RESEARCH ARTICLE

Production of Fructo-oligosaccharides by Purified Inulinase from *Klebseilla pneumoniae* and using it as a Prebiotic agent

Hadel K. Abbas*, Sahira N. Muslim, Rajwa H. Essa

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

Received: 19th September, 2020; Revised: 12th October, 2020; Accepted: 16th November, 2020; Available Online: 25th March, 2021

ABSTRACT

A novel strain of *Klebsiella pneumoniae* among 45 isolates collected from urine samples, had an ability to produce extracellular inulinase when grown on basal medium containing inulin as a substrate. Inulinase was fractionated with ammonium sulfate at 70% saturation at a higher level in comparison with organic solvent (methanol, ethanol, acetone, propanol, and butanol). Inulinase was purified with a recovery yield of 34.5% and 2.73 fold of purification by using ammonium sulfate followed by ion-exchange chromatography on the DEAE-cellulose column. Fructo-oligosaccharide (FOS) is produced by reaction of purified inulinase with its substrate and characterized by UV-spectrophotometer in comparison with standard FOS.

The effect of FOS on the growth and activity of local lactic acid bacterial isolates revealed that three concentrations (1, 2, and 4%) of FOS had stimulated the growth of lactic acid bacteria with two logarithmic cycles for *Lactobacillus plantarum* (*L. plantarum*) and *Lactobacillus pentosus* (*L. pentosus*). At the same time, FOS gave the maximum stimulating growth for *Lactobacillus gasseri* (*L. gasseri*) with three logarithmic cycles. So that FOS has a potential prebiotic property of enhancing of lactic acid bacteria which supports gastrointestinal health.

Keywords: Fructo-oligosaccharide, Inulinase, Prebiotic agent, Purification.

International Journal of Drug Delivery Technology (2021); DOI: 10.25258/ijddt.11.1.8

How to cite this article: Abbas HK, Muslim SN, Essa RH. Production of Fructo-oligosaccharides by Purified Inulinase from *Klebseilla pneumoniae* and using it as a Prebiotic agent. International Journal of Drug Delivery Technology. 2021;11(1):47-51.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is a gram-negative, facultatively anaerobic, rod-shaped, non-motile bacterium, belongs to the family *Enterobacteriaceae.*[1] *K. pneumoniae* are the causative agent of numerous kinds of infections in humans, including urinary tract infections, respiratory tract infections, and bloodstream infections.[2] Clinically, the majority of human *Klebsiella* infections are related to *Klebsiella oxytoca* (*K. oxytoca*) and *K. pneumoniae;* they are recognized as vital human pathogens associated with serious hospital-associated infectious processes, including urinary tract infections (UTI), respiratory tract illnesses such as pneumonia and bloodstream infections (BSI).[3,4]

Inulin is a natural storage polymer located commonly in plants, specifically roots and tubers GaoW et al.[5]such as yacon, asparagus, leek, onion, banana, wheat, and also garlic.[6] It can be produced by microorganisms, including *Streptococcus* and *Aspergillus* species.[7,8] Inulin is one of the most generally utilized substrates for production of inulinases.[9]

Inulinase (EC 3.2.1.7) is inulin hydrolyzing enzyme to produce fructose and fructo-oligosaccharide.[10] Usage of microorganisms to produce inulinases will become a viable choice to get this enzyme in larger amount.[11] Inulinases can be found in microorganisms; among yeasts are *Candida kefyr* and *Pichia polymorpha,*[12] and several bacteria such as *Pseudomonas* sp.[13] *Streptomyces* sp.;[14] *Sphingomonas*sp.JB13;[15] *Bacillus sp.* SG113, ASN 3.[16] Inulinase has obtained considerable interest as it can be widely used to hydrolyze inulin to produce fuel ethanol, fructose, and fructo-oligosaccharides, both of which are necessary components in food and pharmaceutical industry.[17]

Fructo-oligosaccharides (FOS) are oligomeric structures containing linear fructose units that are linked by β(2-1) bonds, varying from 2 to 60 units, a fixed to a terminal glucose residue.[18] Prebiotic FOS are getting rising recognition as agents to modulate the colonic microbiota in humans and animals; these so-called prebiotics were initially defined in 1995 as 'non-digestible food component(s) that beneficially influence host's health by promoting the growth and/or activity of one or a limited number of bacteria in the colon'.[19,20] Thus, this study aims to isolate, diagnose, and screen of the most powerful inulinase producer besides to purification of inulinase, production of FOS, and using it as a prebiotic agent.

MATERIALS AND METHODS

Collection and Identification of Bacterial Isolates

Forty-five isolates from urine were collected from two hospitals at Baghdad city. The isolates were cultured on MacConkey

*Author for Correspondence: hadelkamil84@gmail.com

agar medium for identification depending on the colonies' morphological and cultural characteristics that included the shape, texture, color, and edges.[21] Pure isolated colony of each bacterial isolates streaked on Eosin Methylene Blue agar and incubated aerobically, the appearance of isolates as dark pink in color indicates a positive result.[22] The diagnostic was confirmed by using Vitek 2 system and this system including 47 biochemical tests and one negative control well according to the instruction of bio Merieux, (France).

Primary and Secondary Screening

All bacterial isolates were examined for their ability to produce inulinase by cultured on inulin agar plates that composed of: (inulin 1gm,yeast extract 0.1 gm, pepton 0.1 gm, NH_4NO_3 0.1 gm, KH2PO4 0.14gm, $MgCl_2$ 0.02 gm, and agar-agar 3 gm) an appearance of a transparent zone around the colonies denote or indicate about inulinase production and then the diameters of clear zones around the colonies were measured.[23] Then, the selected isolates that gave higher clear zones around the colonies were inoculated to inulin broth medium and incubated at 37°C for 24 hours, after that the centrifugation at 5000 rpm for 15 minutes. The resulting supernatant was used as the crude extract to establish protein concentration and inulinase activity.

Protein Quantification and Lnulinase Assays

Protein was quantified as described by Bradford MM *et al*.,[24] depending on the standard curve of bovine serum albumin. Protein concentration was determined by adding 0.1 mL of sample to 4.9 mL of Bradford reagent, then the mixture was incubated at 30°C for 10 minutes and after that the absorbency was measured at 595 nm by using spectrophotometer.

Inulinase activity was measured by modification of the method that described by Miller GL *et al*.,[25] 0.5 mL of crude extract was incubated for 30 min at 37°C with 0.5 mL of (1 gm of inulin as substrate dissolved in 50 mL of 0.1 M acetate buffer at pH = 5.0) then stopped the enzyme reaction by putting the mixture in boiling water bath for 15 minutes. After that adding 1 mL of 3,5- Dinitrosalicyclic acid (DNS) reagent to the mixture and the final volume was become 2 mL then measured the absorbency at 540 nm by using spectrophotometer. The standard curve was prepared by using different concentrations of fructose solution. One unit of lnulinase activity was defined as the amount of enzyme, which generated 1µ mole of fructose under the above conditions.

Purification of Inulinase

Two method were used to precipitate of inulinase. In the first the crude solution was precipitated with (20–80%) saturation ammonium sulfate, ammonium sulfate was added gradually to the crude solution at 4°C (in ice bath) with continuous stirring for two hours. After that the solution was centrifugated by cooling centrifuge at 5000 rpm for 15 minutes, then the precipitate was dissolved in a small amount of 0.1 M and pH 5.0 of acetate buffer. In the second method each solvent separately (methanol, ethanol, acetone, propanol and butanol)was added to the crude extract to obtain 20, 30, 40, 50, 60, 70, and 80% solvent concentrations in ice bath with continuous stirring for two hour, then the precipitate was separated by cooling centrifugation at 5000 rpm for 15 minute and dissolved in 0.1 M acetate buffer pH 5.0.The obtained inulinase solution was dialyzed against acetate buffer for 24 hours in a cold condition. Ten mL of produced inulinase solution were loaded on DEAE-cellulose column (30 × 1.5) cm. The column was washed with 0.1 M acetate buffer pH5.0 at flow rate of 30 mL/min and eluted with gradient (0.1–0.5) M NaCl solutions. The fractions of 5 mL/tube were collected and the absorbency at 280 nm was read. Protein concentration and inulinase activity were estimated and the active fractions were pooled for further studies.

Production and Purification of FOS

Preparation of FOS

The preparation of FOS described by method of Oliveira KG *et al*.,[26] with some modifications, which included by the incubation of 0.5 mL of purified inulinase with 0.5 mL of (1 gm of inulin as substrate dissolved in 50 mL of 0.1 M acetate buffer at pH = 5.0) for 30 minutes at 37°C, then stopped the enzyme reaction by putting the mixture in boiling water bath for 15 minutes, with this reaction we got FOS product.

Purification of FOS

The pre-prepared reaction mixture was loaded on sephadex G-25 gel-filtration, which was previously equilibrated with a 0.1 M sodium acetate buffer at pH 5.0; elution was done using the same buffer with flow rate 30 mL in hour. The obtaining fractions were read by using the three wavelengths that obtained from the FOS standard by using UV-spectrophotometer, separately, according to Nobre C.[27]

Effect of (FOS) on Lactic Acid Bacterial Growth

The prebiotic properties of FOS were verified by its effect on the growth of 3 *Lactobacillus* spp. isolates, as described by Hadid M A[28] as follow:

For each isolate, three different FOS concentrations were used (1, 2, and 4%) with a control. The effect of FOS on bacterial growth was identified comparatively by using two tubes of 2 mL MRS medium. A 20 µL of MacFarland solution for Lactic acid bacteria was added to one of them as a control tube while for the other tube 20 µL of MacFarland solution mixed with 20 µL of 1% concentration FOS, 40 µL of 2% concentration FOS and 80 µL of 4% concentration FOS was added, separately. All tubes were incubated at 37°C for 24 hours. anaerobically. After the incubation, 1 mL of each treatment and control were serially diluted from 10^{-1} to 10^{-10} then 0.1 mL of each diluted sample was taken and spreaded on MRS agar plates. The plates were incubated at 37°C for 24–48 hours. The number of cells was calculated according to the following equation for each type of lactic acid bacteria: Number of cells/mL = number of colonies × Inverted dilution × 10.

RESULTS AND DISSCUSION

Detection of lnulinase Production

According to the semi-quantitative analysis there were 11 *K. pneumoniae* isolates out of 45 isolates have an ability to

produce inulinase enzyme with a diameter of inhibition zone ranged between 17–23 mm and larger inhibition zone revealed by *K. pneumoniae* $U_7$with 23 mm. In comparison, the other 34 isolates cannot produce inulinase enzymes. These 11 isolates will be subjected to quantitative analysis and revealed specific activity ranged from 9 to 32 U/mg with maximum specific activity 32 U/mg for *K. pneumoniae* U_7asshown in Figure 1.

The growth of bacterial isolates on inulin agar plates and producing this visible transparent zone surrounding the colony indicates that these isolates produce inulinase enzymes that hydrolyzed inulin to produce fructose fructooligosaccharides led to form a transparent zone around the colonies after prolonged incubation.

An exoinulinase produced from *Bacillus* sp. screened based on hydrolytic zone formation around the colonies and the highest activity level ofinulinasewas recorded in the supernatant with 25.10 U/mL.[17] Earlier studies on *Bacillus subtilis*, *Lactobacillus casei*, *Pseudomonas aeruginosa*, and *Achromobacter* sp. showed that maximum inulinase accumulation (333 U/L) has been observed for the isolate *Achromobacter* sp. and other isolates produced maximum inulinase activities ranging from 221 to 333 U/L in the supernatant.[29]

Inulinase Extraction and Purification

The crude extract of *Klebsiella pneumoniae* $U_{7\,was}$ subjected to two methods of extraction, the first extract included ammonium sulfate precipitation by using different concentrations (20-80%). It was discovered that 70% saturation of ammonium sulfate triggered precipitation of inulinase with inulinase activity of 74 U/mL, while the second method of extraction by organic solvents which gave different levels of inulinase activity, since ethanol gave the highest enzymatic activity of 68 U/mL at concentration 60% followed by methanol and acetone, while propanol and butanol precipitated inulinase with inulinase activity 55 U/ml and 50 U/mL, respectively, at concentration 30 and 40%, respectively, as revealed in Figure 2. So that ammonium sulfate was more effective than organic solvents in inulinase precipitation.

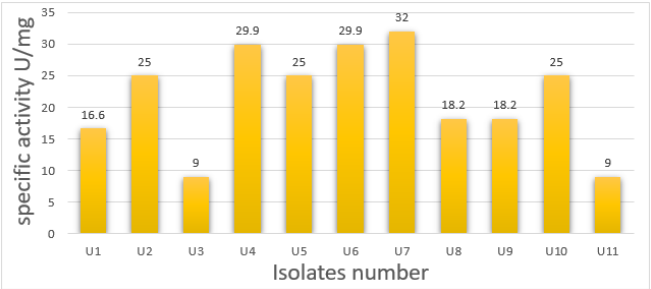

Figure 1: Specific activity of inulinase produced by *K. pneumoniae* isolates.

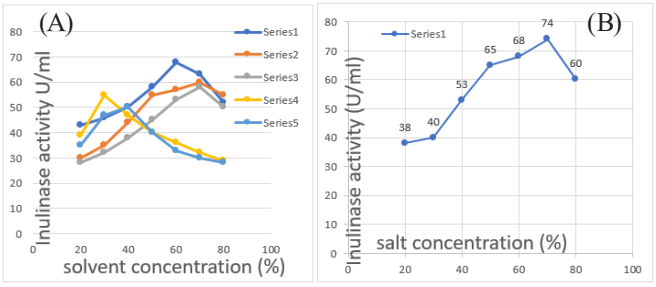

Figure 2: Extraction of Inulinase by using (A) ammonium sulfate (B) organic solvents

The enzyme purification procedure involves three steps included ammonium sulfate precipitation, dialysis and anion exchange chromatography.

After ammonium sulfate precipitation and dialysis step, the dialyzed inulinase was applied to the equilibrated DEAE-cellulose column, then the column was washed with an equal volume of 0.1 M sodium acetate buffer (pH 5.0). The bounded proteins (negatively charged) were then eluted using gradient concentrations of sodium chloride ranging between 0.2–0.6 M, the results indicated in Figure 3 showed that there was one protein peak that appeared in the washing step, while two peaks appeared after elution by gradient concentration of sodium chloride, all these three proteins peaks were detected by measuring the absorbency at 280 nm of each eluted fraction, For three protein peaks, inulinase activity were measured, and found that inulinase activity was located in the third protein peak. The active fractions were collected for further studies, and the specific activity was 87.36 with fold of purification 2.73 ata yield 34.5% as revealed in the Table 1.

The crude inulinase that extracted from *Bacillus* sp. was purified by using ammonium sulphate precipitation, dialysis and ion exchange chromatography on DEAE–sephacel and obtained 1.9 purification fold with total activity 293 U.[17] Research of Golunski S *et al*.,[30] purified inulinase enzyme by

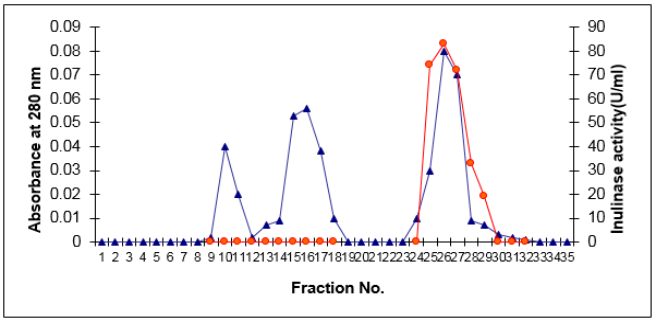

Figure 3: Inulinase Purification from *K. pneumoniae* U_7 by using ion exchange chromatography on DEAE-cellulose column (30–1.5) with flow rate of 30 mL/hour

Table 1: Serial steps for inulinase Purification from *K. pneumoniae* U_7

Purification step	*Size (mL)*	*Inulinase activity (U/mL)*	*Protein conc. (mg/mL)*	*Specific activity (U/mg)*	*Total activity*	*Purification fold*	*Yield (%)*
Crude extract	80	60	1.87	32.0	4800	1	100
$(NH_4)_2SO_4$ precipitation	45	74	1.21	61.15	3330	1.91	69.3
DEAE-cellulose	20	83	0.95	87.36	1660	2.73	34.5

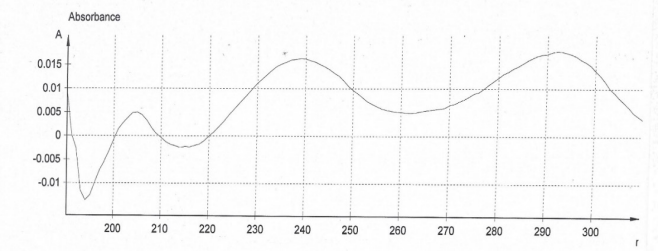

Figure 4: Standard curve of Fructo-oligosaccharide by using UV-spectrophotometer

changing the ionic strength of the medium through addition of NaCl and CaCl2 followed by precipitation with n-propyl alcohol or iso-propyl alcohol, the effects of the concentration of alcohols and the rate of addition of alcohols in the crude extract on the purification yield and purification factor were evaluated, so activation of enzyme and allowed purification factors up to 2.4-fold for both alcohols[31] obtained a specific inulinase activity from *Ulocladium atrum* of 1915 U/mg protein which represented 2.65-fold purification over the crude inulinase extract with 42.8% recovery.

Production of FOS and Preparation of Standard Curve

The standard curve of FOS was prepared and measured by UV-spectrophotometer and the results showed that FOS standard contain three components which are: 1-kestose, Nystose and 1-F-fructofuranosylnystose that showed three peaks in UV-spectrophotometer at wavelengths (205, 240 and 293 nm) as revealed in Figure 4.

Production and Purification of FOS

After reaction of purified inulinase enzyme with the inulin as its substrate, the obtained reaction mixture was loading on sephadex G-25 gel-filtration and washing with 0.1 M sodium acetate buffer pH 5; the fractions were measured in each of wavelength 205, 240, and 290 nm, separately, with UV-spectrophotometer, the active fractions that contain each of FOS components were collected and used for further studies.

Effect of Fructo-oligosaccharide (FOS) on Lactic Acid Bacterial Growth

The effect of FOS on the growth and activity of local lactic acid bacterial isolates was conducted. The results revealed when three concentrations of FOS (1, 2 and 4%) were used as nutritional elements for the growth three kinds of lactic acid bacterial isolates which included *L. plantarum, L. pentosus* and *L.* gasseri had stimulated effect on lactic acid bacteria. According to Table 2, we found that FOS stimulated the growth of *L. plantarum, L. pentosus* with two logarithmic cycles, while FOS gave the maximum stimulating growth for *L.gasseri* with three logarithmic cycles. From these results, we found that the role of FOS prebiotic was more pronounced in supporting and increasing all lactic acid bacteria's growth.

The health benefit Prebiotic agents to the host targets initially on the modulation of intestinal tract microbiota that includes the indigenous gut microflora that contributes in different functions that advanced host health.[32] Dietary fibers

Table 2: Effect of Fructo-oligosaccharide (FOS) on growth of Lactic acid bacterial isolates:

		No. of bacterial cell(cell/mL)	
Isolate	*Prebiotic*	*Before incubation period*	*After incubation period*
L. plantarum	Control	4.3×10^6	2.2×10^7
	1% FOS	4.3×10^6	8.3×10^7
	2% FOS	4.3×10^6	4.6×10^8
	4% FOS	4.3×10^6	9.1×18^8
L. pentosus	Control	2.8×10^7	3.1×10^8
	1% FOS	2.8×10^7	2.5×10^9
	2% FOS	2.8×10^7	1.2×10^{10}
	4% FOS	2.8×10^7	$8.0 \times 1^{01}0$
L. gasseri	Control	8.5×10^6	7.3×10^7
	1% FOS	8.5×10^6	5.6×10^8
	2% FOS	8.5×10^6	3.3×10^9
	4% FOS	8.5×10^6	5.6×10^{10}

and their hydrolysis products are becoming an evolving source of new constituents with strong prebiotic activity.[33]

The health benefits associated with the consumption of FOS that including encourages the growth of favorable bacteria in the colon; this, in turn, discourages the growth of microorganisms in the colon resulting in a healthy gut environment and there was a selective growth of certain groups of bacteria such as B*ifidobacterium* was observed, thus confirming the prebiotic properties of oligofructose.[34] Earlier research by Sabater-Molina M *et al*., and Bibas Bonet ME, *et al*.,[35,36] and Slavin J, *et al*.,[37] displayed that FOS can escape enzymatic digestion in the upper gastrointestinal tract reaching the colon intact before undergoing microbial fermentation. Another study by[38] showed that FOS also favor the growth of health-promoting bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp., while reducing or maintaining pathogenic populations (e.g., *Clostridium* spp. and *Escherichia coli*) at low levels thus, FOS are small soluble dietary fibers that exhibit prebiotic activity. FOS offers physiological benefits that use as a food supplement, particularly in cases of chronic diseases,[39] FOS health benefits in colon cancer, diabetes and obesity.[37]

ACKNOWLEDGMENT

The Authors would like to thank Mustansiryiah University (http://uomustansiriyah.edu.iq/) Baghdad, Iraq for its support in the present work.

REFERENCES

1. Ramos P.I.P, Picão R.C, Almeida D,, Lima L.G.P, Girardello N.C.B, Vivan R, Médigue A.C.P,. Comparative analysis of the complete genome of KPC-2-producing Klebsiella pneumoniae Kp13 reveals remarkable genome plasticity and a wide repertoire of virulence and resistance mechanisms. *BMC genomics.* 2014;*15*(1):54.
2. MartinRM. and Bachman MA. Colonization, Infection, and the Accessory Genome of *Klebsiella pneumoniae*. *Front. Cell. Infect. Microbiol*. 2018;8:1-4.
3. Kareem S.M, Al-Kadmy I.M.S, KazaalS.S, Mohammed A.N, Abduljaleel T.J, Wahaeb Z.M, Abdulhussain Z.A. Antibiotic susceptibility profile and molecular characterization of quinolones resistant klebsiella pneumonia isolates: First report

from Iraq. Indian Journal of Public Health Research and Development. December. 2018;9(12):676-682
4. JandaJ.M . The Genus *Klebsiella*: An Ever-Expanding Panorama of Infections, Disease-Associated Syndromes, and Problems for Clinical Microbiologists. *Clin Microbiol Case Re.* 2015;1(4): 022.
5. GaoW, Bao Y, LiuY, Zhang X, Wang J, & AnL. Characterization of thermo-stable endoinulinase from a new strain Bacillus smithii T7. *Applied biochemistry and biotechnology.* 2009;*157*(3):498-506.
6. ShoaibM, Shehzad A, Omar M, Rakha A, Raza H, Sharif H. R&Niazi S. Inulin: Properties, health benefits and food applications. *Carbohydrate polymers.* 2016;*147*:444-454.
7. BardayT, Cooper M, Petrosky N. Inulin–a versatile polysaccharide with multiple pharmaceutical and food uses. *J Excipients Food Chem.* 2010;*1*:24-42.
8. Glibowski P, &Bukowska A . The effect of pH, temperature and heating time on inulin chemical stability. *Acta Scientiarum Polonorum Technologia Alimentaria.* 2011;*10*(2):189-196.
9. Vijayaraghavan K, Yamini D, Ambika V, &Sowdamini N . Trends in inulinase production–a review. *Critical reviews in biotechnology.* 2009;*29*(1):67-77.
10. Chi Z, Chi Z, Zhang T, LiuG, Li J, & Wang X. Production, characterization and gene cloning of the extracellular enzymes from the marine-derived yeasts and their potential applications. *Biotechnology advances.* 2009;*27*(3):236-255.
11. Singh R. S, & Singh R. P . Production of fructooligosaccharides from inulin by endoinulinases and their prebiotic potential. *Food Technology and Biotechnology.* 2010;*48*(4):435.
12. Liu X. Y, Chi Z, Liu G. L, Wang F, Madzak C, & Chi Z. M . Inulin hydrolysis and citric acid production from inulin using the surface-engineered Yarrowialipolytica displaying inulinase. *Metabolic Engineering*, 2010;*12*(5):469-476.
13. Yun J. W, Kim D. H, Kim B. W, Song SK. Production of inulo-oligosaccharides from inulin by immobilized endoinulinase from Pseudomonas sp. *Journal of fermentation and bioengineering.* 1997;*84*(4):369-371.
14. Sharma A. D, Kainth S, & Gill P. K . Inulinase production using garlic (Allium sativum) powder as a potential substrate in *Streptomyces* sp. *Journal of Food Engineering.* 2006;*77*(3):486-491.
15. Zhou J, Peng M, ZhangR, Li J, Tang X, Xu B& Huang Z . Characterization of Sphingomonas sp. JB13 exoinulinase: a novel detergent-, salt-, and protease-tolerant exoinulinase. *Extremophiles.* 2015;*19*(2):383-393.
16. Gavrailov S, & Ivanova V . Biosynthesis of inulinases by Bacillus bacteria. *Journal of BioScience& Biotechnology.* 2014;12:22-44
17. Ramapriya R, Thirumurugan A, Sathishkumar T, &Manimaran D. R . Partial purification and characterization of exoinulinase produced from Bacillus sp. *Journal of Genetic Engineering and Biotechnology.* 2018;*16*(2):363-367.
18. Korczak R, &Slavin J. L . Fructooligosaccharides and appetite. *Current Opinion in Clinical Nutrition & Metabolic Care.* 2018;*21*(5):377-380.
19. Meyer D, &Stasse-Wolthuis M . The bifidogenic effect of inulin and oligofructose and its consequences for gut health. *European journal of clinical nutrition*, 2009; *63*(11): 1277-1289.
20. Sivieri K, Morales M. L. V, Saad S. M, Adorno M. A. T, Sakamoto I. K, & Rossi E. A . Prebiotic effect of fructooligosaccharide in the simulator of the human intestinal microbial ecosystem (SHIME® model). *Journal of medicinal food.* 2014;*17*(8):894-901.
21. MacFaddin J.F . Biochemical tests for the identification of medical bacteria, 3PrdP ed., The Williams and Wilkins-Baltimor, U.S.A. 2000; .3:55.
22. Leboffe M. J and Pierce B. E . Atlas of microbiology laboratory. 4th edition. P. 62. USA: Morton Publishing Company.2011; 62:56
23. Singh RS, ChauhanK, Kennedy JF. A panorama of bacterial inulinases: production, purification, characterization and industrial applications. *International journal of biological macromolecules.* 2017;*96*:312-322.
24. Bradford M. M . A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry.* 1976;*72*(12):248-254.
25. Miller G. L . Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry.* 1959;*31*(3):426428.
26. Oliveira KG, Silvaa, M. F, Dallagoa, R. M., Dalla Rosaa, C, Treichelb H, Oliveira D, &OliveiracJ. V. Synthesis of Fructo-oligosaccharides from *Aspergillus niger* commercial inulinase immobilized in polyurethane foam and pre-treated in pressurized LGP. 2013;25:981
27. Nobre C, Santos M. J, DominguezA, Torres D, Rodrigues L. R, Rocha I& Ferreira E. C . Fractionation of fructooligosaccharides by gel filtration. Critical reviews in food science and nutrition. 2000;55(10):1444-1455.
28. Hadid M. A . Treatment of bacteria causing burn wound infections by Lactobacillus Reuteri as a probiotic. World Journal of Pharmaceutical Research. 2015;5(2):116-125.
29. AngelS. J, Kavitha C, Vidyadharani G, Roy P, & Dhandapani R. Isolation of inulinase producing bacteria from sugarcane soil. Int J Appl Biol Pharm Technol. 2012;3(4):320-326.
30. Golunski S, Silva MF, Marques CT, Rosseto V, Kaizer RR, Mossi AJ, Treichel H. Purification of inulinases by changing the ionic strength of the medium and precipitation with alcohols. Anais da Academia Brasileira de Ciências. 2017;89(1):57-63.
31. El-souod SMA, Mohamed TM, Ali EM, El-badry MO, El-keiy MM. Partial purification of extracellular exoinulinasefrom Ulocladiumatrum. Journal of Genetic Engineering and Biotechnology. 2014;12(1):15-20.
32. Muslim SN; and kareem SM . Purification of levanase from *Enterobacter aerogenes* and using it as a prebiotic and antibacterial agent, IJDDT. 2019;9(3):389-393.
33. Mohammed AN, Muslim SN. Enhancement the antimicrobial activity of disinfectants by the purified lipase from *coronobacter dublinensis*. Indian J. Forensic Medicine& Toxicology. 2020;14(1):394-400.
34. Khanvilkar S. S, & Arya S. S . Fructooligosaccharides: applications and health benefits. Agro FOOD Industry Hi Tech. 2015;26(6).
35. Sabater-Molina M, Larqué E, Torrella F, & Zamora S . Dietary fructooligosaccharides and potential benefits on health. Journal of physiology and biochemistry. 2009;65(3):315-328.
36. BibasBonet ME, Meson O, Dogi CA, Chaves S, Kortsarz A, Perdigon G. Prebiotic effect of yacon (Smallanthussonchifolius) on intestinal mucosa using a mouse model. Food and agricultural immunology. 2010;21(2):175-189.
37. Caetano BF, De Moura NA, Almeida AP, Dias MC, Sivieri K, Barbisan LF. Yacon (Small anthusson chifolius) as pathogenesis by intestinal short-chain fatty acids. In Advances a food supplement: health-promoting benefits of fructo-oligosaccharides. Nutrients. 2016;8(7):436.
38. Slavin J. Fiber and prebiotics: mechanisms and health. 2013;5(4):1417-1435.
39. Sun Y, & O'Riordan M. X . Regulation of bacterial benefits. in applied microbiology. 2013;85:93-118.