Hyper-production of levansucrase from *Zymomonas mobilis* KIBGE-IB14 using submerged fermentation technique

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Abstract: The industrial utilization of enzymes requires the high yield of enzyme production for the synthesis of polymers by microorganisms. Therefore, it is necessary to optimize different production parameters of levansucrase in order to increase its industrial applications. *Zymomonas mobilis* KIBGE-IB14 was considered as a promising candidate for the large scale production of levan among wide range of microorganisms. The current investigation is aimed to optimize the production parameters of levansucrase by *Z. mobilis* KIBGE-IB14 isolated from molasses. The results indicated that bacterial growth as well as enzyme production was greatly influenced by both physical and chemical conditions. It was revealed that high enzyme titers were achieved at 30°C with pH 6.5 after 24 hours of incubation in a modified medium. Moreover, the enzyme exhibited its induction in the presence of sucrose used as a substrate. Thus, the present study demonstrated that newly isolated *Z. mobilis* KIBGE-IB14 can be used as a plausible producer of levansucrase for industrial applications.

Keywords: Fructosyltransferase, levan, sucrose catabolism, slime production.

INTRODUCTION

There are several aspects that have ability to make biopolymers superior to petrochemical-derived polymers such as biodegradability, biocompatibility as well as human and environmental compatibility (Poli *et al*., 2009). Therefore, industries have been directed to stimulate greater interest in the production of efficient and cost effective biopolymers (Song *et al*., 2000). Levan is one of the industrially important fructopolymer and potential candidate for a wide array of industrial and technological applications in different industries. It has been used as an emulsifier, stabilizer, thickener, surface finishing agent, formulation aid, encapsulating agent and carrier of flavor and fragrances (Han, 1990; Jang *et al*., 2001). Moreover, levan is promising as drug activity prolongator, plasma substitute and antihyperlipidemic agent in the field of medicine. This important polymer is synthesized by the action of microbial levansucrase (β-2, 6-fructan: D-glucose-1-fructosyltransferase E.C.2.4.1.10). It consists of linear or branched chains of fructose units connected through β (2→6) glycosidic bond (Harnandez and Banguela, 2006). Extra cellular levansucrase is produced by various microorganisms including *Zymomonas mobilis* (Chiang *et al*., 2009), *Leuconostoc mesenteroides* (Morales-arrieta *et al*., 2006), *Bacillus subtilis* (Shih *et al*., 2010), *Microbacterium laevaniformans* (Bae *et al*, 2008), *Bacillus amyloliquefaciens* (Tian *et al*, 2011), *Bacillus polymyxa* (Han and Watson, 1992), *Acetobacter xylinum* (Tajima *et al*., 1998), *Lactobacillus sanfranciscensis* (Tieking *et al*., 2004) and *Pseudomonas syringae* (Visnapuu *et al*., 2011).

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Despite the large number of microbial fructosyltransferases producers, only few of them give high yield of levansucrase due to the inefficiency of producer organisms that ultimately limit its industrial usage (Reiss and Hartmeier, 1990). Among many levansucrase producing microorganisms, Olivera, *et al*., (2007) considered *Z. mobilis* as a potential candidate for large scale production of levansucrase. Several authors have emphasized the importance of medium optimization and biochemical strategies for levan production (Jang *et al*., 2001; Ananthalakshmi *et al*., 1999). Considering the importance of *Z. mobilis* in production of levansucrase, this study was designed to optimize physico-chemical condition for hyper-production of this enzyme. The high yield of levansucrase will further expand the way to produce an industrially important polymer, levan.

MATERIALS AND METHODS

Isolation of bacterial isolate

Several bacterial strains were isolated from molasses and soil samples collected from different sugar refineries and vegetative fields of Karachi, Pakistan.

Screening of levansucrase producing bacteria

The isolates from molasses and soil samples showed morphologically different colonies after 24 hours of incubation on Yeast Peptone Dextrose (YPD) medium at 30°C. All of these contrasting colonies were further incubated for 24 hours to screen out maximum levan producing strain by slimy mucoid appearance. After purification, the selected strain was identified on the basis of morphological and biochemical analysis (Holt *et al*., 1994). This strain was further confirmed by 16S rDNA analysis.

Fermentation conditions for levansucrase production
Levansucrase production was carried out with batch fermentation technique using one variable at a time approach in stepwise manner. Different fermentation conditions were optimized for the maximum production of enzyme.

Medium selection
Several previously reported media were used for the production of crude levansucrase which include medium-1 (Ananthalakshmy and Gunasekaran. 1999), medium-2 (Schreder *et al*., 1934), medium-3 (Bekers *et al*., 2002) and medium-4 (Kirk and Doelle 1992). Medium which showed the maximum enzyme production was selected and further modified by incorporation of different ions. The composition of modified medium (medium-5) comprised of (%) sucrose, 15; peptone, 0.2; yeast extract, 1.0; dipotassium hydrogen phosphate, 1.5 and calcium chloride, 0.01. The culture was incubated at 30°C for 24 hours. All media were maintained at pH 6.5 before sterilization and autoclaved at 115°C for 20 minutes prior to inoculation.

Physical conditions
The culture medium was studied over a pH range of 5.0 to 9.0 at 1.0 unit interval adjusted by using 1N HCL and 1N NaOH. The production medium was inoculated with 10% (v/v) inoculum and incubated at 30°C for 24 hours. To observe the effect of temperature and fermentation time on levansucrase production, fermentation medium was kept at 20-60°C for different time intervals ranging from 6 to 96 hours respectively.

Chemical conditions
After optimization of physical parameters, different chemical parameters (carbon, nitrogen source and trace elements) were studied. Various substrate (sucrose) concentrations were examined for their effect on enzyme production ranging from 5 to 25% in fermentation medium. To observe the influence of nitrogen source on levansucrase production, bacterial culture was grown in fermentation medium containing different concentrations of peptone and yeast extract ranging from 0.1 to 0.5% and 0.5 to 2.0%, respectively. The effect of various salts on levansucrase production was determined by incubating the production medium with different concentrations of K_2HPO_4 and $CaCl_2.2H_2O$ ranging from 0.5 to 2.5% and 0.005 to 1.5% respectively.

Enzyme assay and total protein
After fermentation, the culture medium was centrifuged at 40248 $\times g$ for 15 minutes at 4°C and cell free filtrate (CFF) was used for levansucrase activity. The reaction mixture containing 0.5ml CFF, 1.0 ml sucrose (0.5M) and 0.5ml sodium phosphate buffer (0.1M, pH 6.0) was incubated at 37°C for 5 minutes. After incubation, the reaction was stopped by adding 0.5ml NaOH (1N) in the reaction tube. The amount of glucose released in to the medium was determined by glucose oxidase (GOD-PAP) method using glucose as standard (Trinder, 1969a, 1969b). Protein concentration was measured by Lowry's method (Lowry *et al*., 1951) using bovine serum albumin (BSA) as standard.

One unit of levansucrase activity is expressed as "amount of enzyme that catalyzes the formation of 1.0 µmol of glucose as reducing sugar per minute at 37°C in sodium phosphate buffer, pH 6.0".

RESULTS

Identification and characterization of strain
Levansucrase producing strains were isolated from different sources and grown on YPD medium. After 24 hours of incubation at 30°C, twenty-one (21) contrasting colonies were appeared on agar plate. Among them, only five (05) colonies showed slimy mucoid appearance of levan polysaccharide production. After further incubation, a colony that showed maximum levan production was selected for morphological and biochemical analysis (fig. 1a). Pure culture study of selected colony depicted that the colony was of Gram's negative short rod bacterium with catalase positive but oxidase negative test. This strain assigned NCBI GenBank accession # HM102366 and was designated as *Zymomonas mobilis* KIBGE-IB14 from 16S rDNA analysis (fig. 1b).

Selection of an optimum medium
Different reported media were used for the production of levansucrase from *Z. mobilis* KIBGE-IB14 (table 1). Maximum enzyme production has been observed in medium-4. Hence, on the basis of nutrients present in the medium-4, a new medium (i.e. medium-5) was designed by using one variable at a time to observe variation in enzyme production**.**

Effect of Incubation time
The pattern of levansucrase production and bacterial growth with reference to fermentation time period was observed by incubating *Z. mobilis* KIBGE-IB14 for different time intervals. In the present study, maximum cell concentration and enzyme production was monitored at 24 hours (fig. 2a) which clearly declared the direct relationship among time interval, microbial growth and enzyme synthesis. After 24 hours, further increase in fermentation time, microbial growth and enzyme synthesis decreased simultaneously.

Effect of temperature
In the current analysis, the optimum temperature for bacterial growth and levansucrase production was monitored by incubating *Z. mobilis* KIBGE-IB14 at different temperatures ranging from 20-60°C. It was observed that the maximum biomass yield and enzyme production was achieved at 30°C (fig. 2b).

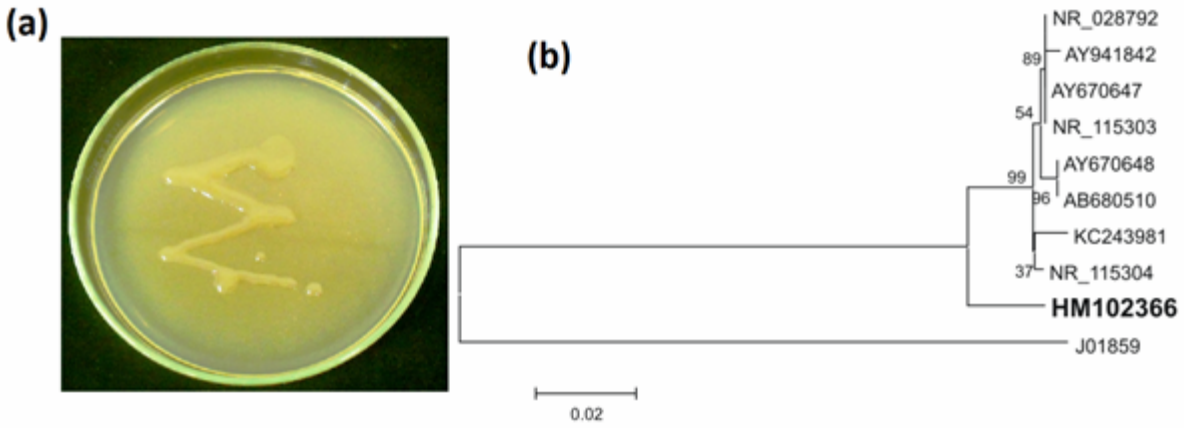

Fig. 1: Growth on agar plate with YPD medium (a) and Pylogeny of *Zymomonas mobilis* KIBGE-IB14 (b).

Table 1: Composition of different media and production of levansucrase in each medium.

S. No.	Medium components	Medium composition (%)	pH	Temperature (°C)	Enzyme Activity (U/mg)
Medium 1	Sucrose Yeast extract KH_2PO_4	5.0 1.0 0.2	6.0	30	1908
Medium 2	Sucrose Yeast extract KH_2PO_4 $MgSO_4.7H_2O$ $(NH_4)_2SO_4$	2.0 0.1 0.1 0.05 0.1	7.2	35	2890
Medium 3	Sucrose Yeast extract KH_2PO_4 $MgSO_4.7H_2O$ $(NH_4)_2SO_4$	15 0.7 0.25 0.1 0.16	4.9	30	2655
Medium 4	Sucrose Yeast extract Casein hydrolysate KH_2PO_4 $MgSO_4.7H_2O$ $(NH_4)_2SO_4$	15 0.2 0.2 0.2 0.2 0.2	6.5	30	3033
Medium 5	Sucrose Yeast extract Peptone K_2HPO_4 $CaCl_2.2H_2O$	15 1 0.2 1.5 0.01	6.5	30	3900

Effect of pH

To observe the effect of pH on cell mass and levansucrase production, *Z. mobilis* KIBGE-IB14 was incubated at different acidic and alkaline conditions (pH: 5.0-9.0). The influence of pH on the growth of bacteria and levansucrase production is depicted in fig. 4c that showed maximum production of levansucrase at pH 6.5.

Effect of Sucrose

The effect of different sucrose concentrations on final cell mass and levansucrase production has been examined (fig. 3a). The maximum enzyme production was observed at 15% of sucrose used in the medium. Afterwards, increase in sucrose concentration showed unfavorable effect on enzyme synthesis.

Effect of yeast extract

The effect of different concentrations of yeast extract on cell mass and enzyme production revealed that both microbial growth and enzyme production yield was directly proportional to the yeast extract concentration up to 1%. In the present study, maximum enzyme production was achieved with 1% of yeast extract present in the medium (fig. 3b). Moreover, further increase in yeast extract concentration caused reduction in enzyme production but increased cell growth.

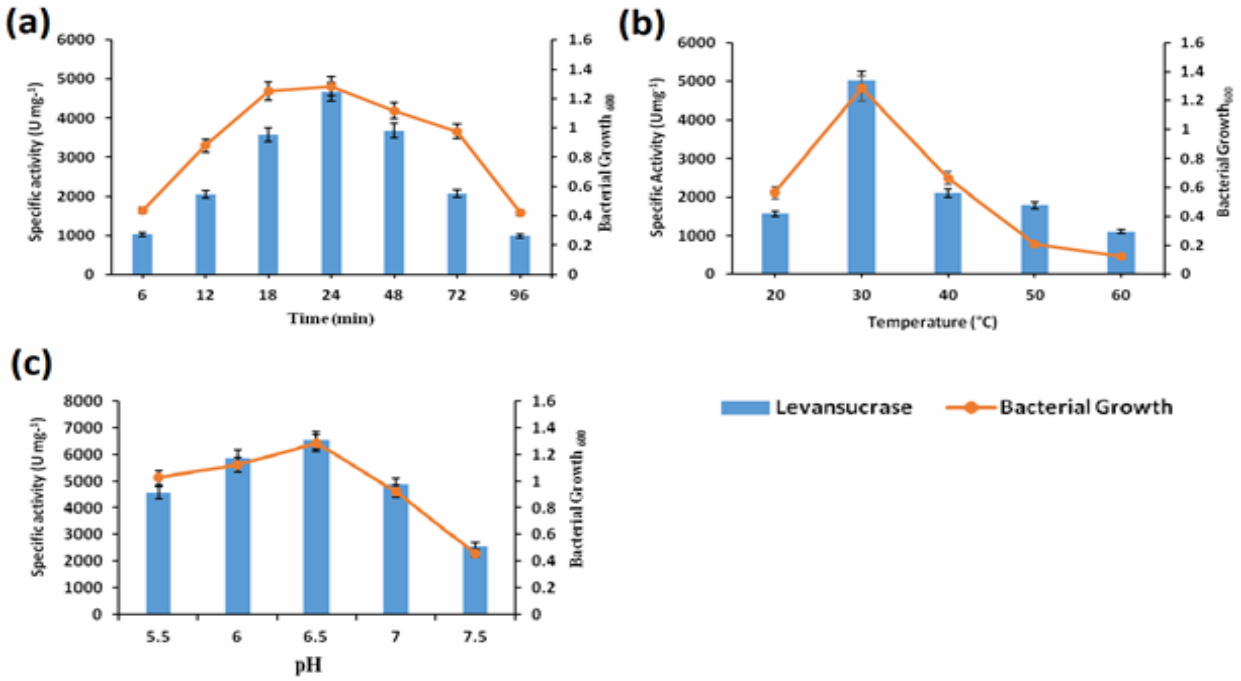

Fig. 2: Levansucrase production from *Z. mobilis* KIBGE-IB14 at various time intervals (a); different temperatures (b) and pH values (c).

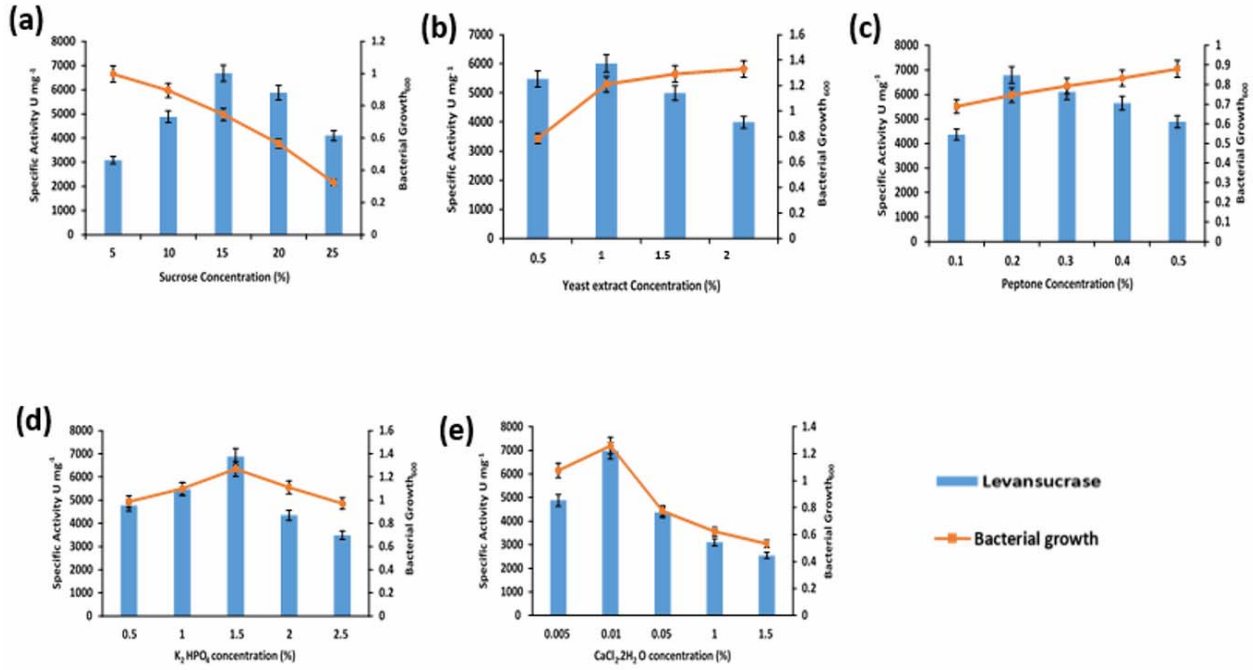

Fig. 3: Production of levansucrase from *Z. mobilis* KIBGE-IB14 at various substrate (a); yeast extract (b); peptone (c) K_2HPO_4 (d) and $CaCl_2.2H_2O$ concentrations (e).

Effect of Peptone

The influence of peptone on cell growth and levansucrase yield by using media containing different concentrations of peptone is demonstrated in fig. 3c. The finding indicated that the maximum enzyme production was achieved at 0.2% of peptone. Furthermore, the enzyme synthesis noticed to be reduced at this peptone concentration, whereas *Z. mobilis* KIBGE IB-14 continued to grow with increased concentration of peptone.

Effect of K_2HPO_4

Influence of different concentrations of K_2HPO_4 in culture medium on biomass concentration and enzyme production were observed. The present data revealed that the microbial growth as well as enzyme production increased with the increase of K_2HPO_4 concentration in medium. K_2HPO_4 (1.5%) was the most favorable concentration for the bacterial growth and production of levansucrase (fig. 3d).

Effect of $CaCl_2.2H_2O$

The influence of different concentrations of diavalent ion (Ca^{+2}) on the growth of *Z. mobilis* KIBGE-IB14 and levansucrase production was examined. The present work represents the increase in the bacterial growth and enzyme production up to the 0.01% concentration of $CaCl_2.2H_2O$. However, subsequently decrease in final cell mass and enzyme yield have been resulted beyond this concentration (fig. 3e).

DISCUSSION

It is a highly crucial step to select a suitable strain capable of producing selective enzyme concomitantly with commercially acceptable yield. The current study revealed that the most active microbe for the production of levansucrase enzyme was isolated from molasses of sugar refineries and was identified by 16S rDNA sequence analysis as *Zymomonas mobilis* KIBGE-IB14. Further standardization of culture conditions for levansucrase production was carried out by using this selected strain. The media optimization is an important aspect to be considered in the development of fermentation technology. Different physico-chemical factors directly influence the microbial growth and production of enzyme. Optimization of growth parameter has an important role in order to achieve high enzyme yield.

Fermentation time of an enzyme has a profound effect on cost of enzyme production and its product formation. The incubation period varies with enzyme production. Short incubation period offers potential for inexpensive production of enzymes (Sonjoy *et al*., 1995) as in present study where maximum incubation time period was noticed at 24 hours.

Temperature is one of the crucial factor which not only influences the metabolic rate of the organism but also determines the amount of end product. The present study recorded optimum temperature for enzyme production as well as cell growth at 30°C, which indicated the mesophilic nature of the organism. However, increase in incubation temperature thereafter resulted in lower cell growth and enzyme synthesis. Previously it has been observed that at high temperatures, large amount of metabolic heat was generated which resulted in cell mass inhibition that in turn declined the levansucrase formation (Babu and Satyanarayana, 1995). In line with current study, the temperature optima for *B. subtilis* NRC33a (Abdel-Fattah *et al*., 2005) and *Acetobacter diazotrophicus* (Hernández *et al*., 1999) were also reported at 30°C. On the contrary, Belghith *et al*., (2012) and Muro *et al*., (2000) observed maximum levansucrase production by mutant strain of *Z. mobilis* and *Bacillus* sp. at 55°C and 70 °C, respectively.

Another important cultivation parameter is pH, which directly affects microbial growth and enzyme production. In a contradistinction to the current data that showed maximum levansucrase production at pH 6.5, pH 5.0 (acidic) and pH 7.5 (alkaline) were also noticed for levanaucrase production from *Z. mobilis 113S* and *B. subtilis* Natto CCT 7712 respectively (Vigants *et al*., 2013; Gonçalves *et al*., 2013). Whereas, several other researchers reported pH 6.0 and 6.5 for the production of levansucrase from *B. subtilis* BB04 and *Bacillus* sp. respectively (Vaidya and Prasad 2012; Belghith *et al*., 2012).

Sucrose was added as a carbon source in the fermentation medium to accomplish necessary requirements of microorganism. The findings of current work exhibited that increase in sucrose concentration resulted in an enhancement of enzyme secretion while caused reduction in microbial growth and maximum enzyme production was observed at 15% of sucrose. As it has been suggested that below 10%, sucrose was mainly used for microbial growth and higher sucrose concentrations resulted in more enzyme production but cell growth inhibition (Chen and Liu., 1996). The current work disagrees with the findings of Belghith *et al*., (2012) where maximum levansucrase production from *Bacillus* sp. was observed with 30% of sucrose present in the medium.

According to Fein *et al*., (1993), the incorporation of yeast extract to the fermentation medium provides vitamins that are necessary for the growth of *Z. mobilis*. Zhang *et al*., (2014) reported the same findings for the production of levansucrase from *B. methylotrophicus* SK 21.002 that is maximum levansucrase production at 1% of yeast extract. On the other hand, some researchers observed an increase in levansucrase production by adding 2% and 5% of yeast extract for *Z. mobilis* and thermophilic *Bacillus* sp. respectively (Gonçalves *et al*., 2013; Belghith *et al*., 2012).

Peptone is most widely used nitrogen source in culture media to grow microorganisms which provides an excellent natural source of amino acids, peptides and proteins. In agreement with present data, Jakob *et al*., (2012) reported the maximum levansucrase production at 0.2% of peptone from *Gluconobacter* species. On contrary, different studies noticed that 5% of peptone

concentration showed maximal enzyme synthesis from *B. methylotrophicus* SK 21.002 (Zhang *et al*., 2014).

The osmotic pressure of the culture propagation medium maintained by the incorporation of micronutrients or trace elements. K_2HPO_4 was added as the source of PO_4^{+2}, as it is known that K^{+2} is necessary for various physiological activities. In contrast to the present study, some researchers found 0.15% and 0.3% of K_2HPO_4 for the maximum yield of levansucrase from *Pediococcus acidilactici* and Erwinia herbicola (Ghada *et al*., 2014; Han and Clarke 1990).

In line with this study, it is reported that the calcium chloride improved the stability of cellular membrane by regulating the entrance and exit of materials. Basically, calcium chloride enhanced the permeability of membrane by making the endocellular pressure consistent with exocellular pressure (Zeng, *et al*., 2010). On contrary, different studies indicated that as the concentration of $CaCl_2$ increased, the biomass concentration also increased (Bajpai and Margaritis 1984).

CONCLUSION

The current study has established the potential of new strain isolated from molasses for the production of levansucrase. This strain was identified as *Zymomonas mobilis* KIBGE-IB14 after its confirmation by 16S rDNA analysis. Medium was also optimized for the maximum production of levansucrase by incorporating different nutrients. Maximum levansucrase production was achieved at 30°C after 24 hours of incubation using modified medium of pH 6.5. Whereas, in the case of chemical parameters, yeast extract (1.0%), peptone (0.2%), K_2HPO_4 (1.5%) and $CaCl_2.2H_2O$ (0.01%) were found to be suitable nitrogen sources and micronutrients. The present work suggested that levansucrase can be produced at higher yields from *Z. mobilis* KIBGE-IB14. Considering such properties, levansucrase from *Z. mobilis* KIBGE-IB14 can be used as a potential candidate for commercial and industrial applications. In future plans, kinetics and characterization of levansucrase will be further investigated.

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