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Characterization of Partially Purified Extracellular Thermostable Invertase by *Streptococcus sp* Isolated from the Date

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ABSTRACT
Production of extracellular invertase in submerged culture of Streptococcus sp has been investigated. Bacterial invertase producers were isolated from fermented date juice. One of the nine isolated strain exhibited a higher invertase activity was selected and identified based on their morphological and biochemical characteristics. The effect of incubation time, pH, temperature, carbon source and nitrogen source for the invertase production was studied. The invertase production was maximum at pH 8, temperature 50°C and incubation time 48 hours by the invertase producing bacteria Streptococcus sp. With a selected carbon source, sucrose 0.4 % was suitable substrate to maximize invertase production (24.4 U/ml).The effect of nitrogen source on invertase production indicated that the yeast extract was suitable substrate for accelerating invertase production (28.19 U/ml). The enzyme was purified by ammonium sulphate precipitation, dialysis and DEAE cellulose column chromatography. A trial for the purification of invertase resulted in an enzyme with specific activity of 82.35, with 43.75 % rcovery of invertase
Keywords: Invertase, Streptococcus, Optimization, Purification, date.

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INTRODUCTION

Invertase (EC: 3:2:1:26) which believed to split terminal β (2-1) fructoside linkages of sucrose, has appreciably gained importance in recent times due to its various biotechnological applications [1].
Invertase is also referred as ß-fructofuranosidaseas it catalyses hydrolysis of the terminal nonreducing residue of ß fructofuranoside [2,3]. 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 [4,1].
The β-D-fructofuranosidases catalyze the hydrolysis of the sucrose molecule to produce an equimolar mixture of D-glucose and D-fructose, known as invert sugar, which has important properties that are interesting for food and beverage [5]. In addition, some microbial β-D-fructofuranosidases are able to perform transfructosilation reaction to produce fructooligosaccharides (FOS). FOS has no considerable caloric value and can be used by diabetic people because they are not metabolized by the human organism. These saccharides also collaborate to reduce the cholesterol and triglycerides levels, and they are beneficial for intestine microorganism [6].
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 [7]. The objective of the present work was to partially purified, characterize and determine optimum production parameters for the invertase production from newly isolated.

MATERIALS AND METHODS

Microorganism

Total 24 bacterial isolates were screened out as invertase producer from several locations. The bacterial isolate used in present investigation designated as S8, was obtained from fermented date juice (Fruit of date, Gars variety). It was identified as *Streptococcus sp* based on morphological and biochemical properties using API 20 NE.

2. 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 sulphate 1.0, and ammonium sulphate 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 at 48 h.

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 [8]. 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.

Optimization of Physical Parameters for Invertase Production

The optimization of growth and invertase production by strain S8 isolate was carried out under various physical conditions viz pH, temperature and period incubation.

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,8h, 24h, 48h and 72h interval and bacterial growth as well as invertase activity in the culture supernatant were determined.

Effect of pH and incubation temperature on invertase production

The effect of pH on growth and invertase production were examined by growing strain S8 in production medium containing (g/l) sucrose 4, ammonium sulphate 3.0, di-potassium phosphate 1.0, and magnesium sulphate 1.0, for 48 h with varying pH (4 – 8). The effect of temperature on growth and invertase secretion were examined by growing the strain in production medium at various temperatures (20 – 70 °C) at a rotary speed of 125 rpm for 48 h.

Optimization of Media Ingredients for 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 sulphate 3.0, di-potassium phosphate 1.0, magnesium sulphate 1.0, pH 7.0. Similarly various nitrogen sources viz ammonium sulphate, 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 sulphate 1.0 at a rotary speed of 125 rpm for 48 h.

Purification of invertase

The extracellular crude extract was precipitated by 70 % saturation with ammonium sulphate and then dialyzed against 50 mM Tris HCl buffer pH 7 for 24 h at 40°C. The filtrate was loaded onto a DEAE-cellulose chromatographic column equilibrated with Tris HCl buffer, 50 mM, and pH 7. The enzyme was eluted with a linear salt concentration gradient (NaCl, 0-0. 5 M) in the same buffer and 3.0 ml fractions were collected at a flow rate of 20ml per hour and used for biochemical characterization.

Characterization of purified invertase of Streptococcus sp

Effect of Temperature on Enzyme Activity and its Stability

For thermostability, the purified enzyme was incubated at 20, 30, 40, 50, 60 and 70 °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 50 °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 AND DISCUSSION

Isolation and screening

Among the total 24 bacterial isolates, 9 potential strains were screened out as potential invertase producers. The bacterial isolate which showed invertase activity was screened for invertase production in the screening medium. In the screening medium the isolate *Streptococcus sp* shows maximum invertase production which produced 19.15U ml-1 was selected for further research (Table 1).

Identification

The bacterial isolate which showed maximum invertase production was further characterized and identified by morphological, biochemical characteristics using API 20 NE as *Streptococcus sp*

Optimization of fermentation

Time incubation of invertase production

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 production of invertase starts only after 24 hours of incubation. Invertase was not produced by the Streptococcus p 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 the fermentation medium was optima for 24 hours for *Streptomyces sp. ALKC* 8 [9].

Effect of pH and incubation temperature on invertase production

The initial pH of the growth medium influences the rate of invertase production. It was inferred from the results that the bacteria is capable of producing invertase from the initial pH of medium from pH 4.0 to pH 8.0. The enzyme production varied considerably from 12.4U/ml to19.35U/ml. The bacteria Streptococcus sp has optimum invertase production at pH 7.0 (19.35U ml-1). However it was noted that the invertase production was declined with increase in pH from pH 7.0 to pH 8.0 (Fig 2). The effect of pH is related to the growth and metabolic activities of the organism. A change in pH affects the ionization of essential active site amino acid residues that are involved in substrate binding and catalysis. The ionization of these residues may cause distortion of the active site cleft and hence may indirectly affect enzyme activity [10].

Similar result was reported that the maximum invertase production was at pH 7 by *Mucor Geophillis EFRL 03* [11]. Temperature is a critical parameter that has to be controlled and it varies from organism to organism. Temperature influences secretion of extracellular enzymes by changing the physical properties of the cell membrane Studies conducted for the optimization of temperature shows that the bacteria produces invertase in wide range of temperature from 20 °C to 70°C.The invertase enzyme produced at different range of temperature was from 8.2 U ml-1 to 24.4U ml-1. The optimum temperature for invertase enzyme production was at 50°C (24.4 U ml-1) and the enzyme production was affected and decreased after increase of temperature above 50°C to 70°C (Fig 3).Similar result was reported that the maximum invertase production was at 55°C by *A. niger* [12].

Effect of carbon source on invertase production

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 (24.40U/ml) were obtained by employing the molasses as carbon source (Fig 4). Similar results were reported for carbone source by *Aspergillus flavus* [13].

Effect of nitrogen source on invertase production

The different organic nitrogen sources (yeast extract, peptone and ammonium nitrate) were evaluated for invertase production by Streptococcus sp in comparison with ammonium sulphate (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 (28.19 µmol/min.ml) while other organic nitrogen sources yielded invertase with all liberating more than 12.08 µmol/min/ml. Similar results was reported by [13] who reported that among all the nitrogen sources 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 *Streptococcus sp* in the culture broth was subjected to a purification protocol. The total invertase activity of crude filtrate was 4800 ± 12.4 U and its corresponding specific activity was 17.39 ± 2.1U/mg (Table 2). Protein was precipitated by ammonium sulphate precipitation method and it was separated by centrifugation under reduced temperature. The total invertase activity of 1500.0 ± 8.12

U and protein content of 80 ± 3.16 mg were measured in the supernatant after 70% ammonium sulphate saturation. Therefore this pellet was further dialyzed. The dialyzed pellet displayed the maximum invertase activity of 1750.0 ± 4.2 U, total protein content of 28 ± 1.2 mg, specific activity of 62.5 ± 3.62 U/mg, purification fold of 3.59 ± 0.42 and invertase recovery of 36.45 %, when compared to that of the pellet obtained in ammonium sulphate precipitation method (Table 2).

The final step of invertase purification through DEAE-cellulose column gave maximum invertase activity (2100.0 ± 6.32 U) and protein content (25.5 ± 0.8 mg). Its corresponding specific activity was 82.35 ± 4.24 U/mg, purification fold of invertase was 4.7 ± 0.8, and the recovery of invertase was 43.75 ± 5.16 % (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 [14]. 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* [15].The enzyme was found to be completely stable at 40 -50°C after 24h. At 60°C, the enzyme lost about 45% after initial activity after 24h.

Effect of metal ions on invertase activity

The effect of different metal ions on activity of the enzyme invertase from Streptococcus sp was examined by incubating various metal ions with purified enzyme in 1mM sodium acetate buffer pH 7 at 50°C for 1 h. Only Mn++ positively modulated invertase activity, whereas enzyme activity was reduced in presence of Ca++ and Cu++ around 42.4 % 54.2 % respectively (Table 3).similar results was reported by [16].

This result suggests that the metal ions protect the enzyme against thermal denaturation at high temperatures.

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 mg/ml	Invertase Activity U/ml
S1	0.251	14.0
S2	0.258	13.5
S3	0.263	14.0
S4	0.094	10
S5	0.276	15.0
S6	0.263	14.0
S7	0.269	14.5
S8	0.364	19.15
S9	0.181	8.0

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

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

Steps	Invertase. Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification (fold) (%)	Recovery
Crud extract	4800 ± 12.4	276 ± 3.4	17.39 ± 2.1	1.00	100
70% $(NH_4)_2SO_4$	1500 ± 8.12	80 ± 3.16	18.75 ± 2.4	1.07 ± 0.06	31.25±2.3
Dialysis	1750± 4.2	28 ± 1.2	62.5 ± 3.62	3.59 ± 0.42	36.45±3.24
DEAE cellulose	2100 ±6.32	25.5 ± 0.8	82.35±4.24	4.73 ± 0.8	43.75±5.16

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

Compound (1m mol)	Relative activity %
Control	100
MnCl2	118
CaCl2	42.4
MgCl2	102
CuCl2	54

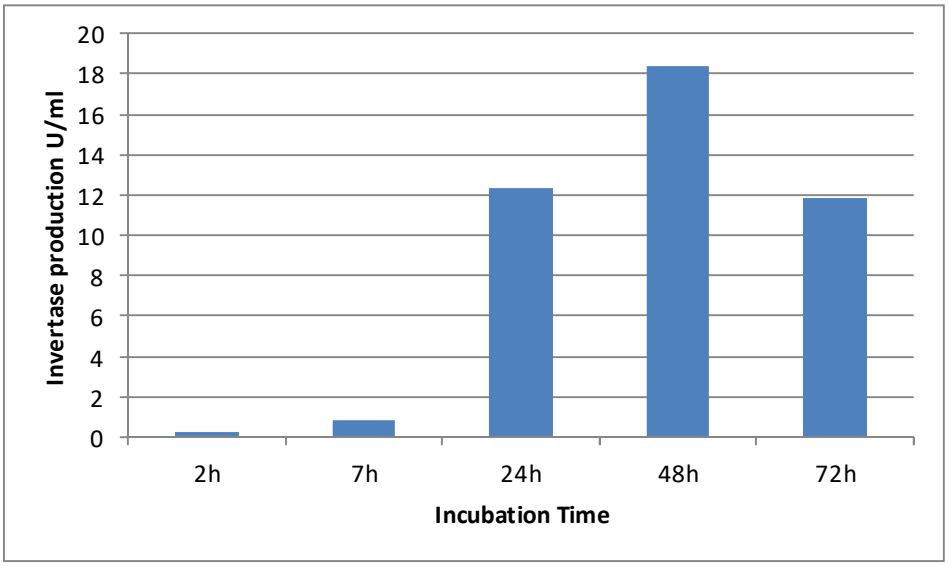

Figure 1. Time course of the invertase production by *Streptococcus sp* using sucrose 0.4% as carbon source

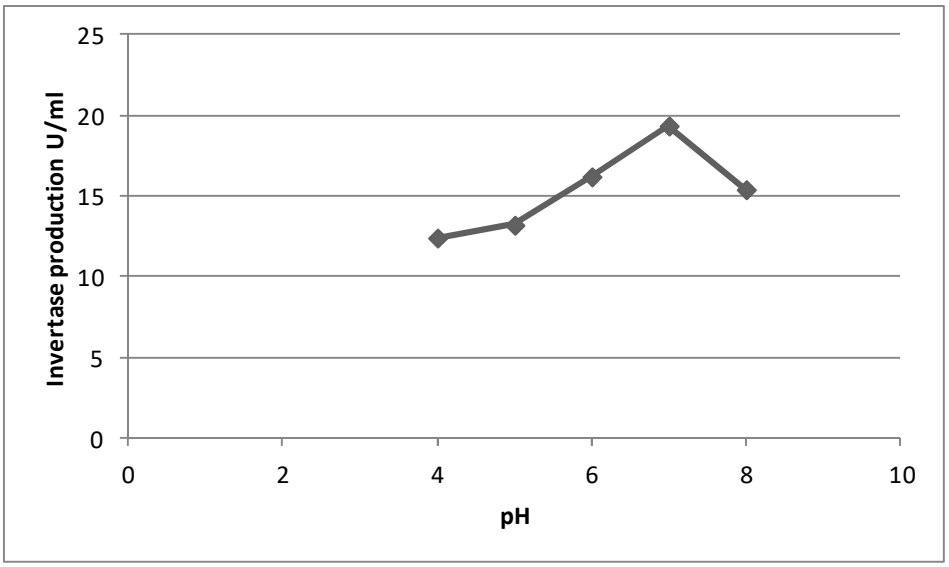

Figure 2. Effect of different pH on the invertase production by *Streptococcus sp*

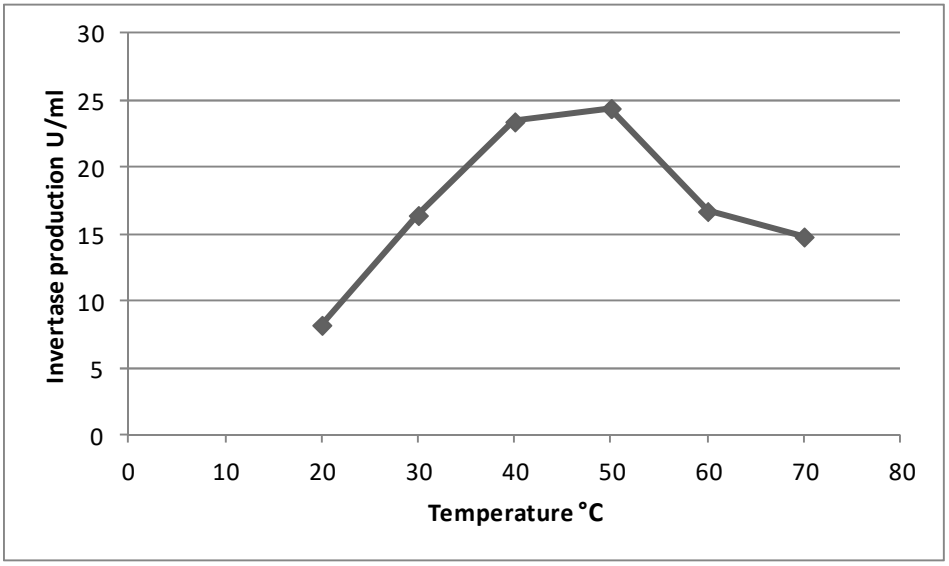

Figure 3. Effect of different temperature on the invertase production by *Streptococcus sp*

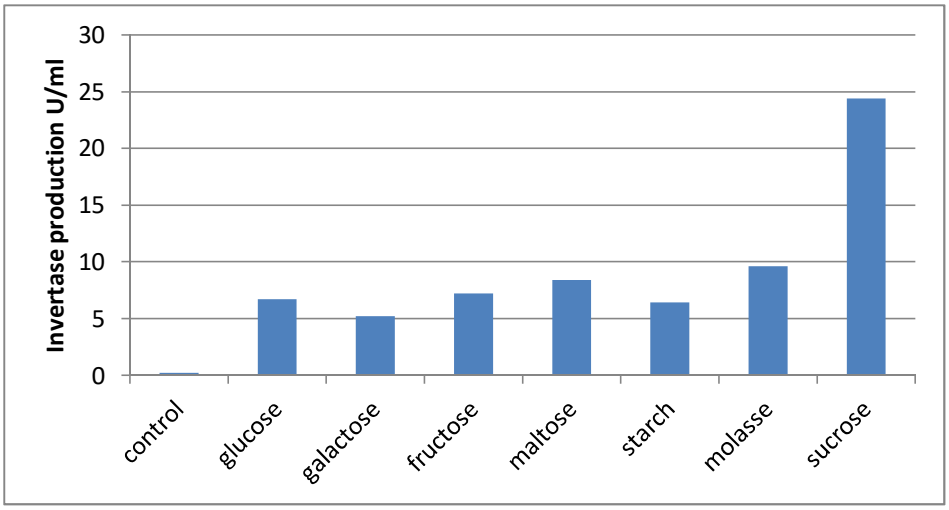

Figure 4. Effect of different carbon source on the production of invertase enzyme by *Streptococcus sp* at 50°C and pH 7 for 48 h with agitation of 125 rpm

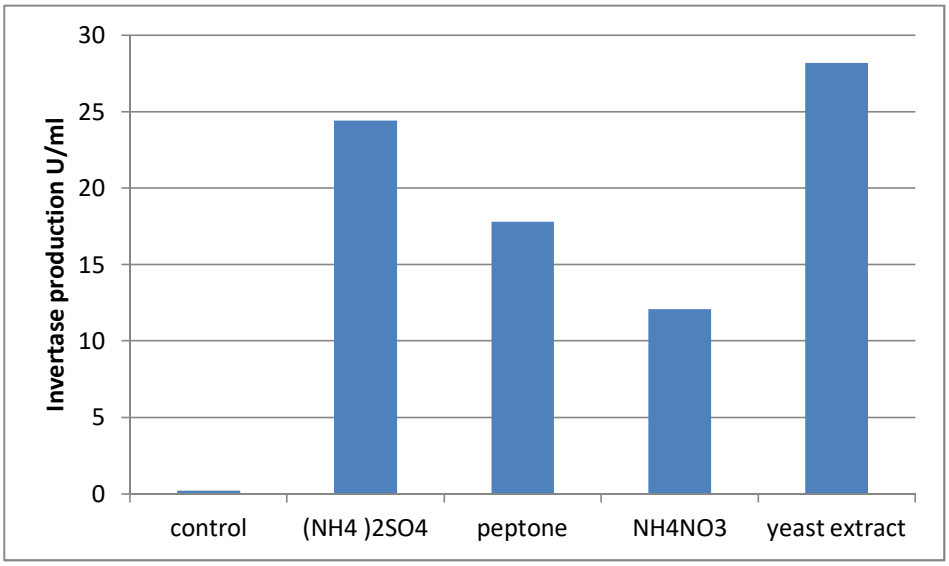

Figure 5. Effect of different nitrogen source on the production of invertase enzyme by *Streptococcus sp* at 50°C and pH 7 for 48 h with agitation of 125 rpm

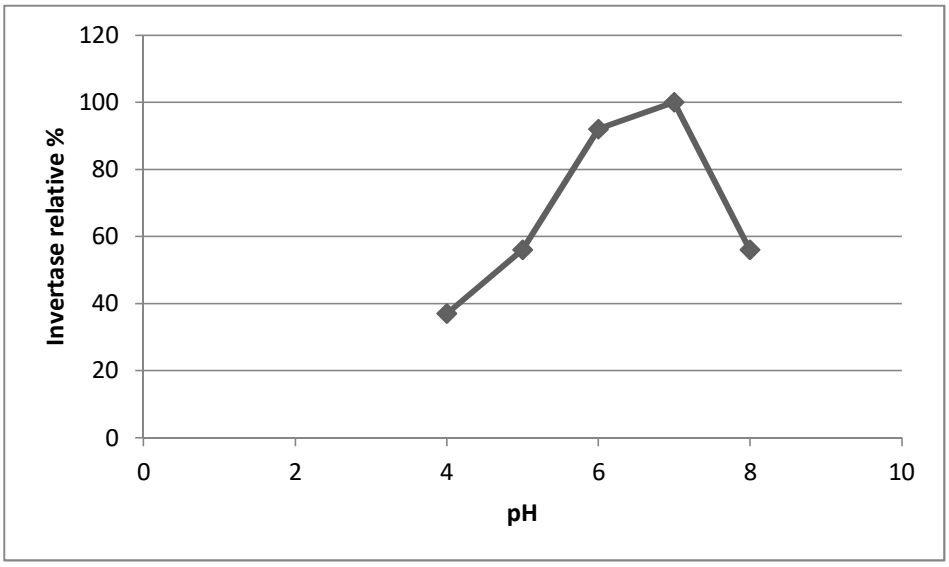

Figure 6. Effect of pH stability on invertase activity by *Streptococcus sp*

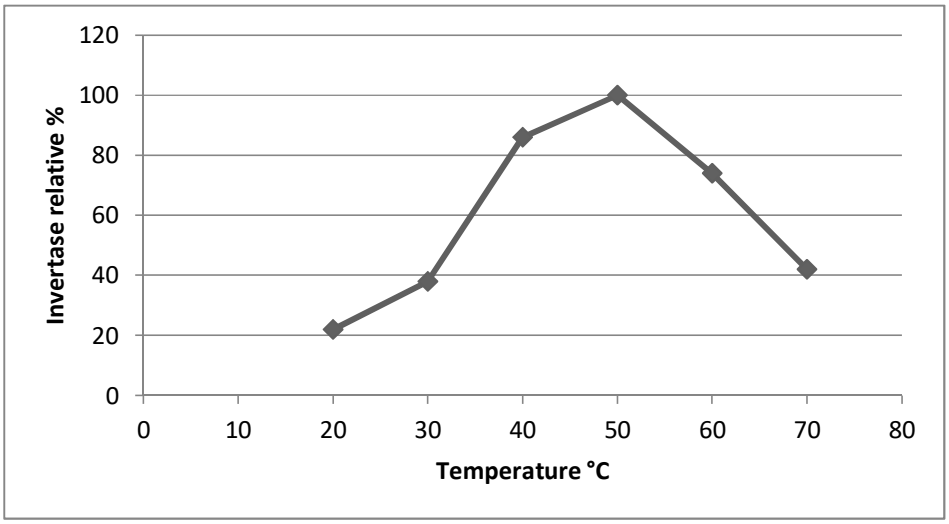

Figure 7. Effect of thermostability on invertase by *Streptococcus sp*

CONCLUSION

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

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