

Research Article

**Renewable Fruit Wastes -Source for Production of Levansucrase by
SSF using *Bacillus megaterium* isolate**

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ABSTRACT:

Bacterial Levansucrase (EC 2.4.1.10) is an extracellular enzyme produced by both Gram positive as well as Gram negative bacteria. Crude enzyme from *Bacillus* sp. rich in levansucrase can be used for the synthesis of levan, hence it can be considered as a satisfactory and promising producer of levansucrases. In present study fruit waste were used for production of Levansucrase enzyme to optimize the by using isolate of *Bacillus megaterium*. Three selective fruit wastes Banana peel, Orange peel and Lemon waste as substrates for different environmental conditions and production carried out by solid state Fermentation (SSF). Enzyme unit were calculated in both purified and crude form and confirmed by GOD method. Maximum production of enzyme was after 72 hrs of incubation at pH 6 and 50°C temperature with orange peels as substrates in SSF.

Keywords: Levansucrase, *B. megaterium*, fruit wastes, SSF, GOD.

[I] INTRODUCTION

Levansucrase is a fructosyl transferase that synthesizes levan and present great biotechnological interest. Levan is a polymer of fructose and forming a non structural carbohydrate. Levansucrase conducts three characteristic reactions: synthesis of levan from sucrose by transfructosylation while releasing glucose, hydrolysis of levan to mono-saccharide of fructose, exchange of glucose in the reaction of fructose-2,1-glucose + [¹⁴C] glucose to fructose-2,1-[¹⁴C] glucose+ glucose [1]. Due to potential usefulness of Levan (produced by Levansucrase enzyme) the development of cheaper production methods for the enzyme is very crucial. Solid state

fermentation (SSF) is one of the alternative low cost and Environmental friendly approaches for production of levansucrase. As levansucrase is an extracellular enzyme and mostly produced by Gram positive bacteria like *Bacillus* sp. [2]. Levan has potential applications in field of food, cosmetic and pharmaceuticals based on its physical properties [3]. It is important that all available by-products can be moved into highly commercial outputs in order to get this renewable resource utilization like Agro industrial wastes. Solid substrates generally provide a good dwelling environment to the microbial flora comprising bacteria, yeast and fungi. SSF is an alternative for

submerged fermentation with simpler cultivation equipments, less capital investment and comparatively higher productive yield than submerged fermentation

[II] MATERIALS AND METHODS

2.1 Isolation and maintenance of microorganism

The bacterial strain was isolated and screened from garden soil of MGM College Campus Nanded, MS INDIA and maintained it on culture medium containing g/L components: peptic digest of animal tissue 5.0 g, NaCl 5.0 g, Beef extract 1.5 g, Yeast extract – 1.5g, pH - 7.4 to 7.6. Medium was autoclaved at 121°C for 15 min. The cultures were kept at 4°C and sub cultured every week. All the media components were used from HiMedia Mumbai.

2.2. Enzyme Production Media

The three substrates namely banana peel, orange peel and lemon wastes were collected from local market, shed dried and powdered it finely. Each substrate was weighed about 5.0 gm and separately added in to 25 ml production media containing g/l D/W with: Sucrose 80 g, MgSO₄.7H₂O 0.3 g, (NH₄)₂SO₄ 0.2 g, Yeast extract 2.0 g, Peptone 2.0 g, K₂HPO₄ 2.0 g, pH 7.0. The medium were autoclaved at 10 lbs pressure for 20 min. About 2 ml of isolated pure culture production strain were separately added in to three flasks and were kept for fermentation in incubator at 37 °C[1].The effect of culture conditions in the present study was carried out at different incubation periods (24, 48, 72 and 96 hrs).

2.3. Extraction and Purification of Enzyme

The extraction was conducted in 50 ml conical flask containing 1 gm of fermented biomass and 5 ml of acetone and kept for 1 h on a rotary shaker at 160 rpm at 30 °C. The crude extract was centrifuged at 10000 rpm for 20 min at 4 °C and the clear supernatant obtained was used in the enzyme assays [5]. The collected supernatant were purified by cellulose membrane cut off nominal 10,000 and dialyzed overnight against 20 mM

potassium phosphate buffer (pH -6.7) at cold conditions [2]. Protein estimation was carried out by Folin-Lowry Method [6, 7].

2.4. Chromatography

The polysaccharide produced (levan) was precipitated with double volume ethanol and indicated after acid hydrolysis (with 0.1 N HCl in a boiling water bath for 1 h) by paper chromatography. The descending technique was adopted using Whatman No.1 paper and the solvent mixture n-butanol: acetone: water (4: 5: 1). The chromatograms were developed with aniline phthalate spray [9]. (Data not shown)

2.5. Cup plate method

Sucrose at various concentrations were mixed with hot (about 85°C) agar solution (1% in 0.05 M sodium phosphate buffer, pH 6.0) and poured into Petri dish, giving a layer approx 0.8 mm thickness. Plates were allowed to solidify and then by using 1 mm diameter cork borer wells are prepared. The extracted levansucrase enzyme from different fruit waste substrates were added in to the wells separately in Petri dishes. The Petri dishes were incubated at 37 °C in incubator for various time periods [10].

2.6. Enzyme Assay

Levansucrase assay were executed by taking 0.5 ml of the enzyme solution, 0.5 ml in 0.2 M acetate buffer of pH 5.2 were incubated with 1 ml 20% sucrose solution at 30 °C for 15 min [8]. The liberated reducing sugar (glucose) were estimated using GOD method and enzyme unit were calculated by using standard formula -

Enzyme unit = concentration of product / time x concentration of protein

One unit of the enzyme was defined as the amount of the enzyme that produces 1µmole of glucose per min [1].

2.7. Effect of pH and temperature on Levansucrase activity and levan formation

Effect of pH on levansucrase activity and levan formation was determined by incubating 50µg of enzyme with buffered sucrose ranging from pH 5.0 to 9.0 (50 mmol of sodium acetate buffer- pH 5.0; sodium phosphate buffer- pH 6.0 and 7.0;

TrisCl buffer-pH 8.0 and 9.0) using the standard assay conditions. Optimum temperature for the activity and levan formation was determined by incubating reaction mixture at 4, 30, 37, 40, 50, 60, 70 and 80 °C. pH stability of levansucrase was examined by pre-incubating the enzyme for 15 min with buffered sucrose ranging from pH 5.0 to

9.0 Temperature stability of the enzyme was tested by pre-incubation for 15 min, pH 6.0 at different temperature up to 80°C and residual activity of levansucrase was measured. The levansucrase activity of the preincubated sample at 50°C was taken as 100%.

[III] RESULTS

3.1. Isolation of organism

From Morphological and biochemical tests it was confirmed the isolate as *Bacillus megaterium*.

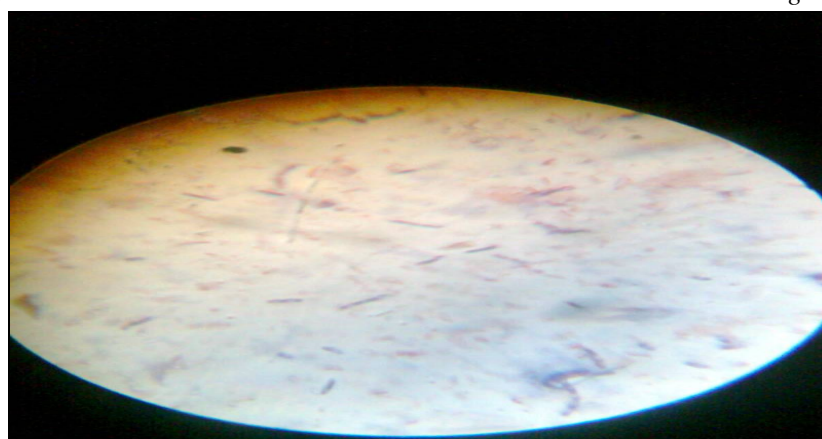
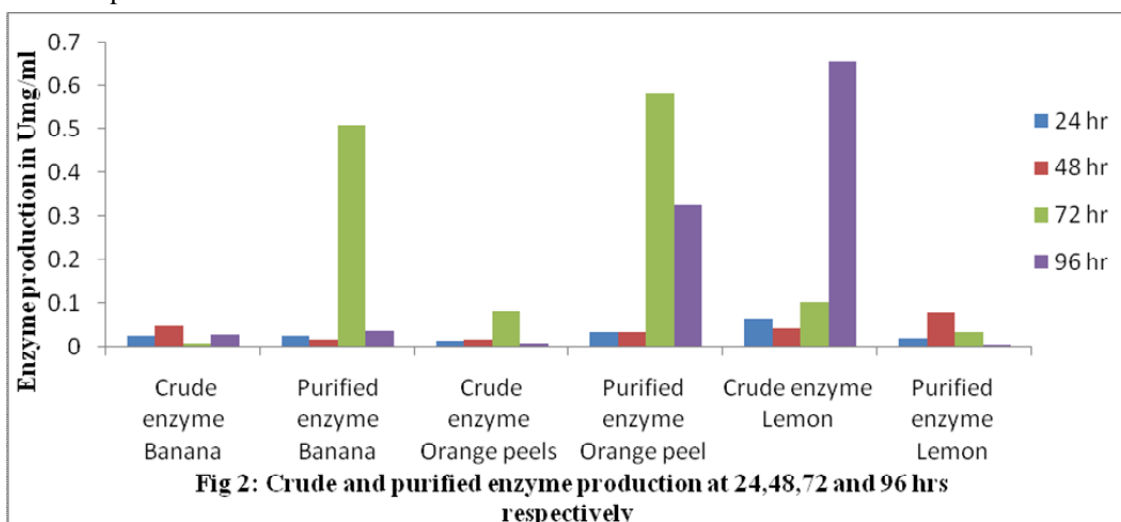


Fig.1. Gram's staining of isolated *B.megaterium*

3.2 Production

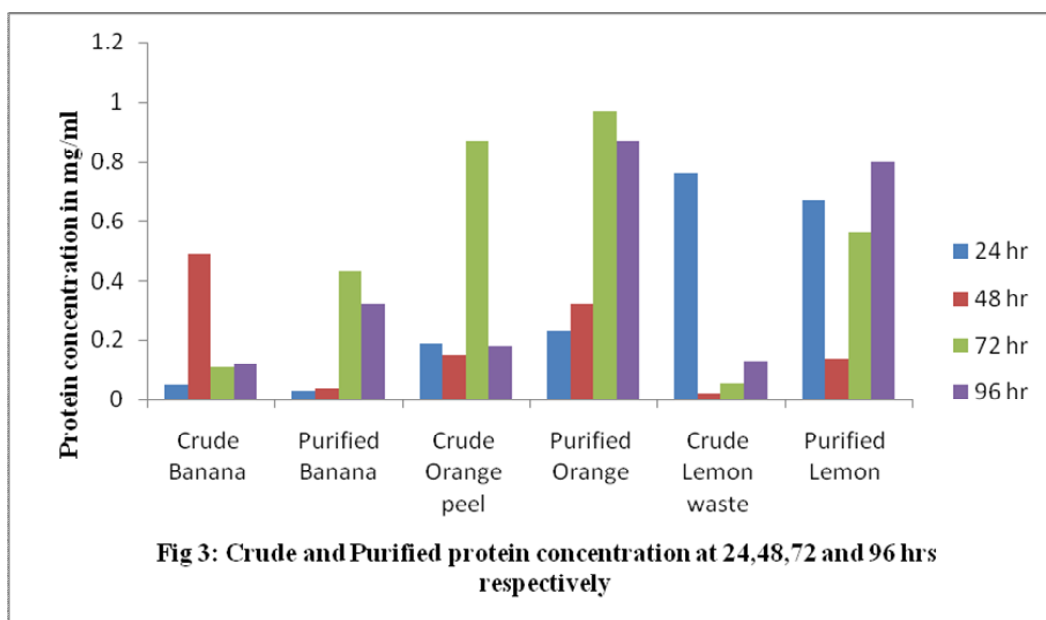
The Fermentation course and levansucrase synthesis by local isolate *Bacillus sp.* was carried out at different incubation periods (24, 48, 72 and 96 hr). The production of levansucrase enzyme were checked in both crude as well purified form.



The levansucrase production was initiated from the early exponential phase and maximal levansucrase activity was reached at 48-72 hr. thereafter the enzyme production were started to decrease. Production of enzyme was also depends upon the substrate used in the study [4].Fermentation time had a profound effect

on enzyme production. Here in purified form, Optimum enzyme production was obtained after using ideal substrate orange peel having optimum production (0.58 Umg/ml) at 72 hrs, 37 °C, pH 7. Thereafter banana peel in purified form (0.50 Umg/ml) (Fig. 2). The highest enzyme production (0.65 U mg/ml) in purified form was obtained after 96 hrs incubation period at 37 °C, pH 7 using lemon waste as a substrate. From above observations and results it reveals that –less production of enzyme was obtained by using substrates at specific time intervals it may be due to reduction in substrate porosity, changes in the structure of substrate particles, reduction of gas volume and decreasing in bacterial growth. If we consider the enzyme production in crude form the ideal substrate is orange peel waste having 0.43 Umg/ml enzyme at 72 hrs, 37 °C, pH 7 were obtained. Thereafter lemon waste produces 0.07 Umg/ml enzyme at 48 hrs, 37 °C, pH 7 and with that of banana it was 0.04 Umg/ml at 48 hrs, 37 °C, pH 7. It reveals that optimum production of levansucrase obtained at 72 hrs. by using banana peel and orange peel as a solid substrate (SS) in purified form of enzyme. While the highest level of levansucrase production was obtained at an optimum time period 72 hrs using Banana and orange peel waste as a SS. While in crude form we may say 48 hrs is the ideal period for optimum production by using banana and lemon waste peels.

The total crude protein content was estimated by Folin –Lowery method using BSA as a standard protein. Fig.3.



Here maximum protein content was observed at 72 hr. incubation in highest range with orange peels (0.97mg/ml) as a substrate in crude form, thereafter by using lemon waste it was 0.67 mg/ml at 24 hrs and with that of banana peel it was 0.49 mg /ml at 48 hrs. After purification the optimum protein content was observed with 96 hrs incubation using orange peel (0.87mg/ml) as a substrate. When Lemon peels were used as a substrate proteins content were reached to 0.67 mg/ml) at 24 hrs. Thereafter by using banana peels it was 0.43 mg/ml at 72 hrs.

3.4. Effect of pH and temperature on levansucrase activity and levan formation

As for levansucrase production is optimum with utilization of orange peel as substrate. The maximum levansucrase activity was observed at pH 6.0 to 7.0 and about 90% activity was retained at pH 9.0 in contrast to 71% at pH 5.0. Optimum yield of levan was in the pH range 6.0 to 7.0, (Fig.4). At 50°C, levansucrase activity was highest; however levan production was higher at 40°C. Levan synthesis was reduced by 32% at 60°C (Fig.5). Enzyme activity was stable in the pH range 6.0 to 7.0. *Bacillus levansucrase* retained less than 20% of its maximum activity at alkaline pH range 8.0-9.0. The enzyme was

stable at 30-50°C and showed steep decrease above 50°C.

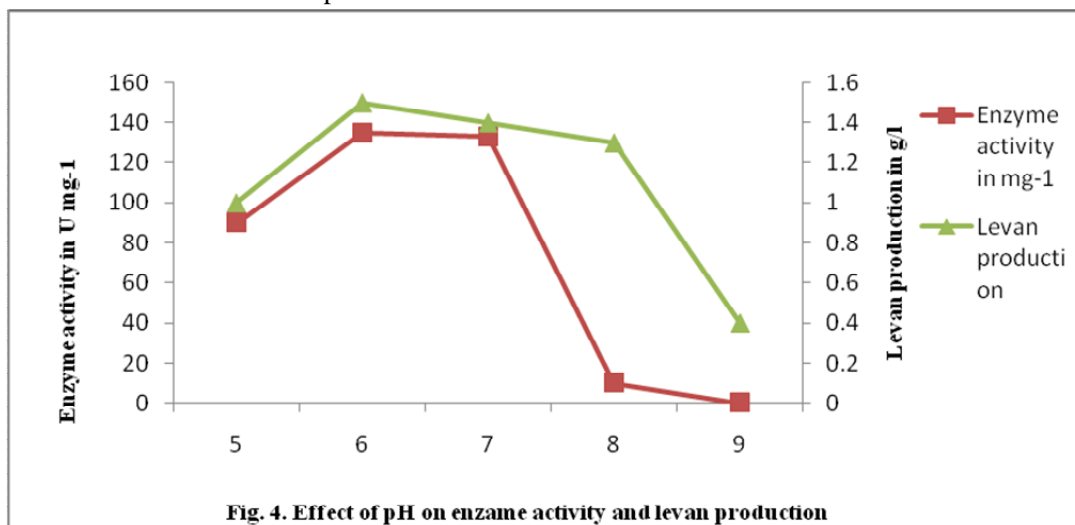


Fig. 4. Effect of pH on enzyme activity and levansucrase production

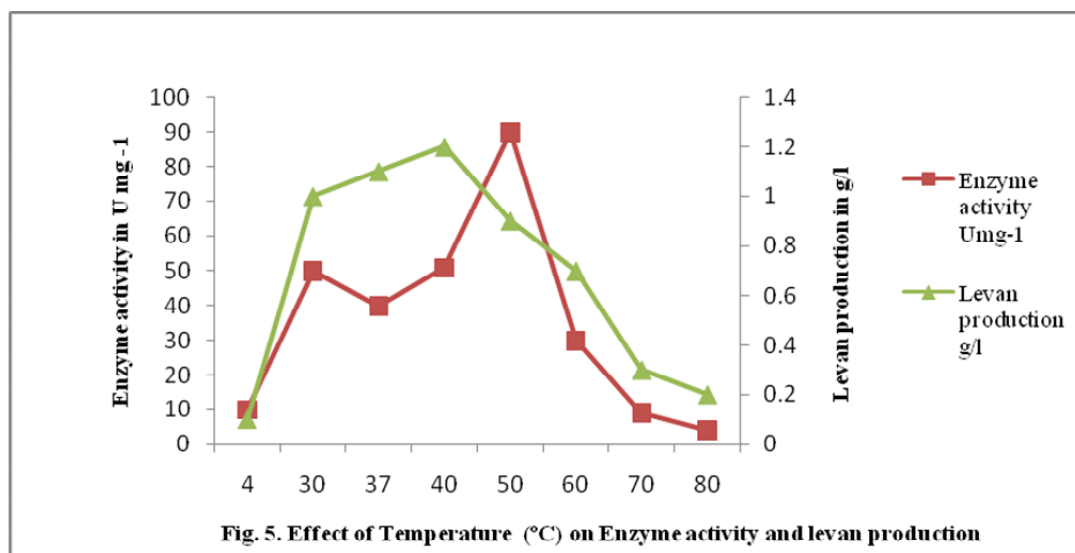


Fig. 5. Effect of Temperature (°C) on enzyme activity and levansucrase production

[IV] DISCUSSION

Due to potential usefulness of levansucrase (produced by levansucrase enzyme) the development methods for cheaper production of enzyme is very crucial. One of the alternative low cost production methods is SSF i.e. solid state fermentation and the nature of Solid Substrate (SS) is the most important factor. SS not only provide the nutrient to culture medium but also give support for the microbial cells [9]. An ideal SS gives all necessary nutrients if not it is necessary to provide externally. Important factors for any fermentation process depends upon microorganism because

each microorganism possesses a range pH for its growth and activity with an optimum value in between the range [11]. The optimum levansucrase production attained at an initial pH 6.0.

From above observations we can conclude, enzyme level declined with prolonged incubation this may be due to loss of moisture or denaturation of the enzyme is resulting from variation in pH during fermentation. As pH is among most important factors for any fermentation process and it depends upon microorganism. It may happen with purification process as we were used potassium phosphate buffer pH 6.7 got better

recovery of enzyme while in case of crude it's not may be due to fluctuation in pH at time of extraction. [12].

Moisture content of the substrate is one of the critical factors influencing the outcome of SSF and is governed by the water holding capacity of the substrate, the type of end product and the requirement of the organism. From above reflection we can say- when moisture content increased beyond a certain limit, the enzyme activity started decreasing. This happens in case of banana peel as a SS. It reveals that banana waste particle's nature is soft and has more moisture content as compared to orange and lemon, requires less time to hold water or moisture. While in case of orange peel's nature is somewhat rough or fierce ultimately it requires more time for inter particle mass transfer within the solid phase hence more time requires for moisture holding for optimum production of levansucrase at 72 hr. incubation period.

[V] CONCLUSION

The present findings reveal that all organic fruit wastes can be used as effective alternative carbon source for the production of levansucrase which is a Cost-effective and high-throughput method. We conclude that cost-saving high-throughput methods are feasible for preliminary characterization of levansucrase. Our current study provides new tools for the isolation, selection and characterization of levansucrases. Here we used Solid state fermentation for the production process having various advantages over liquid cultures; it is cost effective due to the use of simple growth and production media comprising agro-industrial residues, uses little amount of water, which consequently releases negligible or considerably less quantity of effluent, thus reducing pollution concerns. SSF processes are simple, use low volume equipment (lower cost), and are yet effective by providing high product titers (concentrated products). In conclusion remark an attempt has been made to

utilize different organic wastes for the optimum production of levansucrase.

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