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Optimum Condition of Inulinase Production from *Bacillus cereus* (Be9)

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Abstract
Fifty isolates of *Bacillus* spp were obtained from rhizosphere soil of compositae plant roots. The ability of inulinase production by these isolates was screened. *Bacillus* Be9, which isolated from soil of lettuce root, was the highest inulinase producer; it was identified as *Bacillus cereus.* Optimal culture medium and condition for inulinase production were determinatd; the highest inulinase production was obtained when the bacteria was cultured in inulin medium which contained 0.5% inulin, 0.4% peptone as carbon and nitrogen source at pH 7.0 inoculated with 1ml of bacterial suspension and incubated at 40˚C for 48hrs.

Keywords: Inulinase ; Production ; *Bacillus cereus* ; Optimum condition.

الظروف المثلى لانتاج انزيم الانيولينيز من بكتريا *Bacillus cereus* Be9

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الخلاصة:
تم الحصول على 50 عزلة بكتيرية تنتمي الى جنس ال *Bacillus* من التربة المحيطة بجذور نباتات العائلة المركبة واختبرت قدرة هذه العزلات على انتاج انزيم الانيولينيز, وبينت النتائج ان العزلة *Bacillus* Be9 المعزولة من تربة الخس هي اكفأ عزلة منتجة للانزيم وقد شخصت على انها احدى سلالات *Bacillus cereus*. تم تعيين الظروف المثلى لانتاج الانزيم ووجد ان افضل وسط لانتاج الانزيم هو وسط الانيولين اللذي يحتوي على 0.5% من الانيولين و 0.4% من البيتون كمصدر للكاربون وللنتروجين عند الرقم الهيدروجيني 7 ولقح ب 1مل من عالق الخلايا البكتيرية وحضن بدرجة 40 م° ولمدة 48 ساعة.

Introduction

Microbial inulinases are belong to an important class of industrial enzymes that have gained increasing attention in the recent years because of its wide spectrum of applications including: ultra-high fructose syrup obtaining from inulin, bioethanol productio, inulo-oligosaccharide production, single-cell oil and single-cell protein production, citric acid, butanediol, alcohols and lactic acid production [1].

Inulin is a well-known fructan particularly abundant in some plants belonging to families Asteraceae, Campanulaceae, Poaceae, Liliaceae and Amaryllidaceae [2]. It is made of linear chains of d-fructofuranose molecules linked by *β*- 2,1-glycosidic bonds and has a d-glucose moiety at the reducing end. Inulin and its partially hydrolyzed products (fructooligosaccharides) have gained significant importance in food and pharmaceutical industries. Fructooligosaccharides are popular functional food components due to their beneficial health properties, such as bifidogenic nature, low calorie diet and rich source of dietary fibre [3]. Inulinase, as a kind of hydrolases, can be divided into endoinulinase and exoinulinase. The endoinulinases, without invertase activity, can only cut the

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internal linkages in inulin to yield inulooligosaccharides, while the exoinulinases remove the terminal fructose residues from the non-reducing end of the inulin to yield fructose or glucose [4]. Generally, the inulinase activity invertase activity (S) and the enzymatic complex accompany (me) is characterized by I/S ratio. When I/S ratio is higher than 10^{-2}, the enzyme complex has a preponderate inulinase activity, while for invertase activity the I/S ratio is lower than 10^{-4} [5]. The purpose of this study was to determine of the optimum conditions for highest inulinase production by *B.cereus* Be9.

Materials and methods

Collection of Samples: Sixty samples from rhizosphere soils of plant roots included(Lettuce, Cabbages, Cauliflower, Leek and Barley) were collected in sterile containers and transported to the laboratory until usage.

Isolation of *Bacillus* spp.

One gm of each soil sample was mixed with 9 ml of sterile water and shacked to homogenize, then heated to 80˚C for 15 min in water bath. Serial dilutions were made for each sample using sterile water. 0.1ml from each dilution was spreaded on a nutrient agar plates, incubated aerobically at 37^{o}C for 24 hrs. The pronounced colonies were reinoculated several time on nutrient agar to obtain pure culture. The Bacterial isolates were identified as *Bacillus* spp. according to the morphological and microscopic examination.

Screening for inulinase production from *Bacillus* spp.

Semi-quantitative method

The bacterial isolates were cultured on inulin agar plate consisted of (0.5g Inulin, 0.4g Peptone and 10mM $MgSO_4.7H_2O$, 2g Agar-Agar dissolved in 100 ml D.W). The pH was adjusted to 7.0 and sterilized at 121 ^{o}C for 10 min. The plates were incubated at 40˚C for 48hrs. The Colonies were selected for further experiments and transferred to a fresh medium.

Quantitative method [6[

Ten ml of inulin broth medium consisted of (0.5g Inulin, 0.4g Peptone and 10mM $MgSO_4.7H_2O$ dissolved in 100 ml D.W) were inoculated with 0.1ml of the selected isolates(O.D=0.4at 600nm) and incubated at 40ºC for 48 hr. The cell-free supernatants were obtained by cooling centrifuge at 6000 rpm for 30 min. The supernatant were assayed for inulinase and invertase activity by measuring the reducing sugars released from inulin and sucrose, respectively.

Assay of inulinase activity [6]

The inulinase activity was assayed by determining the reducing sugars formed during incubation of soluble enzyme as follow. 0.2ml of cell-free supernatant was mixed with 0.8ml of reaction solution 1% inulin (1g of inulin in 100ml of 0.1M of potasium phosphate buffer pH7.0) the mixture was incubated at 40ºC for 30 minutes. The enzyme reaction was stopped by adding 1ml of DNSA. Then incubated in boiling water bath for 5 minutes and cooled in ice bath. Five milliliter of D.W. was added to each tube and mixed well and the optical density of the solution was measured at 540nm. The enzymatic activity was calculated based on the standard curve of fructose.

One unit (U) of inulinase activity was defined as the amount of enzyme that produces 1µmol of fructose per minute under the specified conditions

Assay of invertase activity [7]

Enzymatic activity of invertase was estimated by the same previous steps as in inulinase assay except the 1% sucrose was used as a substrate instead of 1% inulin and the enzymatic activity was calculated based on the standard curve of mixture of glucose and fructose .One unit (U) of invertase activity was defined as the amount of enzyme that hydrolyzes 1µmol of sucrose per minute under the specified conditions .

Protein concentration in the supernatant was determine by method described by Lowry *et al*.,[8].

Enzyme activity (U/ml)= O.D (540 nm)/(Slope x volume of enzyme xincubation period)

Protein concentration (mg/ml)=O.D(600 nm)/ (Slope x 1000)

Calculation of specific activity

Specific activity (U/mg protein) = Enzyme activity (U/ml)/Protein concentration (mg/ml)

Determination of optimal conditions for inulinase production

Carbon sources.

Inulin broth (100 ml pH 7.0) containing 0.5% of different carbon sources (inulin, glucose, lactose, sucrose, maltose ,inulin +glucose ,inulin +lactose ,inulin +maltose and inulin +sucrose)were

inoculated with 1ml of activated bacterial suspension (O.D=0.4 at 600nm) and incubated at 40˚C for 48hr. Cell- free supernatant were obtained by centrifugation at 6000 rpm for 30 min. Supernatants were assayed for enzyme activity, protein concentration and specific activity was calculated.

Nitrogen sources.

Inulin broth (100ml pH7.0) containing 0.4%of different organic nitrogen sources(peptone, yeast extract, urea) and inorganic nitrogen sources (sodium nitrate and potassium nitrate) and mixture of organic and inorganic nitrogen sourses (peptone +sodium nitrate, yeast extract +sodium nitrate and yeast extract +urea)were inoculated with 1ml of activated bacterial suspension (O.D=0.4 at 600nm)and incubated at 40˚C for 48 hr. Cell- free supernatant were obtained by centrifugation at 6000 rpm for 30 min. Supernatant were assayed for enzyme activity, protein concentration and specific activity was calculated.

Incubation temperature

Inulin broth (100ml pH 7.0) were inoculated with 1ml of activated bacterial isolate (O.D=0.4 at 600nm) and incubated at different temperature (37, 40 and 50 ˚C) for 48hr. Cell- free supernatant were obtained by centrifugation at 6000 rpm for 30 min. Supernatant were assayed for enzyme activity, protein concentration and specific activity was calculated.

pH

Inulin broth (100ml pH 7.0) was prepared at different pH values (4-10) adjusted with 1N HCl or 1N NaOH .The medium were inoculated with 1 ml of activated bacterial suspension (O.D=0.4 at 600nm) and incubated at 40˚C for 48hr. Cell- free supernatant were obtained by centrifugation at 6000 rpm for 30 min. Supernatants were assayed for enzyme activity, protein concentration and specific activity was calculated.

Incubation period

Inulin broth (100ml pH7.0) was inoculated with 1ml of activated bacterial isolate (O.D=0.4 at 600nm) and incubated at 40˚C for different times (24, 48 and 72) hr. Cell- free supernatant were obtained by centrifugation at 6000 rpm for 30 min. Supernatants were assayed for enzyme activity, protein concentration and specific activity was calculated.

Inoculums size

Inulin broth (100ml pH7.0) was inoculated with different inoculums size (1,2 and 3ml) of activated bacterial isolate (O.D=0.4 at 600nm) and incubated at 40˚C for 48 hr. Cell- free supernatant were obtained by centrifugation at 6000 rpm for 30 min. Supernatants were assayed for enzyme activity, protein concentration and specific activity was calculated.

Results and discussion

Isolation of *Bacillus* spp.

Sixty samples were collected from rhizosphere soil of plant roots. Fifty bacterial isolates were obtained table 1 and identified as *Bacillus* spp. according to morphological and microscopic examination.

The result showed that the highest number of isolates (20) was obtained from rhizosphere soil of Lettuce plant roots. Table 1. Rhizosphere soil samples and compositae plant material are common sources of inulinase – producing microorganisms. This might be attributed to the adaptation of some strains to inulin-containing plants, which is generally found in compositae [9]. Inulin can be found in many plant species from mono- and dicotyledonous families, such are Liliaceae, Amaryllidaceae, Gramineae and Compositae [10].

Table 1-*Bacillus* isolates obtained from different plant roots soils

Sources of isolates	Number of *Bacillus* isolates	Code number of isolates
Lettuce	20	Be1,Be2, Be3, Be4, Be5, Be6, Be7, Be8, Be9, Be10, Be11, Be12, Be13 Be14, Be15, Be16, Be17, Be18,Be19,Be20
Cabbages	13	Bc1,Bc2,Bc3,Bc4,Bc5,Bc6,Bc7, Bc8,Bc9,Bc10,Bc11,Bc12,Bc13
Cauliflower	8	Bu1, Bu2, Bu3, Bu4, Bu5, Bu6, Bu7, Bu8
Leek	6	Bl1, Bl2, Bl3, Bl4, Bl5,Bl6
Barley	3	Bb1, Bb2, Bb3
Total	50	

Screening for inulinase producing *Bacillus*

Semi-quantitative screening

Inulin agar medium was used for screening of inulinase production. The results showed that 25 isolates out of 50 isolates were able to produce inulinase by utilization inulin as sole sources of carbon in medium at 40˚C and 48 hr incubation. Fifty bacterial strains were isolated on the basis of their growth on agar plates containing inulin as sole carbon source [11]. The rapid growth and healthy colonies of these isolates on inulin based media indicated positive inulinase activity. This has also been reported as the most common technique used for the isolation of inulinase producing microorganisms [12].

Quantitative screening

The inulinase producer isolates were quantitatively analysed using DNSA method. The obtained indicated that all bacterial isolates were inulinase producer. Among them *Bacillus* (Be9) was the most efficient one, the specific activity was (59.5 U/mg) protein, while the specific activity for the other isolates ranged between (21.5- 58.3) U/mg protein table 2.

Table 2-Production of inulinase by *Bacillus* isolates grown in inulin broth medium pH 7.0, for 48 h. incubation at 40°C.

Isolate symbol	Specific activity (U/mg) towards:		I/S ratio
	Inulinase (I)	Invertase (S)	
Be9	59.5	0.0	-
Be7	58.3	0.0	-
Bl4	58.0	0.0	-
Bc3	56.6	3.5	16.17
Bc1	56.2	0.0	-
Be2	53.7	2.2	24.41
Bc6	52.9	3.1	17.06
Bb3	50.2	2	25.1
Be3	47.9	0	-
Be5	47.3	2	23.65
Bc10	46.0	4	11.5
Bu6	44.7	5	8.94
Bl2	43.0	0.0	-
Bb2	41.8	2	20.9
Bc5	35.7	0.0	-
Be13	33.0	0.0	-
Bu7	30.8	0.0	-
Bl5	25.7	3.4	7.56
Be15	25.2	0.0	-
Be19	25.0	0.0	-
Bc12	23.7	0.0	-
Be20	22.9	0.0	-
Be11	21.9	9	2.43
Be17	21.8	5.9	3.69
Bu3	21.5	0.0	-

The I/S ratio varied between (0.0 - 25.1). The ability of the bacterial isolate to produce high level of extracellular inulinase comparing with the production of invertase has been confirmed. The differences in the inulinase production among these isolates might be due to the activity of genes encoded inulinase synthesis and source of isolation [13]. When I/S ratio is higher than 10^{-2}, the enzyme complex has a preponderate inulinase activity, while for invertase activity the I/S ratio is lower than 10^{-4} [5].

Identification of *Bacillus* Be9 isolate

The isolate (Be9) was subjected to further morphological and biochemical tests according to Bergys Manual of Systematic Bacteriology [14] the results are showen in table 3.

Table 3-Morphological and Biochemical characteristics of the *Bacillus* Be9 isolate.

Bacillus cereus	**Characteristics**
Cell shape	Rod
Spore shape	Ellipsoidal
Spore site	Central
Gram stain	+
Motility	+
Catalase	+
Oxidase	+
Voges-Proskauer	+
Mehyl Red	+
Indol	-
Egg-yolk reaction	+
Growth at 50°C	+
Citrate utilization	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Carbohydrates fermentation	
fructose	+
Glucose	+
Maltose	+
Sucrose	+

Positive result **+ , -** : negative result

The optimum condition for inulinase production from *B. cereus* Be9:

Effect of carbon source on inulinase production

The result showed that the maximum specific activity of enzyme (56 U/mg) was achieved in the presence of 0.5% inulin figure 1.

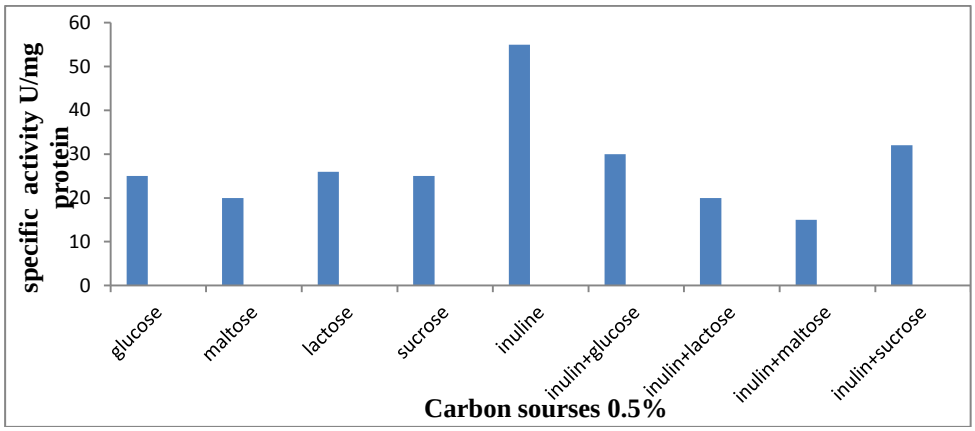

Figure 1-Inulinase production from *B. cereus* Be9 cultured in inulin broth containing different carbon sources incubated. at 40°c for 48 hrs. at pH7.0.

Among the pure substrates (which are mostly sugars of mono-, di-, or polysaccharide nature), inulin and sucrose have been employed as the preferred carbon source. In general, if the microbial strain showed only inulinase activity, inulin served as the best substrate [15]. Inulin was the best carbon source for inulinase production in B. *cereus* MU-31[16].

Effect of nitrogen source on inulinase production

The results showed a highest specific activity (57 U/mg protein), was detected when pepton was used as nitrogen source and the minimum activity was (25 U/mg protein) observed with yeast extract

figure 2. The nitrogen sources are a secondary energy sources for organisms, which play an important role in the growth of the organisms and the production of metabolites, the nature of the compound and the concentration that might stimulate or down modulate the production of enzymes [17]. It was reported that the optimum production of inulinase for *B. sphaericus* was found when pepton was used [5]. The maximum enzyme production from *B. cereus* MU-31 was found with yeast extract as a nitrogen source [16].

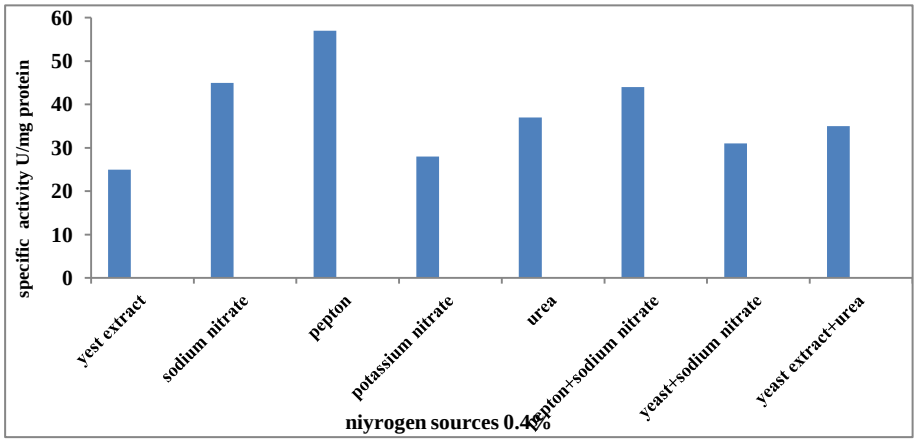

Figure 2-Inulinase production by *B. cereus* Be9 cultured in inulin broth containing different nitrogen sources and incubated at pH 7.0 at 40°c for 48 hrs.

Effect of temperature on inulinase production

The optimum temperature for inulinase production was found to be 40°C (55.3 U/mg proteins). However, the decrease or increase in the incubation temperature lead to decrease enzyme production as it was illustrated in figure 3. Most of the optimum temperature was found between 30 -55 C˚ for inulinase producers [18].

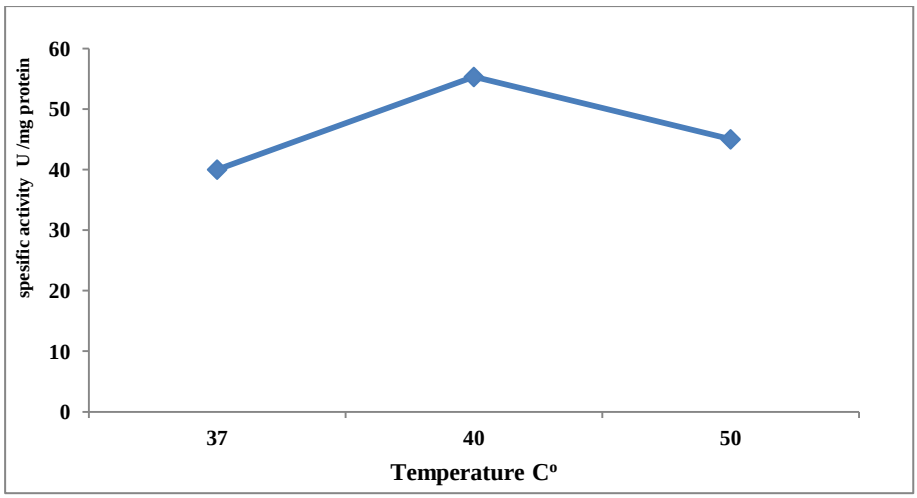

Figure 3-Inulinase production by *B.cereus* Be9 cultured in inulin broth pH7.0 incubated at different temperatures for 48 hr.

Effect of pH on inulinase production

The results showed that the enzyme was produced over pH range from 4-10 with a maximum value at pH 7.0 with specific activity 57.1 U/mg protein figure 4.The most important characteristic of microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production [19]. The pH 7.0 was found to be optimal for inulinase production by *B. polymyxa* [20], *B. subtilis* [21] and *B. cereus* MU-31 [16].

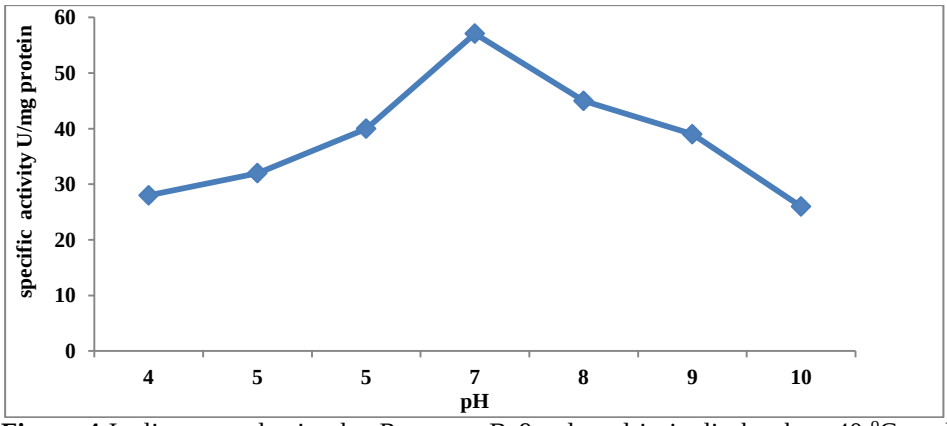

Figure 4-Inulinase production by *B. cereus* Be9 cultured in inulin broth at 40 °C. and incubated for 48hrs. at different pH values**.**

Effect of incubation period on inulinase production

The results in figure 5 showed that the production of inulinase started from 24hrs of the growth and reached its maximum in 48hr, (56U/mg protein) and then decreased with increasing the incubation time. It might be that inulinase is produced during logarithmic phase and reaches its maximum value at stationary phase.

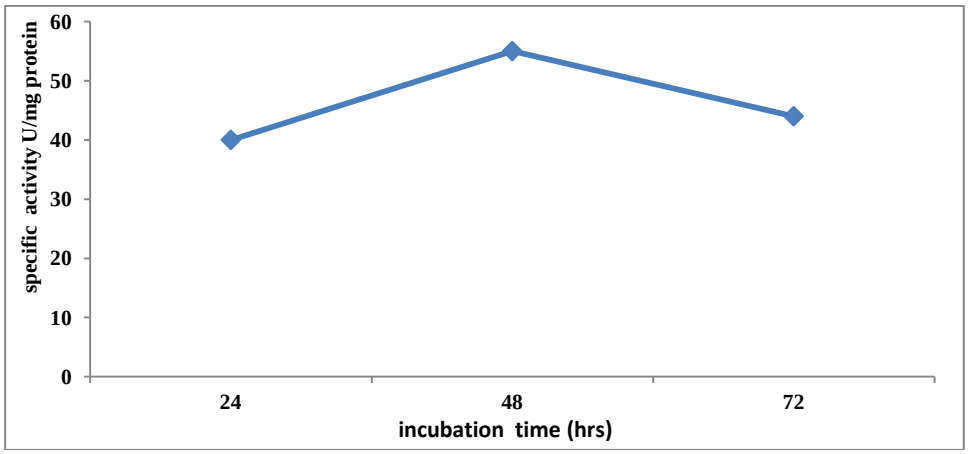

Figure 5-Inulinase production by *B.cereus Be9* cultured in inulin broth at pH 7.0 and incubated at 40°C for different incubation periods

Increase in enzyme activity as well as biomass was observed up to 20 h. incubation, which showed growth-associated production of inulinase in *B. macerans* and *Xanthomonas oryzae* [22]. Decrease in nutrient availability in the medium, or catabolic repression of enzyme could be the main reason of decline in enzyme activity after 72 hr [22].

Effect of inoculum size on inulinase production

The results showed that the production of inulinase is affected by the inoculums size figure 6. The optimum inoculums size for inulinase production was 1 ml (O.D 0.4) .The specific activity was 57U/mg protein. An inoculums concentration higher than the optimum value may produce a high amount of biomass, which rapidly depleted the nutrients necessary for growth and product synthesis. On the other hand, lower inoculums levels may give insufficient biomass and allow the growth of undesirable organisms in the production medium. This increase the necessary time to grow to an optimum number to consume the substrate and synthesize the desired product [23].

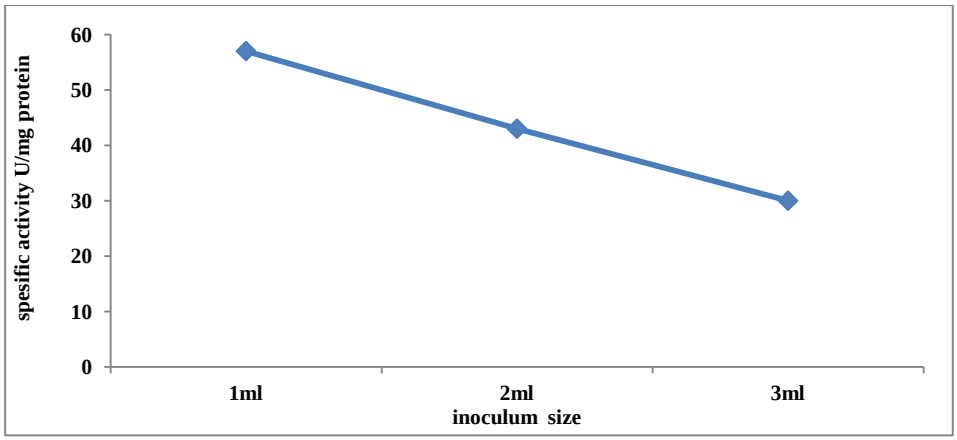

Figure 6-Inulinase production by *B. cereus* Be9 cultured in inulin broth inoculated with different inoculum size at pH 7.0 after incubation at 40^{O}C for 48 hrs.

Conclusions

The local isolate of *Bacillus cereus* Be9 is an efficient inulinase producer. Inulin medium was the best for inulinase production by *B. cereus* Be9 in neutral environment (pH 7.0) after 48h incubation at 40^{o} C.

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