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ISOLATION, CHARACTERIZATION AND STRAIN IMPROVEMENT OF SOIL BACTERIA *Bacillus Subtilis* FOR INVERTASE PRODUCTION

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Enzyme invertase used to hydrolyse sucrose is used in many industries preparing drink, confectionary, bakery, invert sugar, fructose syrup, calf feed and food for honeybees along with several other applications. The extensive uses of the enzyme require their new sources which can tolerate the severe industrial processes. The invertase of improved features to work in extreme industrial process is need of the time. The present investigation deals with isolation of soil bacteria from soil having history of continuous discording of fruits waste. The bacterial isolates identified as *Bacillus subtilis.* The bacteria performed well naturally further subject to strain improvement by physical and chemical mutagenesis resulted in enhanced invertase activity. The strain performs well between pH 6-11 with optimum at pH 7.

Keywords: Globular protein; invert sugar; industrial processes; recombinant invertase; genetic engineering.

1. INTRODUCTION

Carbohydrates are copiously synthesised macromolecules by different types of organisms [1]. They are important structural constituent of cell and also play regulatory role in intracellular signalling pathway. However, the most significant role is as source of energy to the cells [2]. Among many carbohydrates, sucrose is the first carbohydrate produced by organisms [3]. Sucrose is biosynthesized by an irreversible pathway involving two enzymes, sucrose phosphate synthase and sucrose phosphate phosphatase [4]. Sucrose is used as source of energy after its degradation with the help of enzyme named invertase [5]. The enzyme invertase is synthesized by plants, bacteria, fungi, as well as some animal for utilizing sucrose as energy source [6]. The activity of invertase on the sucrose produces D-glucose and D-fructose in the equimolar proportion [7]. The produced monosaccharide mixture is called invert

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sugars. The sucrose acts as primary substrates for generating starch and cellulose, as well as signalling molecules in controlling the stress [8].

Invert sugar is very prevalent in the food industry because of two reasons; first fructose in reverse sugars is sweeter as compare to sucrose second rate of crystallization in fructose is lengthy. In addition to reducing the number of sweeteners applied in the food industry, furthermore it increases the shelf life of foodstuffs [9, 10].

Invertase is capable to degrade α-1,4-glycosidic bond between D-glucose and D-fructose of sucrose and transfer αβ-D-O-fructofuranoside residue to the acceptor substrate [11]. Thus, Invertase behaves as a hydrolytic enzyme with transferase activity, mainly when sucrose concentration is high. This characteristic of invertase place it in the transferase class that known as the fructosyl-transferase [12, 13]. Besides, other oligosaccharides like ketose, raffinose, and stachyose are hydrolyzed by invertase enzyme [14].

Traditional methods to produce invert sugar of sucrose through acid hydrolysis are not economic because of several reasons like low efficiency as well as formation of undesirable products [15]. Comparatively, using living organisms through biotechnology approach are very effective because it produces variety of enzymes in a low cost methods further hydrolyse sucrose through single step [16,17]. The present investigation was conducted to screen soil bacteria for isolating efficient invertase production ability. The identified *Bacillus subtilis* resulted in efficient invertase production.

2. MATERIALS AND METHOD

2.1 Sample Collection

The invertase enzyme producing bacteria were isolated from the soil near the fruit discard sites of near fruit shop.

2.1.1 Isolation of bacteria

Soil sample (1g) was suspended in 10 ml of phosphate buffer saline having pH equivalent to the soil and shake well at rotary shaker for 10 minutes and considered as $10^{-1.}$ Subsequent serial dilutions were prepared up to 10^{-6} by inoculating 1 ml suspension in the 9ml (PBS). From each dilution 0.2 ml suspension were poured and spread well on the solidified agar plates. The plates were marked for their dilution, date and inoculum size and incubated at room temperature and for appearing bacterial colonies. The colonies were counted. The colonies differed in their shape; size, texture margin and colour were picked and streaked on fresh plates for isolating discrete colony as bacterial isolate. These bacterial isolates were screened for their invertase production ability in media having only sucrose as the source of carbon and energy.

2.1.2 DNS assay

Compound 3, 5-dinitro salicylic acid (DNSA), is used for the estimation of sugar having presence of carbonyl(C=O) group. The aldehyde functional of glucose and ketone group of fructose reduces DNSA to 3-amino 5-nitro salicylic acid (ANSA) a reddish brown colour resin under alkaline condition is estimated by measuring optical density of solution at 540 nm using UV visible spectrophotometer. The enzyme activity was estimated based on the invertase released and incubation time.

2.1.2.1 Lowery protein assay

The protein content can be estimated by several methods. One of the commonly used methods is Lowery protein assay. In this assay, copper binds with the peptide bonds under alkaline conditions, the monovalent copper ion react with the Folin reagent, to a coloured blue colour complex which is measured by spectrophotometer for estimating protein content.

2.2 Improvement of Bacterial Strain for Invertase Production

2.2.1 Physical Mutation by UV light treatment

Bacterial isolate containing plates were exposed to UV-A radiation for duration up to 10 minutes and effect of UV exposure was evaluated on invertase production. In solidified nutrient agar plates were inoculated with 20 µl inoculums and properly spread over plate already marked as control, 2min, 4min, 6min, 8min, 10min for UV treatment. The plates were placed under UV light and plates were removed from UV exposure after the time marked on it. These plates after desired treatment of UV light incubate at 37ºC observed at 24 hrs. and 48 hrs.

2.2.2 Chemical mutation by Ethidium Bromide (EtBr) treatment

The UV –Exposed cells were further treated with various concentration and time to Ethidium Bromide treatment. The mutated bacterial isolates were observed for their invertase production.

2.2.3 Strain improvement by Ethidium Bromide treatment

Culture tubes containing nutrient broth (3ml) and marked as control, 1µg/ml, 2µg/ml, 3µg/ml, 4µg/ml, 5µg /ml, were autoclave and cooled. The tubes were removed definite volume of broth and calculated amount of Ethidium Bromide added to it and mix well followed by addition of 20µl of bacterial culture inoculation in each test tube. The tubes were incubated for 24hrs. Nutrient agar plates were prepared and 20µl inoculum was spread on the plates marked similar to the inoculum source culture tube. Bacterial colonies appeared in the plates.

2.2.4 Identification of bacterial strain

The bacterial strain was subjected to series of biochemical test for their identification including *Gram Staining*.

2.2.4.1 Glucose fermentation test

Sugar are metabolised to different metabolic pathway depending on types of microbial species and aerobic and anaerobic environment. With fermenting bacteria are grown in a liquid culture medium containing the carbohydrates they may produce organic acid as by product of the Fermentation. These acids are released into the medium and so lower pH of the medium. If a pH indicator such as phenol red included in the medium, the acids production will change from its original colour to yellow.

2.2.5 Optimization of temperature and pH

Four sterilized test tube containing 7 ml of nutrient broth and marked as pH 5, 7, 9 and11 were inoculated with equal volume of culture and incubated for 24 hrs. followed by OD measurement for evaluating effect of pH whereas optimum temperature was determined by growing the bacteria at optimum pH at different temperatures such as 22ºC, 28ºC, 37ºC and 50ºC, and observing the growth pattern on nutrient agar plate.

2.2.6 Bacterial growth kinetics

In 200 ml of nutrient broth, 1000 µl of fresh inoculums was added to each flask at pH 5, 7 and 11 and OD_{540} recorded at every 24h interval till reaching decline phase.

2.2.7 Optimization of production media for maximum yield

The production media is inoculated with the culture and growth absorbed by the calculating O.D. for the good production the best condition for the growth are identified and the production media is preferred. Different modification was done in the nitrogen sources, concentration of nitrogen sources etc.

2.2.8 Effect of nitrogen source

The 20 ml production media with beef extract as main nitrogen source was modified by using different nitrogen sources (peptone, beef extract , yeast extract and ammonium chloride and urea) modified media (20ml) was prepared, autoclaved and inoculated with 20µl of bacterial culture. The flasks were incubated at orbital shaker for 48 hours. After incubation the enzyme assay was done and best nitrogen source is identified.

2.2.9 Effect of different substrate concentration

In order to find the most appropriate concentration of the starch it was varied for our culture. 8 flasks with 20ml production media with different concentration of starch were prepared and autoclaved. All media were inoculated with 20µl of culture and incubated at room temperature for 48 hours. After incubation, enzyme assay was done and the O.D. was compared with graph to find the best concentration of starch.

2.2.10 Batch fermentation and extraction of crude enzyme

Batch fermentation experiment was carried out in a flask containing 100ml optimized production media inoculated with 1ml of culture in the sterile condition and placed over incubator shaker at 150 rpm at room temperature for 4 days. The 1ml culture media was transferred to the Eppendorf tubes and centrifuged (5000rpm for 5 min at 4ºC). The 0.5 ml of the supernatant enzyme was added in the test tube, added 1 ml distilled water added 0.5 ml of sucrose to it and incubated at room temperature for 15 min. After incubation, 1 ml of DNS was added to all test tubes. Test tube was boils for 15 min in water bath.5 ml distilled water was added to the all test tube.

2.2.11 Dialysis of the salt precipitated protein

The dialysis bags were pre-treated to remove the cellulose acetate jelly. The bag was boiled in 100 ml of distilled water for 10 minutes followed by boiling in 0.1% SDS or 1% $NaHCO_3$ for 10 min. The salt precipitate protein is centrifuged at 10000 rpm for 10 minutes and the pellet is suspended in 20 ml of 100mM Tris buffer. Again boil it in a 100 ml beaker containing the crude enzyme (50ml) which was kept on magnetic stirrer and washed magnetic bead was droop for proper mixing under cold condition water

for 10 minutes. The dissolved pellet in 100mM Tris buffer after 1 and ½ hour was changed to ensure proper osmosis and diffusion, kept overnight at 4ºC. Next day the buffer was changed and after 1 and ½ hour the dialysis bag was opened and protein was collected in the beaker.

2.2.12 Characterization of the purified enzyme

Five test tubes were taken. Four tubes were used for test and one tube used as blank. Each tube contain 0.5 ml of 1% starch and 0.5 ml of the enzyme whereas 1ml distilled was used for the blank tube were incubated at different temperature such as room temperature, 22º C, 28°C,37 º C and 50 º C for 15 min followed by addition of 1ml of DNS before taking OD at 540nm and compared with slandered graph for estimating enzyme activity at varying all the temperature.

2.2.13 Effect of pH on enzyme activity

At 37º C tubes with different pH with substrate and enzyme were incubated for 15 min. followed by addition of 1ml DNS followed OD measurement and comparison with standard graph for estimation of enzyme activity.

2.2.14 Effect of activator and inhibitors

For testing effect of activator, 0.2 ml of activator ($MgCl_2$ or $CaCl_2$) and inhibitors (SDS and EDTA) were used.

3. RESULTS AND DISCUSSION

The bacterial isolate of sucrose waste rich biomass was identified as *Bacillus subtilis* SPCP1910 on the basis of biochemical test performed. The isolated bacterial strain performed better naturally for invertase activity. The strain *Bacillus subtilis* SPCP1910 further improved by exposing to the UV radiation for various duration. The invertase activity determined for UV treatment showed maximum activity in 6 min treatment.

3.1 Growth Curve of Isolated Bacterial Strain

The growth curve of isolated bacterial strain is depicted in the figure given below. The number of bacteria increased initially slowly for 36 h followed by exponential increase in its highest number in 72 hour. Further incubation of bacteria showed stationary phase for about 12 hour and stared decline in 96h.

3.1.1 Effect of mutagen treatment on bacterial invertase production

The isolated bacterial strain grown on the sucrose minimal medium when tested for its invertase production potential, it performed well in DNS assay. The strain subjected to further strain improvement. The strain exposed to UV radiation and treated with Ethidium Bromide and further tested for their invertase producing potential. The effect of Ethidium Bromide showed better effect in improving invertase

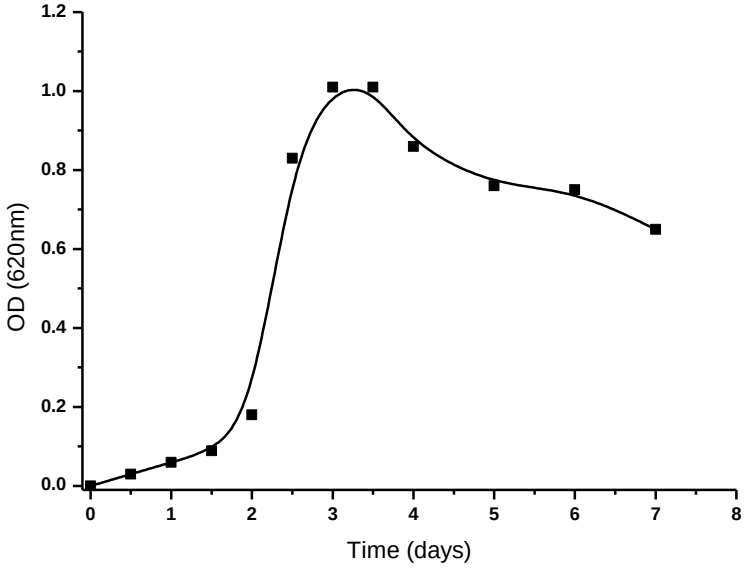

Fig. 1. Growth curve of bacteria

production compared to the UV light treatment. Based on the structural and biochemical the bacteria showed its maximum similarity to the *Bacillus subtilis*. The bacterial was grown at different temperatures showed its optimum growth at 37°C and pH9. The bacterial growth attains stationary state of growth on 3rd day.

3.1.2 Effect of C and N source

Effect of C and N addition to the culture medium has significant effect on the bacterial growth and invertase enzyme activity. The N sources tested during the study were peptone, yeast extract; beef extract and ammonium chloride whereas carbohydrate tested were maltose, lactose, sucrose and mannitol. The better enzyme activity recorded in the beef extract as generalized N source and sucrose as the carbohydrate for the activity of enzyme.

3.2 Effect of pH and Temperature on Invertase Activity

Bacterial invertases are potentially active in acidic, neutral or alkaline condition depending upon the bacteria. The bacteria able to grow in acidic medium [18,19,20], neutral and alkaline [21, 22,23] pH have been previously reported.

The invertase production by bacteria is affected by parameters like incubation period, pH and temperature. The incubation period may vary from 12h to 72 h in the temperature range 25-50 °C in the pH range 4.5-9.5., in general. In some cases of commercial invertase production have been recorded at high temperature of 80 °C as optimum for invertase synthesis using *T. neapolitana* DSM 4359T [20]. In present investigation, optimum pH was neutral for invertase activity.

Then media optimization of different physiochemical factor like pH, different metal ions, substrate concentration was checked for maximum invertase production. It was identified that the enzyme activity of EtBr mutated *B. subtilis* SPCP1910 was enhanced by modifying production media. The invertase enzyme was purified and its activity was determined. The optimum parameters required for its stability and better activity of enzyme were studied. The activity of the enzyme was quite stable in wide range of temperature ranging from 35°C to 50°C whereas activity was optimum at pH 9.

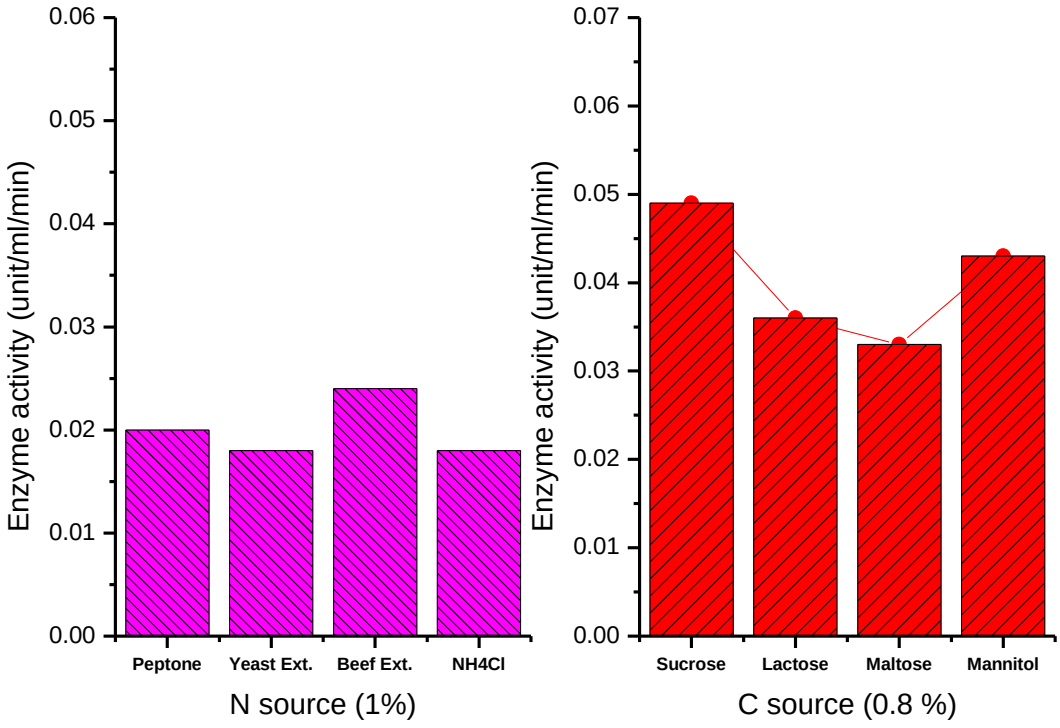

Fig. 2. Effect of carbon and nitrogen source on enzyme activity

Table 1. Optimal conditions for the bacterial invertase production

Bacteria	Incubation time	pH	T (°C)	Agitation rate (rpm)	Inoculum size	C-source (%)	N Source	Reference
Arthrobacter sp. 10137	20	-	30	250	5%	4% Sucrose	4% corn, 0.4% $(NH_4)_2HPO_4$	Xu et al. 2009 [24]
B. maccerans	48	6	30	160	8	3	0.5% peptone, 0.3% yeast extract	Ahmad et al. 2008 [25]
Bacillus cereus TA-11	36	9.5	50	100	--	1%	0.6% Yeast extract	Yoon et al. 2007 [22]
Bacillus subtilis SPCP1910	48	7	37	150	20 µl	0.8% sucrose	1% beef extract	This study

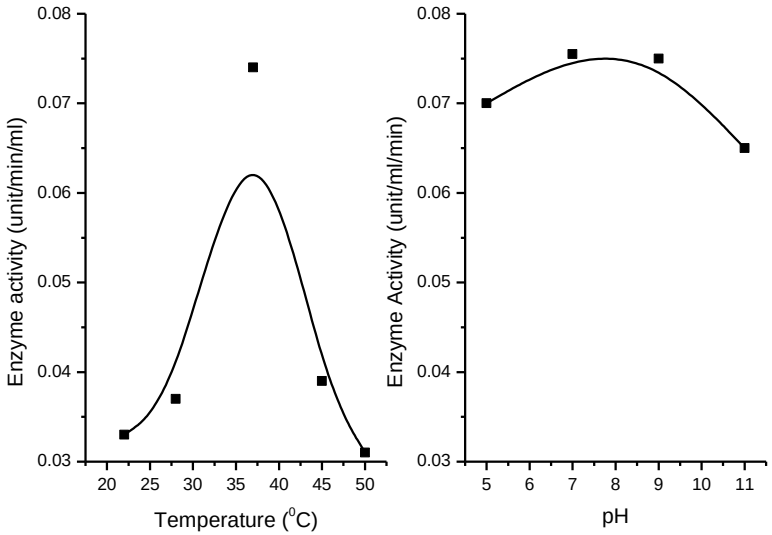

Fig. 3. Effect of pH and temperature on enzyme activity

In previous investigation activity of invertase has been shown to be maximum in medium supplemented with 1.5% sucrose (Mishra, et al, 2008). The bacteria showed optimum growth at 37 C and pH 7 in the present study. Specific activity for partially purified invertase was calculated in the present study to be 0.09 U/mg on when following submerged fermentation. The previously reported value for the enzyme is 0.06U/mg by Khan and Priya (2011) for *Bacillus subtilis whereas* specific activity of this enzyme was reported to be 1.68U/mg by these researchers. In case of *Bacillus megaterium* invertase activity was reported to be maximum at 37^0 C (Mishra, et al., 2008). In the present investigation the invertase production was quite stable in temperature range 20-50 ^{0}C making the bacterial strain more suitable for harsh industrial usage. The optimal condition for invertase production by various bacterial species has been compared in the Table 1 given below.

4. CONCLUSION

Media for submerged fermentation was optimized and fermentation was carried out. From these cultures was used for isolating crude enzyme. The enzymes were partially purified by salt precipitation and dialysis showed a specific activityof 0.09U/mg.

The enzyme characterization resulted in consistent high activity at pH 7 and temperature 37^0C. The activity of enzyme was enhanced by use of activator Ca ions whereas SDS acts as inhibitor of the enzyme. More sophisticated techniques are required to purify the enzyme such as ion exchange and affinity chromatography for better understanding the behavior presently isolated invertase from strain of *B. subtilis*. Bacterial invertases have been mainly purified using ammonium sulphate precipitation, and series of column chromatography and gel filtration followed by desalting or dialysis [22, 21,23, 18]. The estimation of molecular weight of enzyme by SDS PAGE analysis can indicate toward multiplicity of enzyme. The presently isolated invertase may be dimeric or multimeric enzyme. Earlier dimeric and multimeric invertases have been isolated from microbes. The monomeric invertases have been reported from only bacteria. It is necessary to optimize the substrate for invertase production in fermentation media because it ultimately affects production. The presently isolated strain of B. subtilis is quite efficient in terms of pH range and use of substrate for production of invertase and need further investigation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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