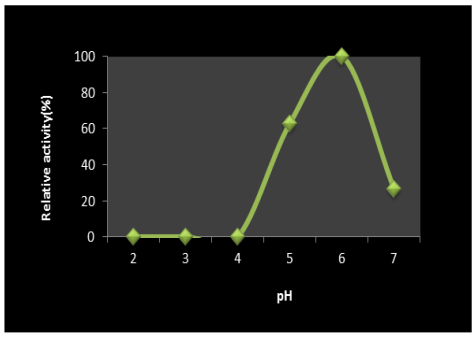

Fig.8: Effect of pH on invertase activity

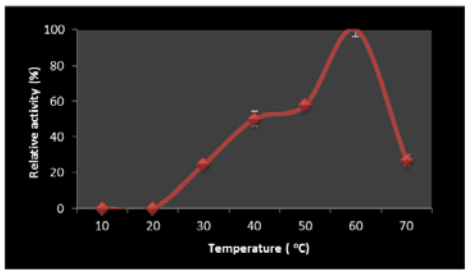

Fig.9: Effect of temperature on invertase activity

Conclusion

The study carried out to bring out a distinct characterization of thermo acidophilic invertase, secreted by newly isolated *Aspergillus spp.* Suitability of widely available molasses as a production medium was also confirmed in present investigation. The thermo-acidophillic invertase will be an ideal enzyme for various commercial applications.

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