

Purification of Levanase from *Enterobacter aerogenes* and using it as a Prebiotic and Antibacterial Agent

Sahira Nsayef Muslim¹, Sawsan Mohammed Kareem¹

¹Department of Biology, College of Science, Mustansiriyah University, Baghdad – Iraq

Received: 06th July, 19; Revised: 11th August, 19, Accepted: 07th September, 19; Available Online: 12th September, 2019

ABSTRACT

A novel strain of *Enterobacter aerogenes* isolated from rhizosphere soil has an ability to produce intracellular levanase when grown on basal medium containing the levan as substrate. Levanase was purified to homogeneity with a recovery yield of 57.85% and 22.5 fold of purification by using ammonium sulfate at 40% saturation followed by ion-exchange chromatography on Sepharose CL-6B column and gel filtration chromatography on Sephadex G-100 column. The purified levanase has a potential prebiotic property of enhancing the growth of lactic acid bacteria while apparently inhibiting the growth of pathogenic bacteria. Typically, prebiotic properties should not only reduce the growth of pathogens but must also promote the growth of desirable and beneficial bacteria, such as *Lactobacillus*. Thus the levanase may be used in animal feed to improve digestibility and support gastrointestinal health.

Keywords: Levanase, Purification, Prebiotic agent.

International Journal of Drug Delivery Technology (2019); DOI: 10.25258/ijddt.v9i3.12

How to cite this article: Muslim, S.N. and Kareem, S.M. (2019). Purification of levanase from *Enterobacter aerogenes* and using it as a prebiotic and antibacterial agent. International Journal of Drug Delivery Technology, 39(3): 390-395.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

They contain a high content of nitrogen, phosphorus, and potassium in the rhizosphere led to enhance of plant growth and stimulation of microorganisms such as *Pseudomonas*, *Listeria*, *Bacillus*, *Paenibacillus*, and *Enterobacter*¹. Enterobacteriaceae are distributed worldwide. They are found in soil, water, fruits, meats, eggs, vegetables, grains, flowering plants and trees, and in animals from insects to man.^{2,3} *Enterobacter aerogenes* is a Gram-negative belonging to the family of Enterobacteriaceae. *Enterobacter* is widely finding in nature since it found in the seeds, plants, soil, marine and freshwater, sewage, and dairy products besides to intestines of animals and humans.^{1,3} *E. aerogenes* is a part of the normal flora of a human and animal gastrointestinal tract, and when host resistance is low, it acts as an opportunistic pathogen and causes different infections such as wound infections, meningitis, respiratory, and urinary tract infections, bacteremia and septicemia, septic shock and abdominal cavity/intestinal infections.⁴⁻⁶

The transfructosylation of a levansucrase (EC 2,4,1,10) for sucrose led to synthesize of levan which is a group of fructans; polymers of fructose forming a non-structural carbohydrate⁷ and produced by microorganisms as an energy reserve and defense in addition to plants.⁸

Fructan that produced by bacteria called levan.⁸

Levanase (2,6-fi-D-fructanohydrolase, EC 3.2.1.65) is a hydrolytic enzyme that cleaves beta-2,6 glycosidic linkage of

levan, producing oligofructose and/or fructose. This enzyme was produced by yeasts, filamentous fungi and some bacteria, including *Streptococcus mutans*, *Clostridium acetobutylicum* and *B. subtilis*.⁷ There are two modes of action for Levanase included an endo-type and exo-type levanase. Endolevanase hydrolyzes within the levan chain, releasing oligofructose with different sizes and fructose as a product, while exolevanase hydrolyzes at end of levan chains, giving only single size product.⁹

In this study, we isolated, screened and identified an effective strain of levanase producing bacteria besides to purification of levanase and investigation of its prebiotic and antibacterial properties.

MATERIALS AND METHODS

Samples collection and primary screening

Twenty rhizosphere soil samples were collected from different locations in the Mustansiriyah University garden. One gram of each sample was suspended in 10 mL of sterile distilled water and shake vigorously for 10 minutes. Then 0.1 of the suspending liquid was spread on the surface of basal medium containing per liter ((NH₄)₂SO₄: 2.1 g, MgSO₄.7H₂O: 0.3 g, CaCl₂.2H₂O: 0.3 g, FeSO₄.7H₂O: 0.5 g, KH₂PO₄: 10.0 g) supplemented with 2% (w/v) levan as the sole carbon source and 20g/l agar-agar. The bacteria growth after 24 hours of incubation at 30°C shows the presence of levanase activity,

and then the diameters of clear zones around the colonies were measured as recommended by.¹⁰

Biological diagnosis

The growing bacterial isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of Systemic Bacteriology and identified by gram staining pattern, characteristic colonies (shiny colonies with entire margins and convex elevation) and standard biochemical reaction like catalase test(+), oxidase test(-), indole test(negative), methyl red test(-), urease test(-) and ability to motile.^{11,12} The diagnostic was confirmed by using Vitek 2 system by using Vitek GNI card (bio Merieux, France).

Extraction and secondary screening

The selected isolates that gave higher clear zones around the colonies were cultivated in the basal medium supplemented with 2% (w/v) levan and incubated at 30°C for 24 hours then the cells were harvested by centrifugation at 8000 ×g for 15min. The cells were washed in 10 mM potassium phosphate buffer (pH 6.0) and resuspended in 1-mL of the same buffer. The cells were disrupted by glass beads (average diameter, 0.1 mm) for 15min at 4°C using the vortex. Residual whole cells and cell membrane fragments were removed by centrifugation 8000rpm for 20 minutes. The resulting supernatant was used as the starting point to establish the levanase activity in crude cell extracts.¹³

Protein quantification and levanase assays

Proteins were quantified as described by Bradford.¹⁴ Levanase activity was assayed by incubating a 2 mL enzyme solution with 2 mL of 1%(w/v) levan in 10 mM sodium acetate buffer pH 6.0 at 30°C for 10 min. After incubation, the reaction was terminated by addition of 2 mL of DNS reagent. The mixture was incubated in a boiling water bath for 10 minutes to stop the enzyme reaction, cooled to room temperature, and the absorbency at 575 nm was measured. Total reducing sugar produced from reaction was quantified by using the dinitrosalicylic acid (DNS method).⁹ The standard curve was prepared by using the different concentrations of fructose solutions. One unit of levanase activity was defined as the amount of enzyme, which generated 1μ mole of fructose under the above conditions.

Purification of levanase

The extracted levanase was purified by conventional techniques as described by.¹³ The crude lysate was first subjected to 20% saturation by the addition of solid ammonium sulfate, and after incubation, for 1 hour the precipitated protein was removed by centrifugation and discarded. To the remaining supernatant, solid ammonium sulfate was added stepwise to 40, 50, 60, 70, and 80% saturation, and the precipitated protein was harvested by centrifugation after each step, and the obtained pellets were dissolved in a small amount of 10 mM sodium acetate buffer pH 6.0. The products were subjected to overnight dialysis at 4°C against the same buffer. The protein concentration and levanase activity were determined in the different protein fractions. The

dialyzed protein solution was loaded on a Sepharose CL-6B anion-exchange column preequilibrated with 10 mM sodium acetate buffer pH 7.0. The column was washed with 50 mL of the same buffer, and proteins were eluted with a gradient of NaCl from 0.1–0.5 M. The active fractions that showing the highest levanase activity were pooled and applied to Sephadex G-100 column that was pre-equilibrated with 5 mM sodium acetate buffer pH 7.0. The column was eluted with the same buffer at a flow rate of 0.5 mL/min. Protein concentration and levanase activity were estimated, and the active fractions were pooled for further studies.

Effect of levanase on lactic acid bacteria growth

The prebiotic properties of levanase were tested by its effect on the growth of three isolates *Lactobacillus reuteri* and three isolates of *Pediococcus* sp. These bacterial isolates were grown in 50 ml of MRS broth for 24 h at 30°C. Later, 1% of the inoculums, which was adjusted to the absorbance of 0.5 at 600 nm was transferred into 5 mL of MRS broth with or without 1% of the purified levanase and incubated for 4 hr at 30°C. After 4 hours of incubation, the number of cells were determined by plating on MRS agar and grown overnight at 30°C. The isolates yielding purified levanase, which showed the promotion of *Lactobacillus reuteri* and *Pediococcus* sp were selected. Enhanced activity (%) was determined as¹⁵:

$$\text{Enhancement activity (\%)} = (\text{SB} - \text{CB}) / \text{CB} \times 100$$

Where SB is the amount of cells in MRS with purified levanase (cfu/ml) and CB is the amount of cells in MRS without purified levanase (CFU/mL).

Effect of levanase on pathogenic bacteria growth

Pathogenic bacteria such as three isolates of *Salmonella* sp. and three isolates of *Escherichia coli* were cultivated in 50 ml of nutrient broth medium under aerobic condition by shaking at 150 rpm for 24 h at 37°C. After that, 1% of the inoculums, which was adjusted to the absorbance of 0.5 at 600 nm were transferred into 5 mL of nutrient broth medium and 5 mL of nutrient broth plus 1% of the purified levanase and grown at 37°C by shaking at 150 rpm for 4 hour. The cell numbers were determined by plating on of nutrient agar and incubated overnight at 37°C. The isolates yielding purified levanase showing inhibition of *Salmonella* sp. and *Escherichia coli* were selected. Inhibition activity (%) was determined as¹⁵:

$$\text{Inhibition activity (\%)} = (\text{CB} - \text{SB}) / \text{CB} \times 100$$

Where SB is the amount of cells in nutrient broth with purified levanase (CFU/mL) and CB is the amount of cells in nutrient broth without purified levanase (CFU/ml).

RESULTS AND DISSCUSION

Detection levanase production

Seven isolates of *Enterobacter aerogenes* were obtained among 20 samples of rhizosphere soil samples (Figure 1) showed an ability to produce the levanase in the primary screening by producing a clear zones around the colonies and *Enterobacter aerogenes* R₂ showed higher level of production of levanase and the diameter of inhibition zone reached to 25 mm.

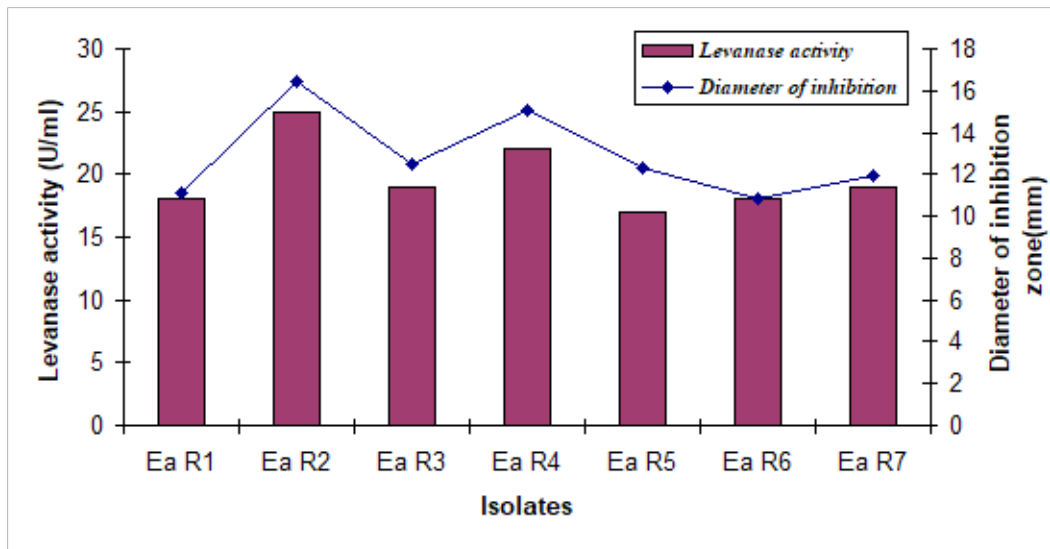


Figure 1: Levansase activity and diameter of inhibition zone for *Enterobacter aerogenes* isolates

Listeria sp. and *Enterobacter* sp were isolated from soil and clinical samples, as reported by.¹⁶ The content of carbon, nitrogen, and other nutritional elements in soil are important for increasing the growth of.¹⁷

In the some ecosystems the presence of levansase lead to hydrolysis of high-molecular-weight levan or inulin to products that would act as inducers for levansase synthesis.¹⁰ Using of levan as a substrate considered as a good inducer for production of levansase enzymes, and hydrolysis of levan to produce fructooligosaccharides with variable degrees of polymerization.¹⁸ The produced by large majority of *Bacillus* sp. isolates had an ability to produce levansase that degraded levan to fructose.¹⁹

Purification of levansase

The extracted enzyme that obtained by centrifugation of a disrupted cells by using glass beads, was used as the crude

enzyme solution and subjected to fractionation step by using ammonium sulfate with 20–80% saturation and found that 40% saturation led to increasing the specific activity from 16.42 to 26.11 U/mL. The dialysis step after ammonium sulfate precipitation was used to remove the impurities and unwanted proteins. The dialyzed solution was applied to the Sepharose CL-6B column (2.5× 25cm) previously equilibrated with 10 mM sodium acetate buffer pH 6.0. The column was thoroughly washed with the same buffer, and the adsorbed enzyme was eluted with a gradient of sodium chloride ranging from 0.1-0.5M with the same buffer. The elution led to appear three protein peaks, and levansase activity was observed in the third peak (Figure 2). The active fractions were collected and loaded on Sephadex G-100 that was previously washed with 5mM sodium acetate buffer pH 7.0. The elution with the same buffer revealed two protein peaks of protein and the activity

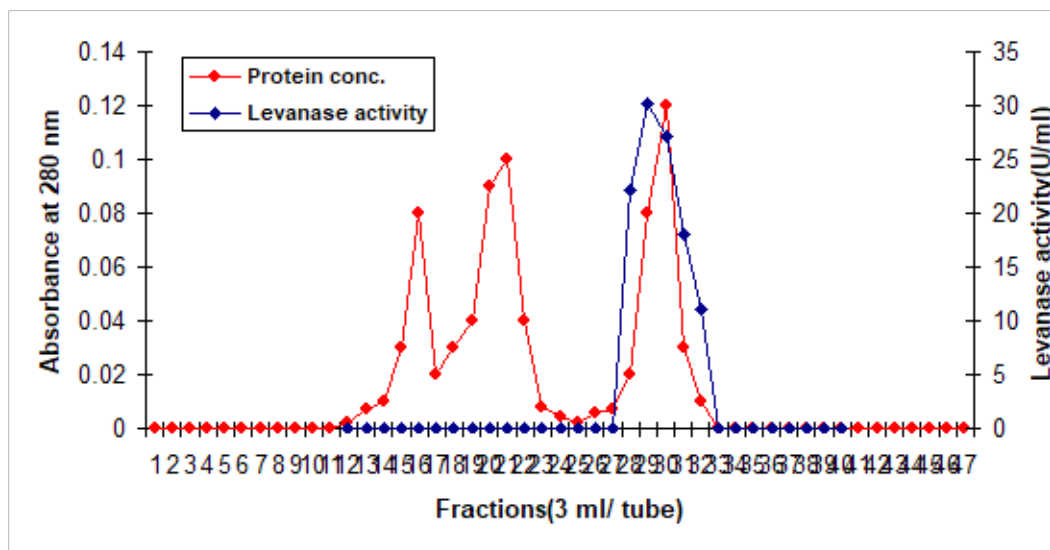


Figure 2: Levansase Purification from *Enterobacter aerogenes* R₂ by using ion-exchange chromatography on sepharose CL-6B column

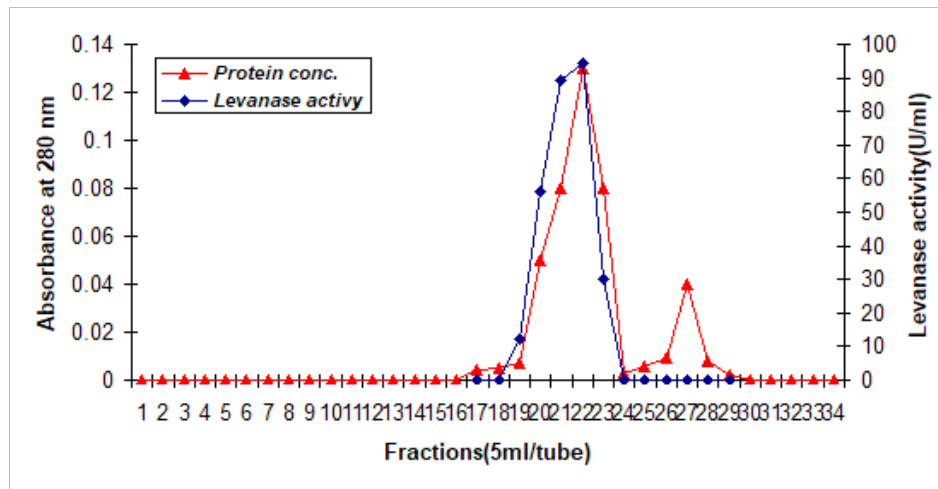


Figure 3: Levanase Purification from *Enterobacter aerogenes* R₂ by using gel filtration chromatography on Sephadex G-100 column

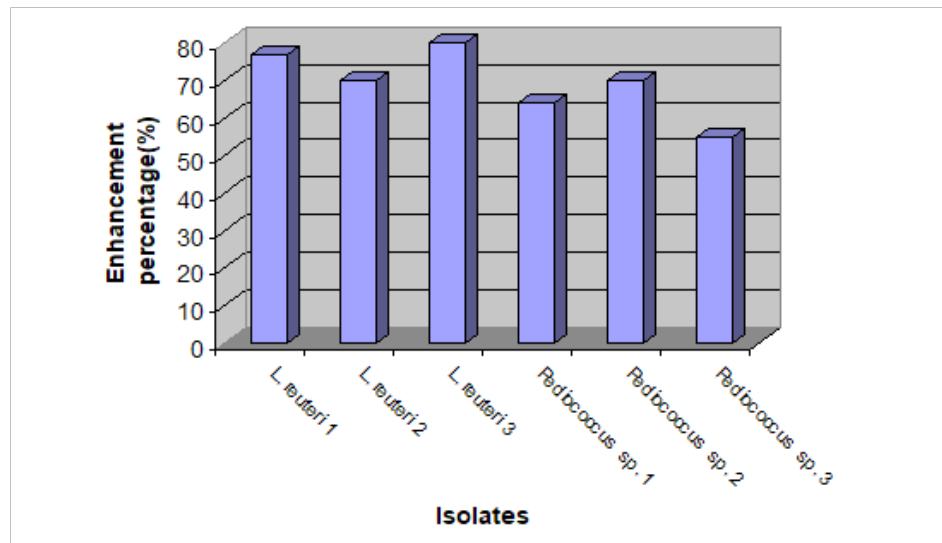


Figure 4: Enhancement of lactic acid bacteria by purified levanase from *Enterobacter aerogenes* R₂

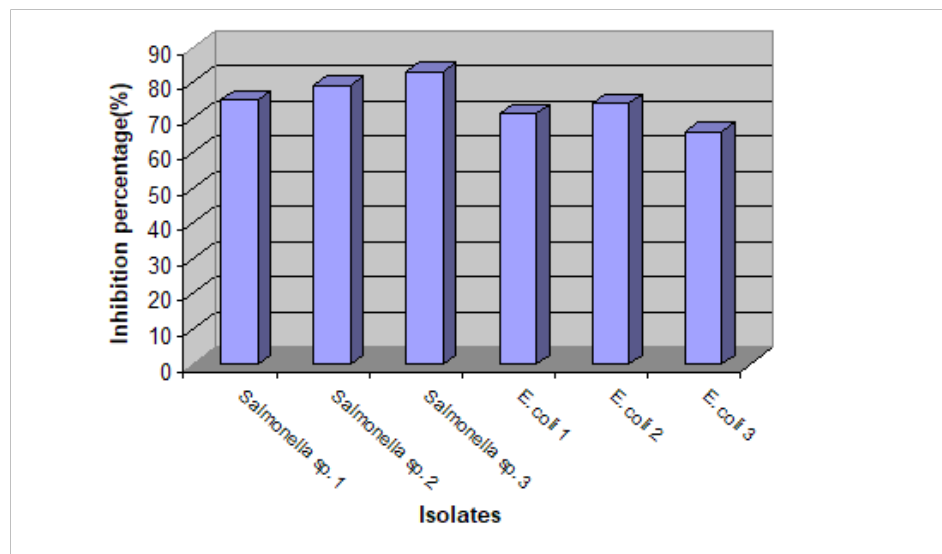


Figure 5: Inhibition of pathogen bacteria by purified levanase from *Enterobacter aerogenes* R₂

Table 1: Serial steps for levanase purification from *Enterobacter aerogenes* R₂

Purification step	Size(ml)	Levanase activity (U/ mL)	Protein conc. (mg/mL)	Specific activity (U/mg)	Total activity	Purification fold	Yield (%)
Crude extract	30	16.42	1.86	8,82	492.6	1	100
(NH ₄) ₂ SO ₄ precipitation	15	26.11	1.55	16.84	391.65	1.9	79.5
Sepharose CL-6B	10	30.11	1.12	26.88	301.1	3.0	61.12
Sephadex G-100	8	32.16	0.34	94.58	257.28	10.7	52.22

appeared at the first peak that contained levanase activity (Figure 3). The levanase was purified with 52.22% yield, 10.7 fold of purification, and specific activity of 94.58U/mg protein, as shown in Table 1.

More than 90% of the inulinase and levanase activity was in a cell-associated form.¹⁹ Column chromatography on DEAE-cellulose, phenyl-Toyopearl 650 M, Sephadex G-100 and hydroxyapatite used after (NH₄)₂SO₄ fractionation for purification of levanase from *Pseudomonas* sp.^{18,20} In contrast, ammonium sulfate precipitation, DEAE, and gel filtration on Sephacryl S-200 column were used for purification of levanase produced by *Rhodotorula* sp.²¹

Evaluation of prebiotic properties

Prebiotic properties of purified levanase were determined by using lactic acid bacteria such as *Lactobacillus reuteri* and *Pediococcus* sp. According to the results, the levanase led to stimulating *Lactobacillus reuteri* isolates with 70–80%, followed by *Pediococcus* sp. isolates with 55–70%. In contrast, the purified levanase when mixed with pathogenic bacteria such as *Salmonella* sp. and *Escherichia coli* revealed an inhibition activity since the inhibition percentage ranged from 75 to 83% for *Salmonella* sp. followed by 66 to 74% for *Escherichia coli* as shown in Figures 4 and 5.

These results indicate levanase has a potential prebiotic property of enhancing the growth of lactic acid bacteria while inhibiting the growth of pathogenic bacteria. Typically, prebiotic properties should not only reduce the growth of pathogens but must also promote the growth of desirable and beneficial bacteria, such as *Lactobacillus*. This is the first paper dealing with levanase purification from *Enterobacter aerogenes* and the elucidation of its prebiotic properties.

Therefore the purified levanase may be used in animal feed to improve digestibility and support gastrointestinal health. Further studies relating to its combination with an appropriate probiotic would lead to synbiotic application will be carried out in the future for determination the type of this synbiotic effect.

ACKNOWLEDGMENT

The authors would like to thank Mustansiriya University (www.uomustansiriya.edu.iq) / Baghdad- Iraq for its support in the present work.

REFERENCES

- 1 Abbas, M.; Atiq-ur-Rahman, M.; Manzoor, F. and Farooq, A. (2012). a quantitative analysis and comparison of nitrogen, potassium and phosphorus in rice husk and wheat bran samples. *Appl. Bio.*, 1(1): 14-15.
- 2 David, G.; Slack, R.C.; Peuthere, J. F.(2002). *Medical Microbiology, a Guide to Microbial Infections: Pathogens,*

- Immunity, Laboratory Diagnosis and Control. Edinburgh: Churchill Livingstone.
- 3 Atlas, R. M. and Richard, B.(1998). *Microbial Ecology Fundamentals and Applications*. 4th ed. Menlo Park, Ca.: Benjammin/Cummings Publishing Company, Inc.
 - 4 Dugauc-Vrncic, N., Vukovic, V., and Nedic, N. (2010). Pathogenicity of some bacterial species isolated from the bee digestive tract. *Acta Vet.* 60, 49-57.
 - 5 Marchi, A., and Utkhede, R.S. (1994). Effect of *Enterobacter aerogenes* on the rhizosphere microflora of Apple trees. *J. Phytopathol.* 141, 127-132.
 - 6 Utkhede, R.S., and Sholberg, P.L. (1986). In vitro inhibition of plant pathogens by *Bacillus subtilis* and *Enterobacter aerogenes* and in vivo control of two postharvest cherry diseases. *Can. J Microbiol.* 32, 963-967.
 - 7 Martin, I.; Debarbouille, M.; Klier, A. and Rapoport, G.(1999). Induction and Metabolite Regulation of Levanase Synthesis in *Bacillus subtilis*. *J. BACTERIOL.*, 171(4): 1885-1892.
 - 8 Cairns, A.J.(2003). Fructan biosynthesis in transgenic plants. *J. Experimental Botany*, 54(382): 549-56.7
 - 9 Miasnikov, A. 1997. Characterization of a novel endo-levanase and its gene from *Bacillus* sp. L7. *FEMS Microbiology Letters*, 154: 23-28.
 - 10 Murakami, H.; Muroi, H.; Kuramoto, T.; Tamura, Y.; Mizutani, K.; Nakano, H. and Kitahata, S.(1999). Purification and Some Properties of a Levanase from *Streptomyces* sp. No. 733. *Agric. Biot. Chem.*, 54 (9) 2247- 2255.
 - 11 Holt, J.G.(1994). *Bergey's manual of determinative bacteriology*. 2nd ed. Vol.2, Williams and Wilkins. Baltimore.
 - 12 Ahmed, R. N.; Sani, A.; Ajijolakewu, G. and Alamu, F.B.(2013). Soil Screening For Antibiotic - Producing Microorganisms. *Advances in Environmental Biology*, 7(1): 7-11.
 - 13 Muslim, S. N.(2015). Broad-Spectrum Biofilm and Adhesion Inhibition by Levanase Purified from *Enterobacter aerogenes*. *Enterobacter aerogenes*. Al-Mustansiriya Journal of Science, 26(2):1-8.
 - 14 Bradford, M.(1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, *Annal . Biochem.* ,72 :248-254
 - 15 Phothichitto, K.; Nitisinprasert, S. and Keawsompong, S.(2006). Isolation, Screening and Identification of Mannanase Producing Microorganisms. *Kasetsart J. (Nat. Sci.)* 40 (Suppl.) : 26 - 38.
 - 16 Sirisena, D. M. and Manamendra, T. P. (1995). Isolation and characterization of cellulolytic bacteria from decomposing rice straw. *J. Natn. Sci. Coun. Srilanka*, 23(1): 25-30.
 - 17 Poorni, K. E.; Manikandan, A.; Geethanjali, S. and Petcy, K. (2011). Production of *Pseudomonas fluorescens* from agricultural wastes and its application in the preservation of selected vegetables. *Advances in Applied Science Research*. 2: (2), 156-160.
 - 18 Lim, Y.S.; Kang, S.K.; Lee, S.O.; Lee, J.D. and Lee, T.H.(1998). Purification and characterization of a levanase from *Streptomyces* sp. 366L. *J. Biotechnol.*, 61(1): 33–41.

- 19 Bergeron, L.J. and Burne, R.A.(2001). Roles of Fructosyltransferase and Levanase- Sucrase of *Actinomyces naeslundii* in Fructan and Sucrose Metabolism. *Inf. Immun.*, 69(9):5395–5402.
- 20 Wanker, E.; Huber, A. and Schwab, H.(2005). Purification and characterization of the *Bacillus subtilis* levanase produced in *Escherichia coli*. *Appl. Environ. Microbiol.* 61(5):953-958.
- 21 Chaudhary, A.; Gupta, L.K.; Gupta, J.K. Banerjee, U.C.(1998). Purification and properties of levanase from *Rhodotorula* sp. *J. Biotechnol.*, 46(1): 55–61.