Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.5, No.8, 2015
www.iiste.org

Improving the antibacterial activity of ceftazidime by inulinase purified from *Staphylococcus aureus*

Sahira Nsayef Muslim, Alaa Naseer Mohammed Ali , Sanaa N. Hussein, Istabreq Muhammed Ali Salman, Suhad Abbas Abid and AL-Maamoon H.Abed
Department of Biology, College of Science, AL-Mustansiryia University

Abstract

Inulinase is an enzyme catalyzing the hydrolysis of inulin, a plant stored polysaccharide, into fructoses and fructo- oligosaccharides that have a large spectrum of applications ranging from food industry to bioethanol production and pharmacology.Eight isolates of *Staphylococcus aureus* were isolated from agricultural rhizosphere soil samples with isolation percentage(32%) and screened for higher inulinase production and found that *Staphylococcus aureus*S_3 was the best producer. Inulinase was partially purified with ammonium sulfate at 70% saturation and the specific activity reached to (7.01)U/mg protein. Inulinase led to enhancement ceftazidime activity against the bacteria and gram positive bacteria more sensitive than negative bacteria to combination of inulinase and ceftazidime. These finding indicate that antistaphylococcal activity of ceftazidime antibiotic has increased in the presence of inulinase enzyme and the inulinase may be useful adjuvant agent for the treatment of *S. aureus* infections in combination with this antibiotic.

Introduction

The rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms. The rhizosphere contains many bacteria that feed on the secretions of plant cells, that called rhizodeposition, and the proteins and sugars released by roots. Protozoa and nematodes that found in this position are also more abundant in the rhizosphere. Thus, much of the nutrient cycling and disease suppression needed by plants occurs immediately adjacent to roots. A large proportion of the root exudates such as sugars, organic acid anions or amino acids are easily degradable by microorganisms in the rhizosphere resulting in high microbial density and activity in the rhizosphere [1]. The most common genera of bacteria are: *Pseudomonas, Arthrobacter, Agrobacterium, Alcaligenes, Azotobacter, Mycobacterium, Flavobacter, Cellulomonas, Micrococcus* and others have been reported to be either abundant or sparse in the rhizosphere[2,3]

Bacteria in the genus *Staphylococcus* are pathogens for human and animals. *S aureus* expresses a variety of extracellular proteins and polysaccharides that assotiated with threi virulence[4]. This bacterium IS frequently related with food poisoning, since the mucous membranes of the human nasopharynx and animal skin are considered the primary habitat of *Staphylococcus aureus*. This bacterium is also found in soil, water sources, dust and air[5].

The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains. *Staphylococcus aureus* is an important pathogen both in community acquired and healthcare associated infections. The organism has successful evolved numerous strategies for resisting the action to practically all antibiotics[6]. Resistance to methicillin is now widely described in the community setting (CMRSA), therefore, the search on new therapies is immediately necessary.

Endoinulinase (2,1-b-D-fructano -hydrolases EC 3.2.1.7) is important microbial enzyme which hydrolyses inulin, usually stored in the roots,bulbs and tubers of composite plants such as Jerusalem artichoke and Chicory[7], to produce fructose and fructooligosaccharides. The inulin and fructooligosaccharides(FOS) are known a non- digestible food ingredients that beneficially affect the health status of human body[8,9] and acting as a prebiotic agents in the intestine[10], in addition to decreasing of total cholesterol and lipid in the serum and [11]. The fructose can be used in many food and beverage industries [9,12].

Filamentous fungi, yeast and bacteria are all capable of producing inulinases and many of them have been successfully used for enzyme production Microbial inulinases are an important class of industrial enzymes, which are usually inducible and extracellular[10].Among the bacteria that producing inulinase, *Bacillus* spp., *Pseudomonas* spp., *Arthrobacter* spp. and *Streptomyces* sp.[13]. The aim of this study was to investigate the ability of *Staphylococcus aureus* to produce inulinase besides to partial purification of inulinase and investigation of antibacterial activity for inulinase with antibiotic combinations.

Materials and Methods

Samples collection

Twenty-five agricultural rhizosphere soil samples were collected from different locations by using pre-sterilized sample bottles and sterile spatula from garden in Al-Mustansiriya university. One gram of each of the samples was suspended in 10 ml of sterile distilled water and shake vigorously for 10 min. later, 0.1 of the

resulting liquid was spread on the surface of blood agar and MacConkeys agar, then incubated at 30 ℃ for 18-24 hour using L- shaped glass rod[14].

Bacteriological analysis

The bacterial isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of Systemic Bacteriology. Suspicious isolates of *Staphylococcus aureus* were identified by characteristic colonies (Round, smooth, white, creamy colonies with beta-hemolysis), Gram staining pattern and standard biochemical reaction like catalase test(+),oxidase test(-), coagulase test(+), citrate utilization test(+), DNase test (-), histamine assimilation test (-), glucose oxidation test, gelatin liquefaction test (-), urease activity(-), unability to motile, cocci in clusters and ability to grow on manitol salt agar[15,16]. Also the selective medium(Baird-Parker Agar) was used for the isolation of *Staphylococcus aureus* and prepared according to the method that described by[17]. Further, the *Staphylococcus aureus* isolate was confirmed by using Vitek 2 system by using Vitek GPI card (bio Merieux, France) according to the manufacturer's instructions.

Primary screening for inulinase activity.

All the bacterial isolates were inoculated in to the inulin agar plates containing 2g/L of inulin, 10g/L of yeast extract , 20 g/L of $MgSo_4.7H_2O$, 2g/L of KCl, 10% of NaCl, 20g/L of agar. Inulin was used as the sole source of carbon in this medium, thus, bacteria growth after 24 hrs of incubation at 30℃ shows this presence of inulinase activity, then the diameters of clear zones around the colonies were measured [18].

Secondary screening for inulinase activity

The selected bacteria isolates were transferred into 10 ml of a medium contained (g/L): inulin 20, yeast extract 20, $(NH_4)_2H_2PO_4$ 5, $NH_4.H_2PO_4$ 2, $MnCl.4H_2O$ 0.5, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, and pH was adjusted to 7.0. The bacteria isolates were incubated on the rotary shaker at 100 rpm at 30℃ for 24 hour. After removal of cells by centrifugation at 10000xg for 20 min. the enzyme activity, protein content and specific activity were assayed[19].

Assay of inulinase

Endoinulinase activity was assayed by incubating 2 ml enzyme solution with 2% (w/v) inulin prepared in 10 mM citrate-phosphate buffer pH 7.0 at 35℃ for 60 min. After incubation, the reaction tubes were kept in a boiling water bath for 10 min. to stop the enzyme reaction and then cooled to room temperature. The reaction mixture was assayed for reducing sugar as fructose by DNS method as described by[18] by reading the absorbance at 575 nm. The calibration curve was prepared with fructose solutions of known concentration and blanks were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme, which produced 1 µ mole of fructose under the assay conditions.

Protein determination estimation

Protein content was determined by the method of [20], using bovine serum albumin as a standard.

Purification of inulinase

Inulinase was purified by modification the method that described by[21]. At the end of incubation period, the cells were separated by centrifugation at 10000 rpm for 20 min (under cooling) and the clear supernatant (crude extract) was partially purified by salting out with ammonium sulphate at concentration (40-80%) saturations. All samples were left overnight at 4℃. The precipitates were collected by centrifugation at 1000 rpm for 15 min, dissolved in 5ml of acetate buffer (0.2M, pH 6.0) and dialyzed overnight against the same buffer.

Determination of minimum inhibitory concentration (MIC)

The ceftazidime antibiotic was screened for antimicrobial activity using the macrodilution method [22] against *S. aureus* and *E. coli*. To determine the minimum inhibitory concentrations (MICs), this antibiotic was dissolved in distilled water to give a stock concentration of 10000 µg/ml. The stock concentration of antibiotic was filter-sterilized using 0.22 µm millipore filter. Twofold serial dilutions of the antibiotic was made with nutrient broth to give concentrations ranging from 1 to 5000 µg/ml. one hundred microliter of 10^4 cfu/ml bacterial suspension(*S. aureus* and *E. coli*, separately) was added to the sterile capped test tubes. 50 µl from each dilution was placed in wells (7 mm in diameter) on Mueller-Hinton agar medium. All tubes and plates were then incubated at 30 ℃ for 18 to 24hour. The lowest concentration of antibiotic showing no visible growth in case of tubes using and inhibition zone in case of plates using was recorded as the minimum inhibitory concentration (MIC).

Determination of interaction between inulinase and antibiotic

Combination of inulinase and ceftazidime was tested by using the same methods as above against *S. aureus* and *E. coli*, since one hundred microliter of bacterial suspension and inulinase were added separately to two fold serial dilutions of the antibiotic. 50µl from each dilution was placed in wells (7 mm in diameter) on Mueller-Hinton agar medium. All tubes and plates were then incubated at 30℃ for 18 to 24 hour. The lowest concentration of antibiotic showing no visible growth in case of tubes using and inhibition zone in case of plates using was recorded as the minimum inhibitory concentration (MIC).

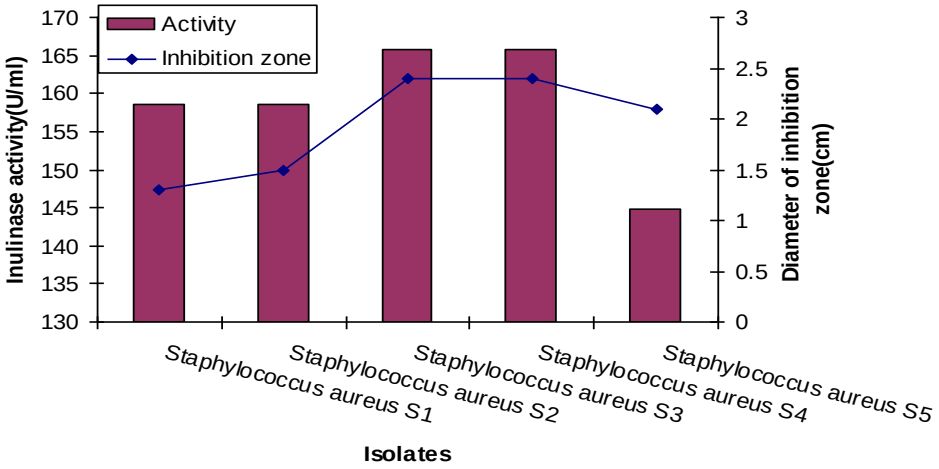
Results and Discussion

Isolation of *Staphylococcus aureus*

In an attempt to determine the distribution of *Staphylococcus* in nature, 25 agricultural rhizosphere soil samples were collected. Eight *Staphylococcus aureus* isolates(32%) were obtained out of 13 *Staphylococcus* spp. isolates. *Staphylococcus aureus* was diagnosed by growing in the selective medium Baird-Parker Agar which is a differential medium for the isolation and enumeration of *Staphylococcus aureus*[17]. The presence of *Staphylococcus aureus* in the soil suggest that these bacteria have an important role in the aerobic mineralization of organic materials[3]. *Staphylococcus aureus* strains are highly distribution in water and soil and do not need the growth factors [5]. The common bacteria, *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* are found in all the soil samples including road side soil, industrial and garden soils[3].

Screening of inulinase producing from *Staphylococcus aureus*

Out of 8 *Staphylococcus aureus* isolates were screened for inulinase production in inulin agar plates, 5 isolates revealed inulinase activity by growing in this production medium. All these five isolates were subjected to secondary screening for inulinase production in liquid culture and the results showed that *Staphylococcus aureus* S_3 was the best inulinase producer as shown in figures(1) and (2). In a study revealed by (23) found that inulinase production by many bacterial species such as *Pseudomonas*sp. and *Lactobacillus casei* reached the maximum level after twenty- two hours hour of incubation. Intracellular inulinase produced by *Flavobacterium multivorum* reached to higher productivity at the end of the growth phase (24). *Bacillus* is also active producer of extracellular inulinase by using the sucrose as substrate(25). on the other hand, the inulin was used as substrate for inulinase production by *Pseudomonas* sp and *Streptomyces* sp. (26, 27).



Figure(1): Diameter of inhibition zone and inulinase activities for *Staphylococcus aureus* isolates

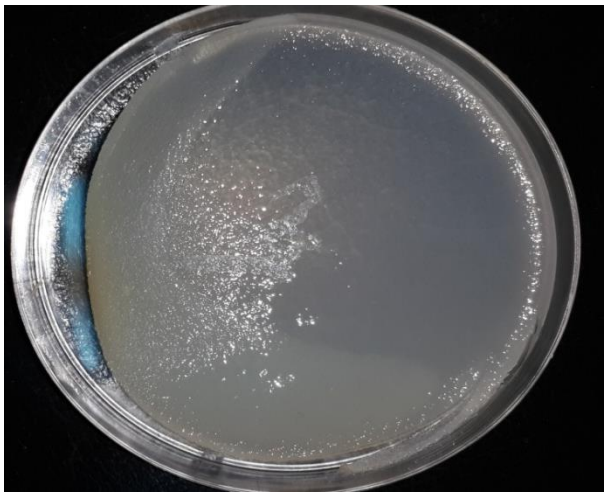


Figure-2: *Staphylococcus aureus* inulinase hydrolyzed the inulin in inulin agar plate

Inulinase Purification

Staphylococcus aureus S_3 was chosen for purification endoinulinase by growing in the broth culture containing the inulin and table (1) showing the results of partial purification. Ammonium sulphate at 70% saturation was led to precipitate the enzyme from the cell-free supernatant with higher specific activity in precipitation step. The crude endoinulinase preparation is directly useful for large scale production of inulooligosaccharides from inulin-containing agricultural crops (28). An inulinase produced by *Aspergillus niveus* was purified by using ammonium sulphate precipitation and ion exchange followed by gel filtration on DEAE- cellulose and sephadex G-15 columns, respectively(14). Also the inulinase produced from from *Streptomyces* sp. was purified salt precipitation and column chromatography. In precipitation step, ammonium sulphate was used frequently because of its high solubility, the cost and the density were low (29). Polyethylene glycols and polyvinyl pyrolidones can have very large molecular masses (i.e. 20,000 Dalton). They are also readily dissolved in water. If the sample in a dialysis bag is coated with dry forms of the above polymers, water will leave the dialysis bag (it can go through the pores) and hydrate the polymers. The result is a decrease in volume of buffer in the dialysis bag (the protein will be concentrated) in addition to removal the salts[29].

Table(1): Summary of treatments used for the partial purification of inulinase from *Staphylococcus aureus* S_3

Purification step	Size (ml)	Protein conc. (mg/ml)	Inulinase activity (U/ml)	Specific activity (U/mg)
Crud extract	150	112.6	165.8	1.47
$(NH_4)SO_4$ 70%	70	34.3	240.7	7.01

Antibacterial activity of ceftazidime

The results that shown in table(2) revealed that ceftazidime antibiotic had low antibacterial activity against the tested isolates, The MICs value for *S. aureus* was 256 µg/ml and for *E. coli* was 1024 µg/ml. On the other hand, an inulinase and ceftazidime mixture had high antibacterial activity against the tested isolates. The minimum inhibition concentration of ceftazidime decreased to 64 µg/ml in the case of *S. aureus* and 16 µg/ml in the case of *E. coli*. Similar results were showed by using agar diffusion method since in this method at a concentration of 16 µg/ml the diameter of inhibition zone for *S. aureus* reached to 19 mm and at a concentration 64 µg/ml the diameter of inhibition zone reached to 22 mm for *E. coli* (figure-3 and 4). According to the result we can see that gram positive bacteria more sensitive than negative bacteria in combination of inulinase and ceftazidime in comparison with control. These finding indicate that β-lactam antibiotics have increased antistaphylococcal activity in the presence of inulinase enzyme and may be useful adjuvant agent for the treatment of *S. aureus* infections in addition to β-lactam antibiotics.

Table-2: MICs(µg/ml)of ceftazidime for *S. aureus* and *E. coli* strains in tubes and plates with or without inulinase

Antibiotic	Bacteria	MIC for antibiotic alone (µg/ml)	Diameter of inhibition zone(mm)	MIC for antibiotic+ inulinase (µg/ml)	Diameter of inhibition zone(mm)
Ceftazidime	*E. coli*	1024	28	64	22
	S. aureus	256	26	16	19

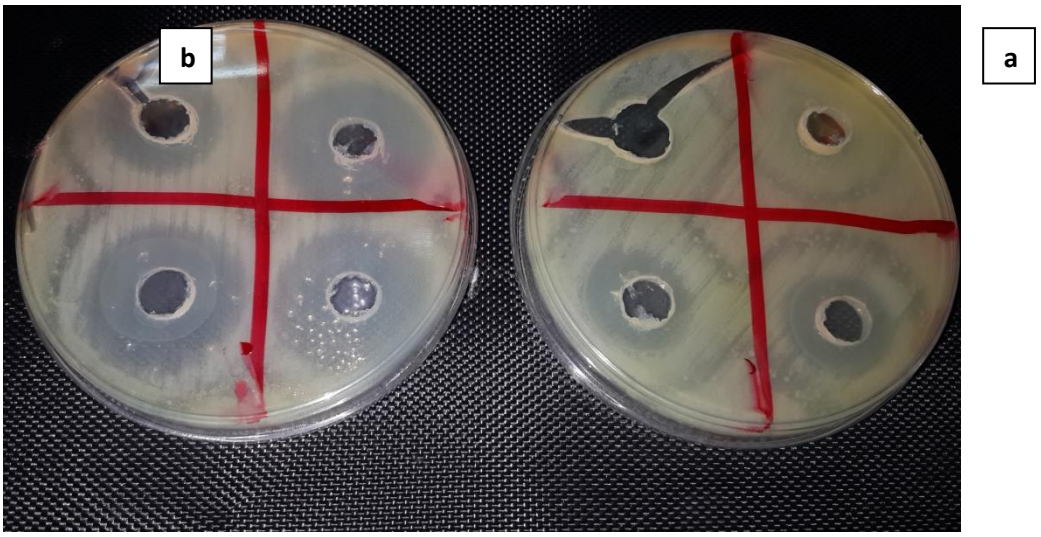


Figure-3: a)Diameter of inhibition zones of ceftazidime against *E. coli.*

b)Diameter of inhibition zones of ceftazidime and inulinase combination against *E. coli*

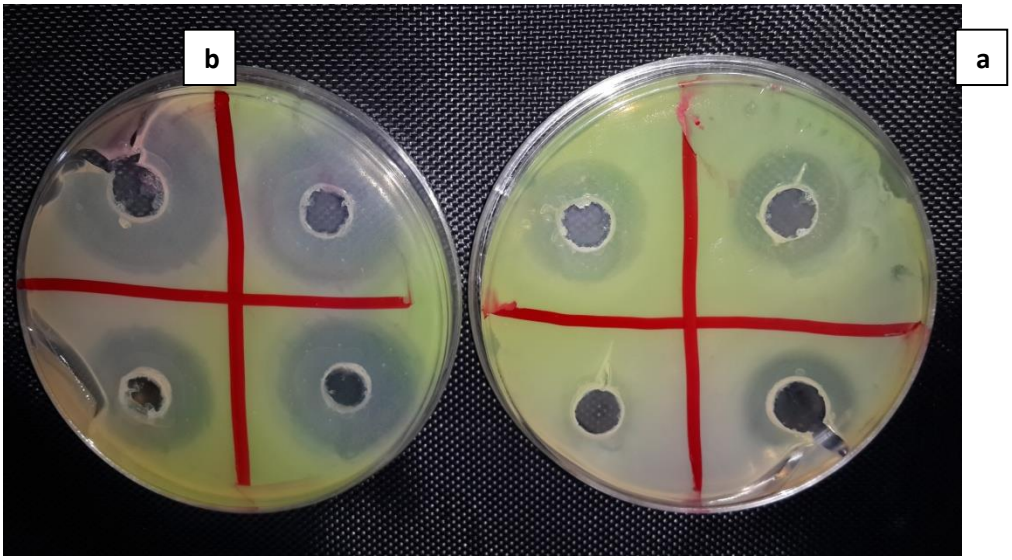


Figure-4: a)Diameter of inhibition zones of ceftazidime against *S. aureus*

b)Diameter of inhibition zones of ceftazidime and inulinase combination against *S. aureus*

References

1- Rawat, S.; Izhari A. and Khan, A.(2011).Bacterial Diversity in Wheat Rhizosphere and their Characterization. Advan. Appli. Scien. Res.2 (2): 351-356.

2- Sahin, F., R. Cakmakci and F. Kantar.(2004). Sugar beet and barley yields in relation to inoculation with N2-fixing and phosphate solubilizing bacteria. *Plant and Soil*.265: 123-129.

3- Abdulkadir, M., and Waliyu,S.(2012).Screening and Isolation of the Soil Bacteria for Ability to Produce Antibiotics. European Journal of Applied Sciences. 4(5:211-215.
4-Easmon, C.S. and Adlam, C.(1983).Staphylococci and staphylococcal infections. Vol 1 and 2. Academic Press, London.
5- Kumar,N.; Singh, R.K. Mishra, S.K. and Singh, A.K. Pachouri U.C.(2010).Isolation and screening of soil *Actinomycetes* as source of antibiotics active against bacteria. Intern. J. Microbiol. Res. 2(2):12-16.
6-Chung,P.Y.; Navaratnam,P. and Chung,L.Y.(2011).Synergistic antimicrobial activity betweenpentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains. Ann. Clin. Microbiol. Antimicrob.10:25.
7-Fawzi, E.M.(2011).Comparative Study Of Two Purified Inulinases From thermophile *Thielavia Terrestris* Nrrl 8126 and mesophile *Aspergillus Foetidus* Nrrl 337 Grown On *Cichorium Intybus* L. Brazilian J. Microbiol. 42: 633-649.
8-Gao,L.; Chi, Z.; Sheng, J.; Wang, L.; Li ,J. and Gong, F.(2007). Inulinase producing Marine Yeasts: Evaluation of their Diversity and Inulin Hydrolysis by Their Crude Enzymes. Microbial Ecology. 54:722–729.
9-Dilipkumar, D.; Rajasimman, D.; and Rajamohan, N.(2011).Responce surface methodology for the optimization of inulinase production by K. *Marxianus var. marixianus*. J. Appl. Scien. Environ. Sanit. 6 (1): 85-95.
10-Bharathi, S.; Saravanan, D.; Radhakrishnan, M.; Balagurunathan, R.(2011). Bioprospecting of Marine Yeast with special reference to Inulinase production. International J. Chem.Tech. Research. 3(3):1514-1519.
11- Nascimento, D.; Jiunio,V.; Fernades,P.; Ribero, G.; Danyo, M.; and Sandra, A.(2012). Production, characterization and application of inulinase from fungal endophyte CCMB 328. Ann. Brazilian Acade. Scien.84(2):443-453.
12-Nandagopal, S. and Kumari, B.D. (2006). Enhancment of inulinase production from Chicory and Fenugreek rhizosphere soil. American-Eurasian J. Agrric. Environ.Sci., 1(3):225-228.
13-Singh, R.S. and Singh, R.P.(2010). Production of Fructo-oligosaccharides from Inulin by Endoinulinases and Their Prebiotic Potential. Food Technol. Biotechnol., *48* (4):435–450.
14- Kamble, K. D.; Bidwe, P. R.; Muley, V. Y.; Kamble, L. H.; Bhadange, D. G. and Musaddiq, M.(2012). Characterization of l-asparaginase producing bacteria from water, farm and saline soil. Bioscience Discovery, 3(1):116-119.
15- Holt, J.G.(1994). Bergey's manual of determinative bacteriology. 2nd ed. Vol.2, Williams and Wilkins. Baltimore.
16-Ahmed, R. N.; Sani, A.; Ajijolakewu,G. andAlamu, f.b.(2013).Soil Screening For Antibiotic - Producing Microrgansms. Advances in Environmental Biology, 7(1): 7-11.
17-Baird-Parker, A. C. (1962). An improved diagnostic and selective medium for isolating coagulase-positive staphylococci. J. Appl. Bacteriol. 25:12-19.
18- Jenny, S.; Kavitha1, C.; Vidyadharani1, G. ; Priya, R. and Dhandapanil, R.(2012).Isolation of inulinase producing bacteria from sugarcane soil. Internat. J. appli. biol. pharmaceut. technol.3(4):320-326.
19- S. Jenny Angel1, C. Kavitha1, G. Vidyadharani1, Priya Roy1 and R. Dhandapani1. (2012). Isolation of inulinase producing bacteria from sugarcane soil. International Journal of applied biology and pharmaceutical technology.3(4):320-326.
20- Bradford, M.(1976). A rapid and sensitive method for the quantitation of microgram quantitities of protein utilizing the principle of protein dye binding, Annal . Biochem.72:248-254.
21- Fawzi, E.M. (2011) . Comparative study of two purified inulinases from thermophile *Thielavia terresteris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337 grown on *Cichorium intybus* L. Brazilian Journal of Microbiology . 42: 633-649.
22- National Committee for Clinical Laboratory Standards (NCCLS): Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. NCCLS document; 2000, M7-A5.
23- Jenny Angel1, S.; Kavitha1, C.; Vidyadharani1, G.; Royl. P. and Dhandapani1. R.(2012).Isolation of inulinase producing bacteria from sugarcane soil. International Journal of applied biology and pharmaceutical technology.3(4):320-326.
24- Kim, D.; Choi, Y.; Song, K. and Yun, W. (1997). Production of inulo-oligosaccharides using endo-inulinase from a *pseudomonas* sp. Biotechnology Letters. Vol.19(4): pp. 369–371.
25- Camelia, N.; and Gabriela, B. (2011). Inulinase- a veasatile tool for biotechnology. Innovative Romanian Food Biotechnology Vol. 9(2): 1-11.
26- Fawzi, E.M. (2011) . Comparative study of two purified inulinases from thermophile *Thielavia terresteris* NRRL 8126 and mesophile *Aspergillus foetidus* NRRL 337 grown on *Cichorium intybus* L. Brazilian Journal of Microbiology . 42: 633-649.
27- Laowklom, N.; Chantanaphan, R.; and Pinphanichakarn,P. (2012). Production, Purification and Characterization of Inulinase from a Newly Isolated *Streptomyces* sp. CP01. *Natural Resources*, 3: p. 137-144.
28- Cho, Y.; andYun, J.W. (2002). Purification and characterization of an endoinulinase from *Xanthomonas oryzae* No.5. Process Biochemistry 37: pp.1325–1331.
29- Scopes, R.K.(1982). Protein purification principles and practice.2 nd ed. Springer-Verlag New York Inc. U.S.A.