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Production of Levansucrase from a Soil Isolate *Bacillus Subtilis* (DQ922949)

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Abstract
Levansucrase is a fructosyl transeferase, which brings hydrolytic breakdown of sucrose to glucose and fructose. The fructose was polymerized to form levan. Levan a biologically active polymer has potential application in the field of food as a low calorie sweetener, cosmetics and in pharmaceuticals as antiviral, anti- diabetic and antitumor agent. In this context the present work brought screening of 11 soil isolates for levan production. Maximum levan producing isolate (25.8g/L) was identified by 16S rRNA as *Bacillus subtilis* (DQ922949). Maximum enzyme activity was found to be 1250 U/ml. The optimum temperature and pH for levansucrase activity were 60^{0}C and 7.0 respectively. Enzyme showed maximum activity at 0.8M sucrose concentration. Different metal ions K^+, Mg^{+2}, Fe^{+3}, Mn^+, $Hg^{+2}$and Ca^{+2} showed inhibitory effect on enzyme activity. The enzyme was partially purified by ammonium sulfate precipitation.
Keywords: Sucrose, Levansucrase, transferase, levan, *Bacillus subtilis* (DQ922949)

Introduction

Levansucrase (**EC 2.4.1.10)** is an extracellular enzyme and produced by both Gram +ve bacteria like *Bacillus sp.* **(Dedonder, 1966**), *Lactobacillus sp* (**Van Hijum *et al.*, 2001**) and Gram –ve bacteria such as *Zymomonas mobilis* (**Dawes *et al.*, 1966**), *Halomonas sp.*(**Poli *et al.*, 2009**), *Erviniamyl ovora* **(Ordax *et al.*, 2010),** *Pseudomonas syringae* (**Visnapuu *et al.*, 2011);** *Streptomyces sp* (**Carlsson *et al.*, 1970**) and *Actinomyces viscosus* (**Pabst *et al.*, 1977**). The enzyme catalyses the synthesis of polysaccharide levan (β-2, 6 linked fructan) from sucrose. Levan is a bioactive polymer with high market value. It has potential application in the field of food industry as viscofier, stabilizer, emulsifier, gelling and binding agent (**Yoo et al, 2004**); in pharmaceuticals as low calorie sweetener, antiviral **(Hermandez *et al.*, 2006),** antidiabetic (**Esawy *et al.*, 2011**) and antitumor (**Dahech I *et al*, 2011**) agent and in cosmetic industry as carrier for fragrances and surface finishing agent (**Beine *et al***. 2008 and **Shih *et al***. 2005). It has prebiotic properties and excellent water holding capacity. Hence microbial levansucrases are of high interest as biocatalyst for catalytic synthesis of levan.

Levansucrase is grouped into glycoside hydrolase 68 family due to its substrate specificity, by the data-base 'carbohydrate-active enzymes' **(Chambert *et al.*, 1976).** It catalyzes two different reactions- hydrolysis of sucrose when water is used as the acceptor and transglycosylation to form fructose polymers releasing glucose **(Ozimic *et al*, 2006**). It has ability to directly use the free energy of cleavage of non activated sucrose to transfer β-D fructosyl units to different acceptors (eg. sucrose, levan) with (β-2, 6) linkage.

The present study is aimed at screening of high levansucrase producer from soil and to study levansucrase activity by optimizing the different parameters like pH, temperature, sucrose concentration and effect of metal ions.

Materials and Methods

Screening of Levansucrase producing bacteria

Soil samples were collected from sugarcane field and the area where jaggery was prepared from sugarcane. Soil samples were inoculated in 5% and 10% sucrose containing medium. The tubes were incubated at room temperature for 24 hrs. A loop full from the tubes showing turbidity was streak inoculated on 10 % sucrose agar plates. Plates were incubated at 30^0C for 24 hrs. Organisms producing mucoid colonies were selected as levansucrase producer. The isolates were sub cultured on nutrient agar slants and incubated at 30^0C for 24 hrs .The slants were maintained and preserved at low temperature and used for further studies.

Levansucrase production

Inoculum preparation

24 hr. old cultures of all the isolates were prepared from preserved slants. A loop full of culture from 24 hr. old nutrient agar slant of each isolate was inoculated in 5 ml of inoculum medium containing (g/L) sucrose 100, yeast extract 2, $(NH_4)_2SO_4$ 1, KH_2PO_4 2, $MgSO_4$ 0.5. The inoculated medium was incubated at 30^0C for 24 hr.

Production medium

5% of inoculum was transferred aseptically to 50ml production medium in 250ml Erlenmeyer flask. The production medium had following composition (g/L) sucrose 100, yeast extract 2, $(NH_4)_2SO_4$ 3, KH_2PO_4 1, $MgSO_4$ 0.6, $MnSo_4$ 0.2.. The pH was adjusted to 7 before autoclaving. The inoculated production medium was incubated at 30^0C for 48 h on rotary shaker at 100rpm.

Extraction of Levansucrase

After incubation period the fermented broth was centrifuged at 10000 rpm for 10min at 4^0C in a cooling centrifuge. The clear supernatant was considered as crude enzyme source and used for levansucrase assay (**Senthikumar and Gunasekaran , 2005).**

Levansucrase assay

The enzyme reaction was initiated by adding 250µl enzyme extract to 250µl of 1M sucrose suspension prepared in 0.2M sodium phosphate buffer **(Senthikumar and Gunasekaran, 2005).** To this 500µl o of sodium phosphate buffer of pH 7 was added. The mixture was incubated at 40^0C for 60min. Two blank assays without the substrate or without the enzyme were prepared in tandem for the trials. All assays were run in triplicate. The concentration of released glucose was measured by dinitrosalicylic acid method **(Somogyi, 1952).**

Levan production

The clear broth obtained was then checked for presence of exopolysaccharide Levan by adding four volumes of chilled aqueous ethanol (70%). The precipitate formed was allowed to settle down at 4^0C for 24hrs. The pellet washed twice with aqueous ethanol and transferred to vial and dried at 60^0C till it was dried. **(MC-Neial and Kristiansen, 1990; Thomas and Reedhamer, 1994).**

Levan detection

2ml of 0.1N HCL was mixed with 2ml of levan solution and boiled at 100^0C for 60min. The hydrolysate obtained was analyzed for fructose and glucose estimation. Fructose estimation was carried by Resorcinol method **(Ashwell , 1957; Vikari and Gisler, 1986)** and glucose was estimated by **Somogyi** method.

TLC for levan

Acid hydrolyzed sample of levan was prepared and used for TLC. Silica Gel plates saturated with solvent 1 Butanol: 2 propanol: water: acetic acid (7:5:4:2) were used. Sample was spotted along with standards, Fructose (Hi media Pvt. Ltd.) and Glucose (Hi media). Plates were sprayed with 5% sulfuric acid in methanol, air dried and then heated at 110^0 C until spots appeared.

Identification of high levan producing strain by 16S rRNA sequencing

The high levan producing strain was identified by 16S rRNA from **ARI Pune**. Genomic DNA of isolate was isolated using Gene Elute Genomic DNA Isolation Kit (**Sigma, USA**) used as template for PCR. Each reaction mixture contained approximately 10ng of DNA; 2.5mM $MgCl_2$; 1x PCR buffer (**Bangalore Genei, Bangalore, India**); 200µM each dCTP, dGTP, dAPT, and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (**Bangalore Genei, Bangalore, India**) in a final volume of 20µl. FDD2 and RPP2 primers were

used to amplify almost entire 16S rRNA gene, as described previously (**Rawlings 1995**). The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94^0 C for 5 min; 30 cycles of 94^0 C, 60^0C and 72^0 C for 1min each; and final extension at 72^0 C for 10min, and the mixture was held at 4^0 C. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%), washed thrice using 70% ethanol and dissolved in Tris-HCL (10mM, pH 8). Samples were run on ABI Prism 3100 Genetic Analyser. The sequencing output was analyzed using the accompanying DNA Sequence Analyzer computer software (**Applied Biosystems**). The sequence was compared with **NCBI GeneBank** entries by using the **BLAST** algorithm.

Optimization of levansucrase activity

Effect of pH on levansucrase activity

Effect of pH on levansucrase activity was determined by incubating 250µl Levansucrase extract suspension to 250µl of 1M sucrose suspension prepared in 0.2M sodium phosphate buffer (**Senthikumar and Gunasekaran, 2005**). To this 500µl of sodium phosphate buffer of pH 4, 5, 6, 7, 8 and 9 were added. All the tubes were incubated at 40^0C for 60min using standard assay conditions.

Effect of temperature on levansucrase activity

Optimum temperature for levansucrase activity was determined by incubating the reaction mixture at different temperatures 10^0C, 20^0C, 30^0C, 40^0C, 50^0C, 60^0C and 70^0C at pH 5 for 60min. in 0.2M sodium phosphate buffer.

Effect of sucrose concentration on levansucrase activity

250µl of enzyme mixture was incubated with different sucrose concentrations ranging from 0.2M, 0.4M, 0.6M, 0.8M, 1.0M, 1.2M and 1.4M in 0.2M sodium phosphate buffer at pH 5, temperature 40^0 C for 60min. The glucose released was then quantified.

Effect of metal ions on levansucrase activity

Enzyme 250µl was incubated with one mmol/l solution of K^+, Mg^{+2}, Fe^{+3}, Mn^+, $Hg^{+2}$and Ca^{+2} in 0.2M sodium phosphate buffer at pH 5 was pre-incubated with the enzyme for 10min. Cations were applied as chloride salts. The reaction was started by addition of buffered sucrose. Control was incubated with distilled water.

Purification of Levansucrase:

Purification was carried by using ammonium sulfate. Different concentrations of ammonium sulfate used were 20%, 35%, 55%, and 70%, 90%. The saturated solution kept overnight at 4^0 C and precipitate obtained was collected by centrifugation at 10000 rpm for 20min.

Estimation of protein content

The protein content of the enzyme was determined by **Folin-Lowry** method (**Lowry, 1951).**

Results and Discussion

In the current study 11 isolates producing mucoid colonies on 10% sucrose agar plates were selected as levansucrase producer. All the isolates were mucoid colony producers and Gram positive in nature. **Vaidya and Prasad (2012**) and **Ghale *et al* (2007)** isolated levan producers reported similar type of colonies on sucrose agar plates. Levan production from all these isolates was carried in levan production medium. After 48hr of incubation at 100rpm, the fermented broth centrifuged and used to precipitate levan. The obtained levan dried at 60^0C till constant weight and measured **(Table-1).** Concentration of levan produced by all the isolates were above 10mg/ml. Isolate SC1, SC3, SC4, SC9, and SC12 isolated from sugarcane field produced 11.5mg/ml, 10mg/ml, 12mg/ml, 10.7mg/ml, and 13.3mg/ml levan while the isolates SJ1, SJ2, SJ3, SJ5, SJ7 and SJ9 obtained from area where jaggery was prepared produced 10.9mg/ml, 11.6 mg/ml, 14mg/ml, 15.4 mg/ml, 12.4 mg/ml and 25.8mg/ml. Levan produced by these isolates was confirmed by fructose and glucose estimation (**Table -1**). High levan producer SJ9 was selected for levansucrase production and identified by 16S rRNA sequencing as *Bacillus subtilis* (DQ922949). **Esawy *et al* (2011)** isolated six strains of *Bacillus subtilis* from honey samples and carried levansucrase production at optimum conditions, the highest levansucrase activity observed was between 62 and 59 U/ml. **Goncalves *et al*** studied levansucrase production from *Bacillus subtilis* Natto CCT7712, and reported enzyme activity 8.53 AU/ml at optimized conditions.

Table: 1 Levan production from screened iosolates

Sr. No.	Isolate	Levan (mg/ml)	Fructose (µg/ml)	Glucose (µg/ml)
1	SC1	11.5.	266	00
2	SC3	10.0	220	00
3	SC4	12.0	285	00
4	SC9	10.7	230	00
5	SC12	13.3	310	00
6	SJ1	10.9	238	00
7	SJ2	11.6	275	00
8	SJ3	14.0	345	00
9	SJ5	15.4	375	00
10	SJ7	12.4	300	00
11	SJ9	25.8	580	00

Each value represents the mean SD of 3 replications

TLC of levan

Sugar components of levan were identified by TLC analysis of levan hydrolyzate. The Rf value of acid hydrolyzed levan from *Bacillus subtilis* (DQ922949) was identical to that of standard fructose (Hi Media Pvt. Ltd.) under laboratory conditions. This result indicated that the obtained compound was levan.

Optimization of levansucrase activity:

Effect of pH on levansucrase activity

Initially at pH 4 the levansucrase activity was lowest as shown in **Fig 1**. Enzyme activity increased steadily at pH 5 and reached to more than 72% of its maximum activity, while at pH 6 activity became more than 85%; the activity reached to highest at pH 7. Beyond pH 8 it reduced markedly. Similar results were reported by **Vinity and Theertha (2012)** who observed maximum levansucrase activity from *Bacillus subtilis* BB04 in pH range of 6-7. Also **Belgith *et al* (2012)** reported optimum pH of 6.5 and **Goncalves *et al* (2013)** reported optimum pH 7.5 for levansucrase from *Bacillus* species. The current study reports activity range of levansucrase at pH (5.5 -7.3) was similar to pH range reported in literature to levansucrase from *Bacillus* species **(Homan *et al,* 2007).** Optimum pH range for most levansucrase studied was between 5.5 and 6.0. **(Yanase *et al,* 1992; Ammar *et al,* 2002)** but the enzyme reported in current study showed activity at broad pH 7 range.

Fig.1

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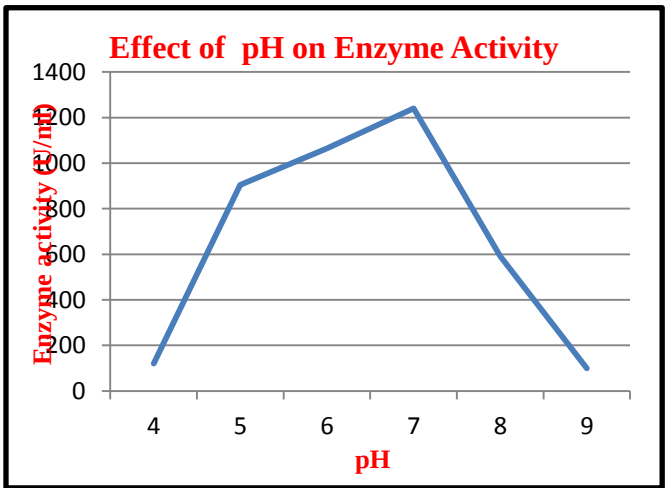

Effect of temperature on levansucrase activity

At 10^0C the activity was very low and reached to 52% of its highest activity at 20^0C. It increased gradually from 40^0C and 50^0C. The activity became highest at 60^0C **(Fig. 2)** and beyond this it falls to 58%. Similar results were reported by **Ammar *et al* (2002)** who found highest levansucrase activity at 60^0C from *Bacillus* sp TH4-2. **Goncalves *et al* (2013)** reported highest levansucrase activity of 5.38U/ml at 50^0C which found much less than the activity reported in this study. The optimum temperature for most of the levansucrase reported ranges between 30^0C to 50^0C, the current study reports enzyme activity range from 40^0C to 60^0C which allows it to work

from mesophilic to thermophilic range. Thus the enzyme works at broader temperature range as compare to other enzymes reported.

Fig.2

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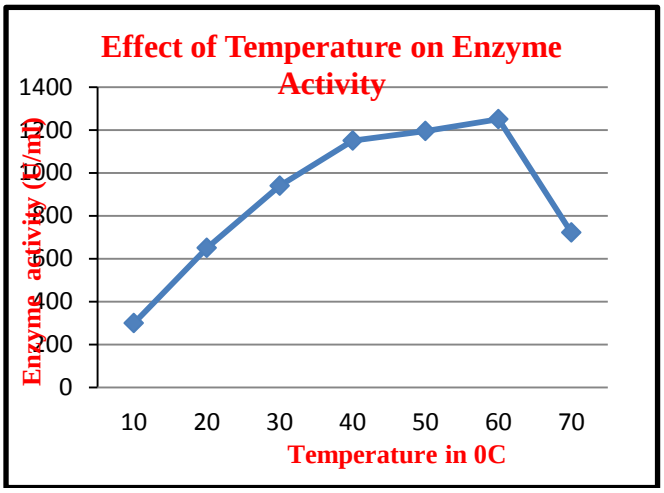
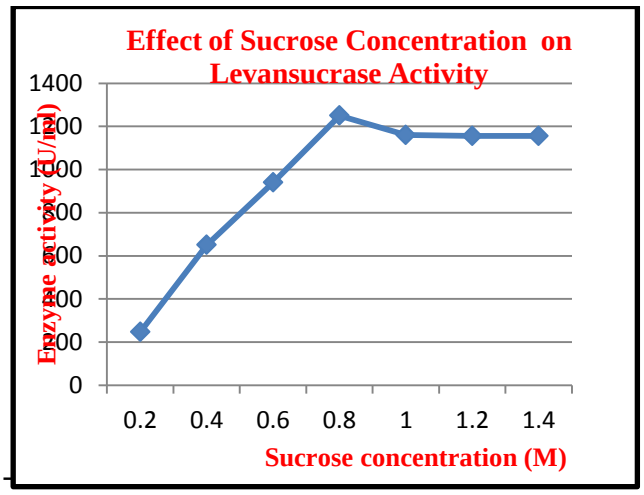


Effect of sucrose concentration on levansucrase activity

At substrate concentration of 0.2M the enzyme activity was 44% of its maximum activity; it became 76% at 0.6M and became highest at 0.8M sucrose. From **(Fig.3)** it is clear that the kinetics of levansucrase from *Bacillus subtilis* (DQ922949) follows **Michaelis - Menten** model. The **Michaelis** constant (Km) for sucrose hydrolyzing activity was determined under optimal conditions and found to be 186mM sucrose. These results was higher than that reported by **Gay *et al* (1983)** and **Homann *et al* (2007)** who studied levansucrase and reported Km values for sucrose hydrolysis was 160mM from *B. subtilis* and 4.0mM for *B. megaterium* respectively.

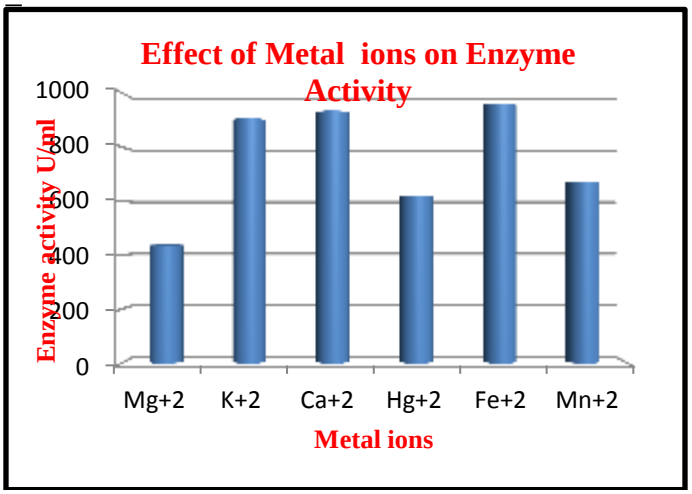
Fig-3



Effect of metal ion on levansucrase activity

As depicted in **(Fig 4)** about 75% enzyme activity was restored with both monovalent cation K^{+} and divalent cations Ca^{+2} and Fe^{+2}. While with Mn^{+2} and Hg^{+2} enzyme levansucrase restored more than 50% of its highest activity. Divalent cation Mg^{+2} inhibited levansucrase activity greatly. However the results reported by **Belgith *et al* (2012)** were different from the results of this study; the levansucrase activity from *Bacillus* species was fore fold times increased with Fe^{+}. **Vinity and Teertha (2012)** reported inhibition of enzyme activity by Fe^{+3}, Mn^{+2}, Ca^{+2} and Hg^{+2} as reported in this study. Thus the literature shows conflicting results regarding the effect of different salts on enzyme activity. This might be due to minor difference in active site composition of enzyme depending on the source.

Fig.4



Partial purification of Levansucrase:

The several protein fractions were obtained after adding ammonium sulfate. Maximum activity of enzyme was obtained in the fraction of 70% ammonium sulfate saturation. The specific activity of levansucrase in fractions obtained after 70% ammonium sulfate saturation was 126.26 U/mg. Hettwer et al (1995) obtained 153 U/mg specific activity for levansucrase obtained from *Pseudomonas syringae.*

Conclusion

Bacillus subtilis (DQ922949) found to be an efficient levansucrase producer. The optimal conditions for enzyme activity were pH 7 and temperature 60^0C with 0.8M sucrose. The enzyme activity found to be 1250 U/ml. Different metal ions like chloride salts of K^+, Mg^{+2}, Fe^{+3}, Mn^+, $Hg^{+2}$and Ca^{+2} showed inhibitory effect on levansucrase activity; Mg^{+2} found more inhibitory while K^+ was least inhibitory. The organism showed efficient levan conversion and the enzyme showed activity in broad temperature and pH range. In addition the levansucrase from *Bacillus subtilis* (DQ922949) can be considered thermostable.

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