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Optimization, purification and characterization of invertase by *Pseudomonas* sp, isolated from the cane molasse

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Abstract

A *Pseudomonas* sp was isolated from cane molasse sample. The effect of different production parameters such as pH, temperature, incubation time, carbon source, nitrogen source (organic and inorganic), on invertase production by the isolated *Pseudomonas sp* strain were studied. The enzyme production was assayed in submerged fermentation (SmF). Maximum invertase activity was found at pH 6, 40°C, 48 hours, sucrose, yeast extract. The enzyme was purified by ammonium sulfate precipitation, dialysis and DEAE cellulose column chromatography. A trial for the purification of invertase resulted in an enzyme with specific activity of 39.56, with 25.7% recovery of invertase. More over this study gives us a hint of microbial wealth of invertase production which can be harnessed for biotechnological processes.

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Introduction

Microbial invertase (EC 3.2.1.26) with transfructosylating activity can catalyse the transfructosylation of sucrose and synthesize fructooligosaccharides (Su *et al.* 1991**).** Invertase is also referred as ß fructofur-anosidaseas it catalyses hydrolysis of the terminal nonreducing residue of ß fructofuranoside **(**Gascon *et al.* 1968; Chen *et al* 1995**)**. 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 *et al.* 1982; Gehlawae 2001**).** The production of the extra cellular invertase shows a cyclic behavior that coincides with the budding cycle. The invertase activity increases during bud development and ceases at bud maturation and cell scission (Rouwenhorst *et al.* 1991**).** Fructose and glucose are competitive and noncompetitive inhibitors of the enzyme, respectively. Percentage of these monosaccharides varies with cultural conditions **(**Sayago *et al.* 2002**).** Biologically enzymes active may be extracted from living organisms like plant, animals and microorganisms. Microbes are preferred to plants and animals as sources of enzymes because of less harmful materials than plant and animal tissues. The majority of enzymes used in industrial biotechnological applications are derived from particular fungi and bacteria **(**Hussain *et al.* 2009**).** In the present study we report the isolation of *P pseudomonas* sp for the production of invertase and kinetic analysis of shake flask fermentation. Five strains of *bacteria* were isolated from cane molasses and tested for invertase activity. The effect of sucrose concentration, incubation period, initial pH and different nitrogen sources was studied.

Materials and methods

Microorganisms

Total 32 bacterial isolates were used for the production of invertase in the present study. The organism was isolated from cane molasse, cultured and maintained on the medium containing g/l sucrose 20.0; agar 20.0; peptone 5.0 and yeast extract 3.0 at pH 7.0. The cultures were stored at 4°C.

Screening for Invertase Producer

The bacterial isolates were obtained by suspending the various samples in medium containing (g/l) sucrose 4.0, di-potassium phosphate 1.0, magnesium sulfate 1.0, ammonium sulfate 3.0 (pH 7.0). All the isolates were grown at 37°C for 24h, 48h and 72h with agitation (125 rpm). Efficient invertase producers were screened out by estimating the enzyme activities and bacterial growth.

Invertase Assay

Invertase activity was assayed by measuring the amount of reducing sugars released from sucrose. The assay mixture for invertase contained enzyme extract 0.1ml and sucrose (0.9ml of 1.1% w/v) in 100 mM sodium acetate buffer (pH 7). The mixture was incubated at 60°C for 1h, and then reaction was stopped by 1 ml of dinitrosalicylic acid reagent. Amount of reducing sugars thus released was meseared (Nelson 1944**).** Finally the absorbance was read at 540 nm in spectrophotometer. One unit of invertase (IU) is defined as the amount of enzyme which liberates 1µ moles of glucose/minute/ml under the assay condition.

Bacterial 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.

Time course of invertase production

The time course of invertase production was studied in the enzyme production medium in shake flasks incubated at 37°C and pH 7 for 72 h. Samples were removed periodically at 2h,7h, 24h, 48h and 72h interval and bacterial growth as well as invertase activity in the culture supernatant were determined

Effect of pH on invertase production

The effect of optimum pH for invertase production by Pseudomonas sp was determined by culturing the bacterial in the production media with different pH.

The experiment was carried out individually at various pH such as 4, 5, 6, 7 and 8. The enzyme assay was carried out after 48 hours of incubation at 37°C (Mahendran *et al.* 2010).

Effect of Temperature on Invertase Production

Temperature plays an important role for the production of the invertase by *Pseudomonas* sp. The effect of temperature on invertase production was studied by incubating the culture media at various temperatures such as 20, 30, 40, 50 and 60°C (Shafiq *et al.* 2004**)**.

Effect of Carbon and Nitrogen Sources on Invertase Production

Various carbon sources viz sucrose, glucose, galactose, maltose, fructose, starch, and molasse were examined for optimum invertase production. Each carbon source (0.4%) was separately added in basal medium containing (g/l), ammonium sulfate 3.0, dipotassium phosphate 1.0, magnesium sulfate 1.0, pH 6.0. Similarly various nitrogen sources viz ammonium sulfate, peptone, yeast extract, and ammonium nitrate were examined for optimum invertase production. Each nitrogenous component (0.3 %) was separately added in basal medium containing (g/l) sucrose 4, di-potassium phosphate 1.0, and magnesium sulfate 1.0 at arotary speed of 125 rpm for 48 h.

Purification of invertase

-Ammonium Sulfate Fractionation and Dialysis Against Buffer (Desalting)

Pseudomonas sp cells were cultured aerobically in 250 ml production medium containing (g/l) sucrose 4.0, di-potassium phosphate 1.0, magnesium sulfate 1.0, ammonium sulfate 3.0 (pH 6.0). All the isolates were grown for 48 hours at 40°C with agitation at 125 rpm. The culture was then centrifuged at 10,000 rpm for 15 minutes at 4°C.

Solid ammonium sulfate was added to the fraction I at 20% saturation and allowed to stand for 30 min. The precipitate obtained was separated by centrifugation (Fraction II) and the resulting supernatant was further treated with solid ammonium sulfate at 40% saturation.

The precipitate obtained was collected by centrifugation (Fraction III). The supernatant was similarly treated with ammonium sulfate at 60 and 80% saturation and the precipitates obtained were termed as fractions IV, V respectively. All the precipitates (II-V) were resuspended in a minimal amount of buffer (100 mM Tris-HCl, pH 7.0) and dialyzed against the same buffer by using successive large volume of buffer. The process was continued till the last trace of ammonium sulfate was removed. The desalted fractions were used for further process. All the concentrated fractions (II-V) were subjected to protein and invertase activity assay to choose the fraction containing maximum activity.

-Ion-exchange chromatography (DEAE-Cellulose Column)

The dialyzed sample was removed from the tubing and filtered through a 0.45µm filter. Then applied to a column previously equilibrated with 100 mM Tris HCl buffer (pH 6) slowly percolating large volume of buffer through packed material. A sample of desalted enzyme preparation (fraction III, precipitate by 40 % saturation with ammonium sulfate) was loaded onto a DEAE cellulose chromatographic column. The enzyme was eluted with a enzyme characterization linear salt concentration gradient (NaCl, 0-0.5M) in the same buffer and 3ml fractions were collected and analyzed for protein, invertase activity and used for further.

Characterization of purified invertase of Pseudomonas sp

pH stability and thermostability on enzyme activity

For thermostability, the purified enzyme was incubated at 20, 30, 40, 50 and 60°C for a period of 24 hours.

After incubation, the reaction was stopped and the residual activity was determined. For pH stability, the purified enzyme was incubated in a buffer solution at different pH values, ranging from 4 to 8 at 40°C for 24 hours. After that, the residual activity was assayed.

Effect of Metal Ions on Invertase Activity

The partially purified enzyme was mixed with 1 mM concentration of various salts such as Ca ++, Mn ++, Mg ++ and Cu ++ for 1 h and subsequently invertase activity were determined. Blank was taken showing relative activity (100 %) before adding the metals

Results

Effect of various incubation time on invertase production

The effect of different kinds of incubation time was tested on invertase production.

The maximum amount of invertase production was observed in 48 hours incubation time (28.0 ± 0.23 IU/ml). The minimum amount of invertase production was obtained in 72 hours of incubation time (11.8 ± 0.32 IU IU/ml) (Table 1. Fig. 1).

Table 1. Microbial screening of selected bacterial isolates for invertase production. They were grown on mineral salt medium supplemented with sucrose (4 g/L) and incubated at 37 ± 2°C for 48h.

Organisms	Biomass	Invertase Activity mg/ml U/ml
S1	0.325	25.0
S2	0.270	20.5
S3	0.367	28.0
S4	0.282	21.5
S5	0.352	27.0
S6	0.282	21.5
S7	0.291	22.5

Cultures were grown in Erlenmeyer flasks 500ml containing 100ml of medium (pH 7) with sucrose 0.4% and ammonium sulfate 0.3% at 37°C for 48 h.

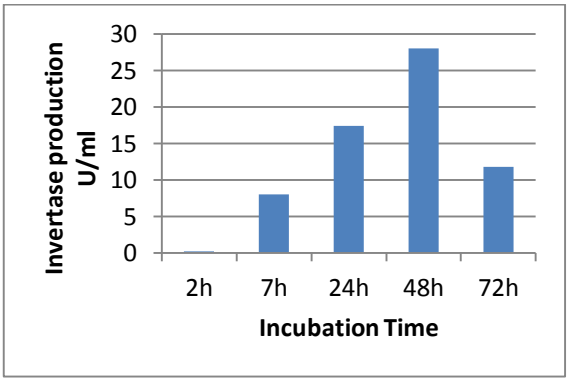

Fig. 1. Time course of the invertase production by *Pseudomonas* sp using sucrose 0.4% as carbon source.

Effect of pH on invertase production

The effectof different pHon invertase production after48 hours of incubationperiod at 37°C showed-maximum amount of invertaseproduction at pH *6.0 (30.6* ± 0.12). The minimum invertase production was recorded at pH 4.0 (12.4 ± 0.23). (Fig. 2).

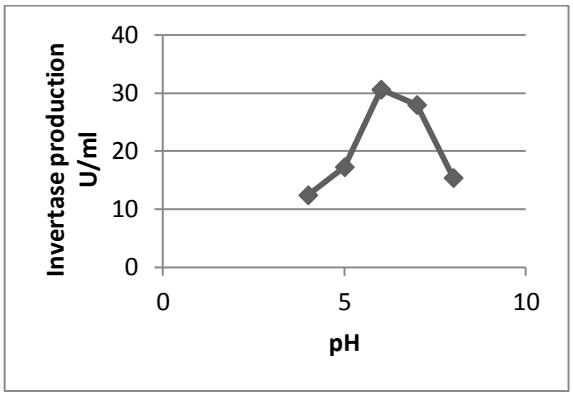

Fig. 2. Effect of different pH on the invertase production by *Pseudomonas* sp.

Effect of temperature on invertase production

Among the various temperatures tested the maximum invertase production was obtained at 40°C (30.8 ± 0.16 IU/ml). On the other hand, the minimum amount of invertase production was observed at temperature 20°C (8.22 ± 0.08 IU/ml) (Fig.3).

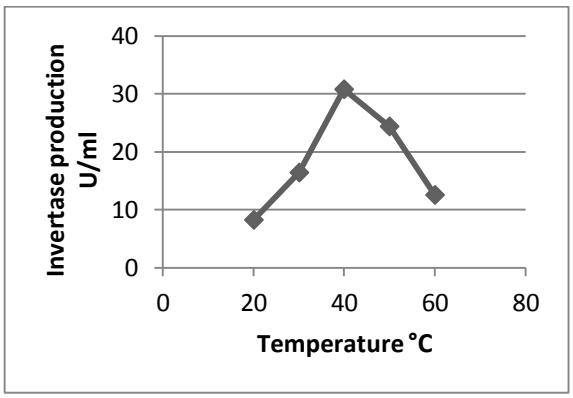

Fig. 3. Effect of different temperature on the invertase production by *Pseudomonas* sp.

Effect of carbon sources on invertase production

The effect of carbon sources on invertase production by Pseudomonas sp after 48 hours of incubation time at 40°C is given in (Fig. 4). Here the maximum invertase production was recorded in sucrose (30.7 ± 0.12 IU/ml) supplemented medium.

The minimum invertase production was recorded in maltose (14.0 ± 0.05 IU/ml) added medium.

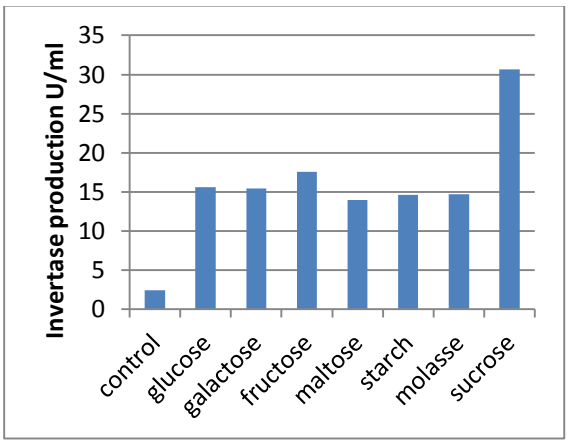

Figure 4. Effect of different carbon source on the production of invertase enzyme by *Seudomonas* sp at 40°C and pH 6 for 48 h with agitation of 125 rpm.

Effect of nitrogen sources on invertase production

The effect of different kinds of nitrogen sources on invertase production after 48 hours of incubation time at 40°C showed maximum amount of enzyme production in yeast extract (30.7 ± 0.26 IU/ml) supplemented medium and minimum amount of invertase production in ammonium nitrate (12.08 ± 0.04 IU/ml) supplemented medium (Fig.5).

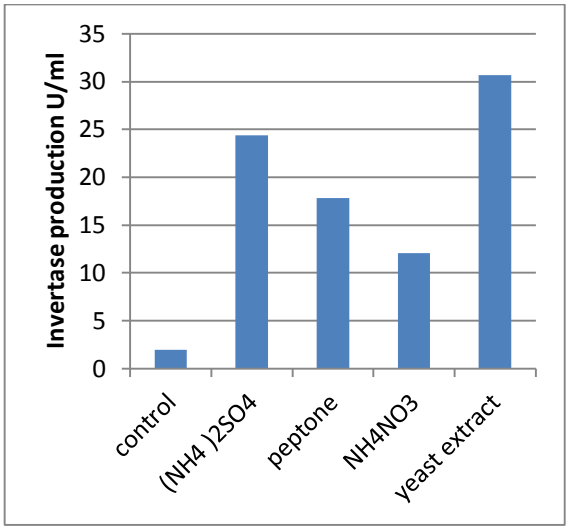

Fig. 5. Effect of different nitrogen source on the production of invertase enzyme by *Pseudomonas* sp at 40°C and pH 6 for 48 h with agitation of 125 rpm.

Purification of invertase

The crude enzyme of *Pseudomonas* sp was precipitated at 40% saturation of ammonium sulfate with 0.98 fold purification and 30% recovery with a specific activity 23.07 U/mg. After dialysis, passage from DEAE cellulose column further purifies the enzyme to 1.69 fold with 25.7% recovery (Table 2). Partially purified enzyme was used for characterization of invertase.

Table 2. Purification and recovery of invertase from *Pseudomonas* sp.

Steps	Invertase Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification (fold) (%)	Recovery
Crudextract	2800	120	23.33	1.00	100
40% $(NH_4)_2SO_4$	840	36.4	23.07	0.98	30
Dialysis	920	28.6	32.16	1.37	32.8
DEAE cellulose	720	18.2	39.56	1.69	25.7

Effect of metal ions on purified invertase activity

Among the tested metal ions, the maximum amount of enzyme relative activity was recorded in $MnSO_4$ (108%) added medium.

Followed by this, sulfate magnesium (102%) was the second best metal ions on invertase production, whereas the minimum amount of invertase activity was observed in calcium chlorure (51.4%) (Table. 3).

Table 3. Effect of metal ions on activity of Invertase of *Pseudomonas* sp.

Compound (1m mol)	Relative activity %
Control	100
$MnSO_4$	108
$MgSO_4$	102
$CuSO_4$	74.2
$CaCl_2$	51.4

pH stability and thermostability of purified enzyme activity

The stability of invertase at various pH levels is shown in (Fig 6). Invertase actiity was very stable in the pH range of 6 to 7 for 24 hours andmaximal thermo stability of the invertase activity was observed in the temperature range of 40 to 50°C (Fig 7).

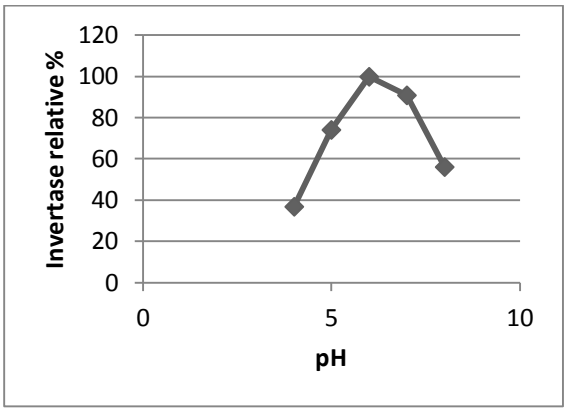

Fig. 6. Effect of pH stability on invertase activity by *Pseudomonas* sp.

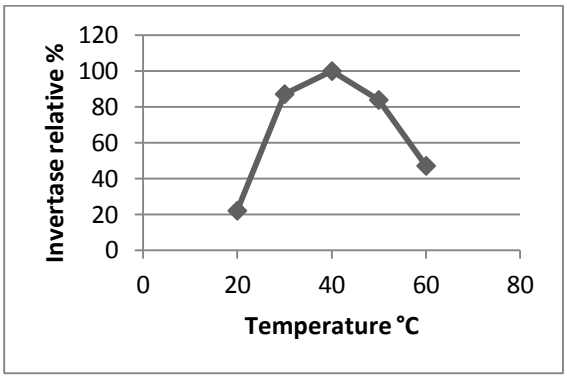

Fig. 7. Effect of the rmostability on invertase by *Pseudomonas* sp.

Discussion

Isolation screening and optimization

Among the total 32 bacterial isolates, sevenpotential strains were screened out as potential invertase producers based on their invertase activity and biomass. The strain described here was designated as S_3, gave high invertase activity among all 7 bacterial isolates. The bacterial isolate S_3 was identified as *Pseudomonas spp* based on morphological and biochemical characteristics. *Pseudomonas spp* S_3 grows in range pH 4-8 and showed an optimum growth and maximum invertase secretion at pH 6 (Fig 2).

The optimum temperature for invertase production and growth was 40°C (Fig 3). The incubation time for enzyme production is governed by the characteristics of the culture and is based on growth rate. In the present study the maximum amount of invertase production was observed at 48 hours incubation time. Invertase was not produced by the *Pseudomonas* sp at 2 hr and 7 hr of incubation time. The invertase production decreases after 48 hours (Fig 1).

It was reported that maximum invertase activity was obtained when the physical environment of thefermentation medium was optimum for 24 hours for *Streptomyces* sp. *ALKC* 8 **(**Kaur and Sharma, 2005).

Invertase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth, temperature, and the dissolved oxygen concentration. The maximum invertase production was detected when culture grown in static condition. The best carbon source for optimum growth and invertase production was sucrose 0.4 % and comparable results (30.7 ± 0.12 U/ml) were obtained by employing the fructose as carbon source (Fig 4). Similar results was reported for Carbone source by *Aspergillus flavus* Uma *et al.* (2010). Kim *et al.* (2000) also investigated that sucrose was good carbon source for invertase production by *Bacillus macerans*-EG-6.

The different organic nitrogen sources (yeast extract, peptone and ammonium nitrate) were evaluated for invertase production by *Pseudomonas* sp in comparison with ammonium sulfate (inorganic) (Fig 5). The fermentation medium was supplemented with each of these nitrogen sources at a level of 0.3 %. Among all the organic sources tested, yeast extract was observed to give the optimum invertase production (30.7 ± 0.26 µmol/min.ml) while other organic nitrogen sources yielded invertase with all liberating more than 24.4 µmol/min/ml.

Similar results was reported by Uma *et al.* **(**2010**)** who reported that among all the nitrogensources yeast extract gave maximum production of invertase activity using *Aspergillus flavus*.

These results show clearly that invertase producing bacteria are widespread in fermented date juice. The optimized growth conditions developed in this study can be used for a large scale in industrial purposes.

Purification of invertase

The invertase produced by *Pseudomonas sp* in the culture broth was subjected to a purification protocol. The total invertase activity of crude filtrate was 2800 U and its corresponding specific activity was 23.33U/mg (Table 2). Protein was precipitated by ammonium sulfate precipitation method and it was separated by centrifugation under reduced temperature. The total invertase activity of 840 U and protein content of 36.4 mg were measured in the supernatant after 40% ammonium sulfate saturation. Therefore this pellet was further dialyzed. The dialyzed pellet displayed the maximum invertase activity of 920.0 U, total protein content of 28.6 mg, specific activity of 32.16 U/mg, purification fold of 1.37 and invertase recovery of 32.8%, when compared to that of the pellet obtained in ammonium sulfate precipitation method (Table 2).

The final step of invertase purification through DEAE-cellulose column gave maximum invertase activity (720.0 U) and protein content (18.2 mg). Its corresponding specific activity was 39.56 U/mg, purification fold of invertase was 1.69 and the recovery of invertase was 25.7% (Table 2).

pH stability and thermostability of enzyme activity

The stability of invertase at various pH levels is shown in (Fig 6). Invertase was very stable in the pH range of 6 to 7 for 24 hours and it retained almost 100% of its initial activity. However, at pH 8 the enzyme lost about 56 %. Out results were in concordance with a number of authors, which reported a maximum activity of invertase at a pH stability 5 to7 (Mona and Mohamed, 2009**).**

Maximal thermo stability of the invertase was observed in the temperature range of 40 to 50°C (Fig 7). Similar result was reported at 45°C by *Torulaspora pretoriensis* (Oda and Tonomara, 1994**).** The enzyme was found to be completely stable at 30 - 50°C after 24h. At 60°C, the enzyme lost about 53% after initial activity after 24h.

Effect of metal ions on invertase activity

The effect of different metal ions on relative activity of the enzyme invertase from *Pseudomonas* sp was examined by incubating various metal ions with purified enzyme in 1mM sodium acetate buffer pH 6 at 40°C for 1 h. Only Mn++ positively modulated invertase activity, whereas enzyme activity was reduced in presence of Ca ++ and Cu ++ around 49.1% 25.8 % respectively (Table 3). similar results was reported by (Kumas and Kesavapilai, 2012**).** This result suggests that the metal ions protect the enzyme against thermal denaturation at high temperatures.

Conclusion

From the screening of invertase producing microorganisms from cane molasse samples, *Pseudomonas sp* showed the highest activity. The optimization of growth and invertase production by *Pseudomonas sp* was carried out under various conditions such as pH, temperature, period incubation, carbon source and nitrogen sources. The enzyme was purified by ammonium sulfate precipitation, dialysis and DEAE cellulose column chromatography.

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