Production of inulinase from *Rhizopusoryzae* by solid state fermentation

انتاج انزيم الانيولينيز من الفطر *Rhizopusoryzae* بواسطة تخمرات الحالة الصلبة

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Abstract

Fifty Rhizopus spp. isolates were obtained from local natural habitat. The ability of inulinase production by these isolates was screened. The isolate RC-2 which isolated from composite plants was the highest inulinase producer on modified Czapek-Dox agar in primary screening. Secondary screening revealed that the same isolate was the highest production on Nakamura broth medium. Isolate was identified as *Rhizopusoryzae*. Measurements of reducing sugars in crude filtrate which represent the products of enzyme revealed that *Rhizopusoryzae* RC-2 produced inulinaseextracellular. The optimum culture conditions for inulinase production by solid state fermentation included a mixture of Jerusalem artichoke *Helianthus tuberosus* tuber with sugarcane Saccharum sp. bagasse with a ratio (1:1) as carbon source, 2.5% of corn steep liquor as nitrogen source, moisturizing ratio (3:1) (v:w) with tap water, the best inoculums rate was 2.5×105 spore/ml and incubation at (30)ºC for 4 days.

Key wards: Inulinase, *Rhizopusoryzae*, Solid state fermentation

الملخص

تم الحصول على 50 عزلة من العفن *Rhizopus* Spp. من البيئات الطبيعية المحلية. اختبرت قابلية العزلات على انتاج الأنيولينيز، وبينت نتائج الغربله الاوليه ان العزلة RC-2 المعزولة من بقايا نباتات تعود الى العائلة المركبه Compositae كانت الأكثرانتاجا لانزيم الانيولينيز من باقي العزلات على وسط Czapek- Dox agarالمحور، كما بينت نتائج الغربله الثانويه ان نفس العزله كانت الأغزر أنتاجا لهذا الأنزيم في الوسط الزرعي السائل Nakamura broth medium وشخصت هذه العزلة فيما بعد على انها *Rhizopusoryzae*. بينت نتائج فعالية الأنزيم على أساس تقدير السكريات المختزلة ان هذا العفن ينتج أنزيم الأنيولينيز في الوسط الزرعي أي أن الأنزيم خارج خلوي extracellular عند وجود سكر الأنيولين كمصدر للكاربون في هذا الوسط. تبين أن الوسط الزرعي الصلب الأفضل لأنتاج الأنيولينيز هو الوسط المكون من خليط مسحوق درنات نبات الألمازة مع مخلفات قصب السكر بنسبة 1:1 كمصدر للكاربون و 2.5% من منقوع الذرة كمصدر للنايتروجين ونسبة ترطيب 1:3(حجم: وزن) باستخدام ماء الحنفية ،وكانت أفضل نسبة لقاح من العفن هي $2.5x10^5$ سبور/ مل والحضن بدرجة 30م لمدة 4 أيام.

الكلمات المفتاحية: انيولينيز، رايزوبس اوريزي، تخمرات الحالة الصلبة

Introduction

Microbial inulinases belong to an important class of industrial enzymes that have gained increasing attention in the recent years [1]. Inulinases have been characterized from inulin storing tissues of plants, but microorganisms considered were the best sources of inulinase for commercial production because of their easy cultivation, rapid multiplication and high production yields [2]. A number of filamentous fungi, yeast and bacterial strains have been reported for the production of inulinase, which were usually inducible and extracellular [3]. There were two different types of inulinase found in microorganisms classified by the mode of action on inulin, exoinulinase and endoinulinase: exoinulinase liberate the terminal fructose from the inulin chain, whereas endoinulinase reduces the long chain of inulin into smaller oligosaccharides, which were suggested to have similar physiological functions to those of fructo oligosaccharides [4]. Few studies on the production of inulinase by solid state fermentation (SSF) have been recently reported [5]. This system offers numerous advantages over submerged fermentation system, including production of bulk chemicals and enzymes, high volumetric productivity, relatively higher concentration of products, etc. [6].

The ability of fungi to produce inulinase has not been locally studied yet in sufficient manner, so this study aimed to produce of inulinase at high level from local isolates of fungi .

البحث مستل من اطروحة الدكتوراه للباحث الاول

Materials and Methods

Samples collection

Sixty three samples of rhizosphere soil, decomposing composite plant materials, decaying fruits, vegetables and grains were collected from Baghdad city in sterile plastic bags and transported to the laboratory and preserved at 4ºC until using.

Isolation of fungi

Isolation from the soil

The samples of rhizosphere soil 25gm were suspended in 225ml of sterilized distilled water (1:10 dilution) and subsequently 10ml of this suspention was added in to 990ml of sterilized distilled water. Petri dishes containing the Sabouraud agar medium were inoculated with 1ml of the 1:1000 diluted soil suspension. The plates were incubated at 28ºC and the growth of the colonies were accompained up to 48h fragments of the individual colonies were transferred separately to the same medium and the growth was accompanied for 24h [7].

Isolation from decaying fruits, vegetables and other plant materials

After washing of the infected fruits, vegetables and other plant materials thoroughly by sterile distilled water, the fungi are isolated from plant tissues exhibiting clear symptoms. The infected tissues long with adjacent small unaffected tissues are cut in to small pieces (2-5 mm2) by using sterilized forceps, they are transferred to sterile petri dishes containing 1%sodium hypochlorite used for surface sterilization of plant tissues. The sterilized pieces was washed at many times with sterilized distilled water to remove sterilizer solution, then one pieces transferred to each Pertri dish containing SDA supplemented only with chloramphenicol, and incubated at 28ºC for 3 days. A protion of mycelium developing was transferred to the same medium for purification and storage for further examination [8].

Isolation from infected grains.

The infected grains (wheat, barley, rice, corn) were external sterilized by 1% sodium hypochlorite for 1min., then washed by sterilized distilled water. Two grains were placed in each petri dish containing SDA and incubated for 3 days at 25ºC[9].

Genus identification

All isolates were identified to the genus level on the basis of macromorphological and micromorphological characteristic using SDA, Czapek-Dox agar and slide culture technique. One potential isolate was identified according to [10].

Screening of inulinase producers

Primary screening (semi-quantitative method)

Rhizopus isolates were point inoculated on modified Czapek-Dox agar. The plates were incubated at 30 ºC for 48 h. Colonies that displayed rapid growth per unit time, were selected for further experiments and transferred to fresh plates. Relative growth (%) was calculated as following:

$$\textbf{Relative growth } \% = \frac{\textbf{diameterofthecolonyininulinmedium}}{\textbf{diameterofthecolonyinglucosemedium}} \times 100 \quad \textbf{[8]}$$

Inoculum preparation

Nine isolates of *Rhizopus*spp were subcultured on the PDA and incubated at 30ºC for 72h. Spores from the slants were suspended in sterile saline (0.85% NaCl) containing 0.01% Tween 80 to obtain $2.5x10^9$spore/ml determined by haemocytometer.

Secondary screening (quantitative method)

isolates of *Rhizopus* spp. were inoculated into Nakamura broth medium with 0.5ml of $2.5x10^4$spore/ml. Fungi were cultured in 250 ml Erlenmeyer flask containing 50 ml of the medium and incubated in rotary shaker incubator (140rpm) at 30ºC for 5 days. Inulinase activities were assayed by measuring reducing sugars released from inulin.

Crude enzyme extraction

After fermentation process, whole sample of each flask was extracted by addition of 50ml of sodium acetate buffer (0.1M, pH4.8), Following incubation at 30ºC and 150 rpm for 30 min . Enzyme activity was assayed in the supernatant after filtrated by filter Paper Whatman No.1 [11].

Inulinase assay

The inulinase activity was assayed by determining the reducing sugars formed during incubation of soluble enzyme, as follows:

enzyme extract (0.1ml) was added to (0.9ml) of 1% inulin in 0.1M sodium acetate buffer with pH 4.8 and the mixture was incubated at 40ºC for 30minutes. The enzyme reaction was stopped by adding 1ml of DNSA to each tube. The tubes were incubated in boiling water bath for 5 minutes, then directly cooled in ice bath. Five milliliter of D.W. was added to each tube and mixed well. The optical densities of the solution were measured at 540nm.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 [12].

Protein assay

The protein concentration in the unknown samples was assayed according to (20) depending onthe standard curve of bovine serum albumin .

Calculation of specific activity

Enzyme specific activity was calculated as following :

$$\text{Specific activity (U/mg protein)} = \frac{\text{Enzyme activity(U/ml)}}{\text{Protein concentration (mg/ml)}}$$ [13]

Solid substrates used in solid state fermentation(SSF)

Different vegetal sources were used as solid substrates for inulinase production by SSF as Jerusalem artichoke tuber (J.a.t.), sugarcane bagasse (S.c.b.), dendilion tap root (D.t.r.), wheat bran (W.b.), rice husk (R.h.), banana peel (B.p.), orange peel (O.p.).

Preparation of solid substrates

All of the previous materials in (2.4.10) were washed with tap water and sliced with blender, then dried in oven at 50ºC. The dried slices was milled to homogenize and sieved through no.20 (850µm) mesh [14].

Determination of cultural conditions effect on inulinase Production.

1. **Effect of different inulin- containing plants and agro-waste as carbon sources.**

Seven different solid substrates each on separately were used for inulinase production. Fermentation process was carried out in conical flask (250 ml) containing 10g of dry solid substrate. Moisture ratio was adjusted to 4:1(V:W) with D.W. and autoclaved at 121ºC,15 psi for 20 minutes. After cooling , each flask was inoculated with 0.5ml of 2.5x104 spore/ml and incubated at 30ºC for 4 days.

2. **Effect of mixing Jerusalem artichoke and sugarcane bagasse.**

Five grams of J.a.t. were mixed with 5g of S.c.b. to obtain (1:1) ratio. Fermentation process was carried out with the same conditions.

3. **Effect of different moisturizing agents.**

Four moisturizing agents (tap water, distilled water, sodium acetate buffer (pH5) andminerals solution (pH5) were used.To choose the appropriate moisturizing agent, fermentation medium that contains J.a.t. and S.c.b. (1:1) (W:W) was selected and humid with four moisturizing agents each on separately with ratio 4:1(v:w) in conical flask (250ml), then sterilized and cooling, each flask was inoculated with 0.5ml of $2.5x10^4$ spore/ml and incubated at 30ºC for 4 days.

4. **Effect of moisture ratio of the substrate.**

The effect of the moisture ratio of the substrates on the inulinase production was determined at ten different levels using tap water (0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1 and 5:1) (V:W), the set up were sterilized and cooling, each flask was inoculated with 0.5ml of $2.5x10^4$ spore /ml and incubated at 30ºCfor 4 days.

5. **Effect of different nitrogen sources (1%).**

The organic nitrogen sources as (urea, peptone, yeast extract, tryptone, corn steep liquor (CSL) and inorganic nitrogen sources as (sodium nitrate, ammonium phosphate, ammonium chloride, potassium nitrate) and mixture of organic + inorganic (CSL + ammonium phosphate, yeastextract + sodium nitrate) were investigated. These were added to solid substrate at the concentration 1%. Moisture ratio

was adjusted to 3:1(V:W), then sterilized and cooling, each flask was inoculated with 0.5ml of $2.5x10^4$ spore/ml and incubated at 30ºC for 4 days.

6. Effect of different concentration of Corn steep liquor (CSL)

The following concentrations of selected nitrogen source (CSL) (0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4%) were used to determine the optimal concentration for enzyme production.

7. Effect of inoculum rate

To determine the effect of inoculum rate on enzyme production, different concentrations ($2.5x10^2$ - $2.5x10^9$)spore/ml were used. Corn steep liquor (CSL) of 2.5% was added to solid substrate as nitrogen source and all previous conditions were applied.

8. Effect temprature for inulinase production.

Six different tempratures (20, 25, 30, 35, 40, 45)ºC were used to determine the optimum temperature for inulinase production. Each flask was inoculated with 0.5ml of $2.5x10^5$ spore/ml and incubated at specific temprature for 4 days.

9. Effect incubation period for inulinase production.

Eight different time period (1-8) days were used to determine the optimum incubation period for inulinase production. Each flask was inoculated with 0.5ml of $2.5x10^5$ spore/ml and incubated at 30ºC for specific day.

Results and discussion

Optimum conditions for inulinase production by *Rhizopusoryzae* RC-2 with solid state fermentation.

1. Effect of different inulin-containing plants and agro-waste as carbon sources on inulinase production:

All the substrates which have been used as carbon source able to support the growth and stimulate the production of inulinase by *Rhizopusoryzae*strain RC-2. The results in figure (1) revealed that the substrate varied in their ability to induce inulinase production.

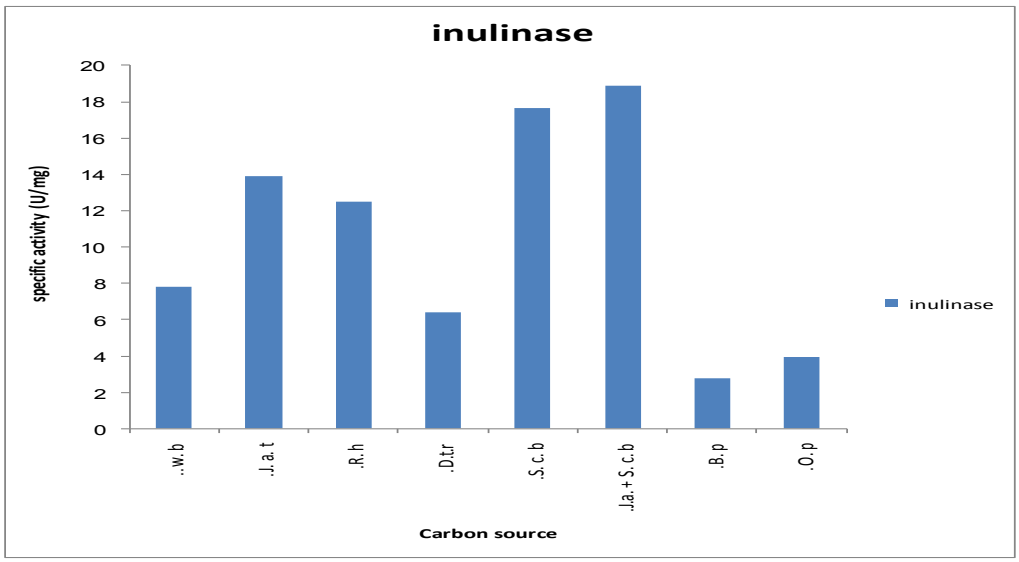

Fig.(1): Effect of different agro-waste as carbon sources on inulinase production from *R. oryzae*RC-2 Moisture ratio 1:4 (W/V) inoculum size $2.5x10^4$ spore /ml and incubated at 30°c for 4 days.

In current study high level of inulinase production occurs in Jerusalem artichoke tuber in combination with sugar cane bagasse at ratio 1:1 (W/W) with specific activity (18.89 U/mg), while low level of inulinase production has been recovered in Banana peel (2.76 U/mg).

Inulinase was produced over the different sources used, but some of these sources reduced the enzyme biosynthesis while others induced it greatly. This mean that, this enzyme is constitutive in nature and induced with its substrate. These results are in agreement with the data obtained by [14]. Also, the results are in agreement with those obtained by [15] which reported that maximum inulinase production using sugarcane bagasse was 391.9 U/g, and this production is higher than that reported in literature for production of inulinase by SSF using wheat bran 122.9 U/g.

Sugarcane bagasse proved to be the best solid substratum with the highest titre of inulinase production. This might be because of the high moisture content in the sugarcane fibers and also presence of satisfactory amount of residual sugars [15].

Jerusalem artichoke *Helianthus tuberosus* attracted scientists attention because of their availability, they present cold and drought tolerance, saline tolerance, wind and sand resistance, they have strong fecundity and they are resistant to pests and diseases [5].

The selection of suitable substrate for SSF has mainly centered around more efficient utilization of different agro-industrial residues for enzyme production, and also due to the potential advantages for the filamentous fungi, which are capable of penetrating into the hardest of solid substrates aided by the presence of turgor pressure at the tip of the mycelium and on the other hand, helps in solving pollution problems, which otherwise may cause their disposal [16].

1. Effect of different moisturizing agents on inulinase production:

The combination of substrates of Jerusalem artichoke with sugarcane bagasse (1:1 w/w) was supplemented with suitable moisturizing agents (mineral salts solution, sodium acetate buffer, distilled water and tap water) with moisture ratio 4:1 (V/W) to meet the water requirement and additional nutrients to the growing cultures. Figure (2) shows that the maximum inulinase production was obtained using mineral salts solution (23.51 U/mg), while distilled water gave (18.82 U/mg).

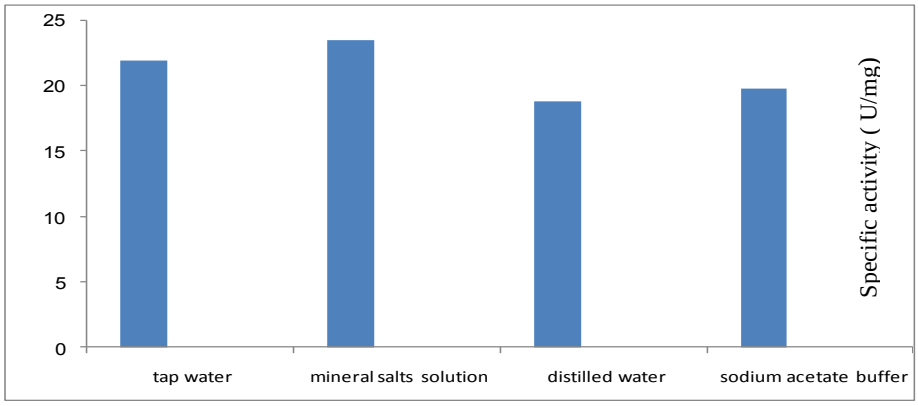

Fig.(2): Effect of different moisturizing agents on inulinase production from *R. oryzae*RC-2, moisturizing ratio 4:1 (V/W), inoculum rate 0.5 ml of 2.5x10^4 spore/ml and incubated at 30°c for 4 days.

The result obtained in this study revealed that mineral salts solution was the best moistening agent for the production of inulinase by *R. oryzae* RC-2. These minerals provide and fulfill the basic requirements for fungal growth like phosphate, sulphate, potassium, chloride, ammonium, magnesium and nitrogen. All these were considered to be essential in fermentation process for the growth of microbial strains and ultimate production and secretion of various hydrolytic proteins [17].

Low level of enzyme specific activity was obtained with distilled water. It might be due to the fact that distilled water has no micro-or macro nutrients, keeping the pH variable as an unpredictable variable which did not support microbial growth [18].

Mineral salts solution and tap water resulted in higher inulinase production compared to distilled water and sodium acetate buffer. However, inulinase production using tap water (21.94 U/mg) showed difference from mineral salts solution (23.51 U/mg). thus, tap water was chosen as the moistening agent for economical reasons.

3. Effect of moisture ratio of the substrate on inulinase production:

To check the influence of moisture content in inulinase production under SSF, the combination of solid substrate J.a.t.-Sc.b. (1:1 W/W) was moistened with different ratio of tap water ranged between 0.5 : 1 – 5 : 1 (V/W). Results summarized in figure (3) shows that moisture ratio 3:1 (V/W)was the optimal for maximum inulinase production (25.66 U/mg).

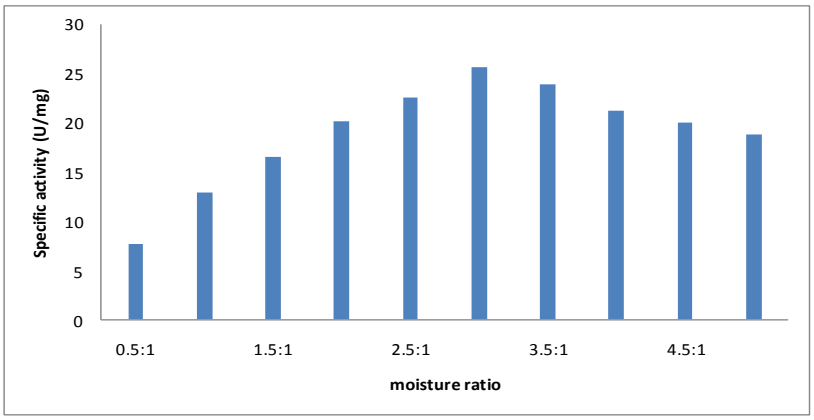

Fig. (3): Effect of different moisture ratios on inulinase production from *R. oryzae*RC-2, in J.a.t. and S.c.b. media (1:1) , inoculum size was 0.5 ml contains 2.5x10^4 spore/ml, incubated at 30°c for 4 days.

The moisture level in SSF has a great impact on the physical properties of the substrate. Solid substrates used in SSF are insoluble in water therefore, water will have to be absorbed on to the substrate particles, which could be used by the microorganisms for growth and metabolic activity. Thus the degree of hydration of the substrate plays an important role on the growth of the fungi and subsequently the enzyme production [19]. The importance of water for SSF is attributed to the fact that the majority of microbial cells require about 70-80% moisture content for new cell biosynthesis. Furthermore, moisture level is very limiting factors affecting stability, biosynthesis and secretion of fungal enzymes [7].

The optimum moisture is closely depended on some other parameters such as nature of substrate, organism ad studied enzyme. Low moisture may reduce the solubility and swelling capacity of substrate causing high-water tension, decreasing growth and enzyme production. A reduction in enzyme biosynthesis at higher moisture than the optimum is due to steric hindrance of microorganisms growth through reduction in inter particle space, decreased porosity, gummy texture, alteration in particles of substrate structure and impaired oxygen transfer [20]**.**

4. Effect of different nitrogen sources on inulinase production:

The effect of supplementation of different organic and inorganic nitrogen sources in inulinase production was evaluated. Data obtained revealed that corn steep liquor (CSL) and yeast extract were potent inducers for inulinase production by *Rhizopusoryzae* RC-2, in which the productivities were 26.68 U/mg and 25.71 U/mg, respectively. On the other hand, ammonium chloride 19.55 U/mg and urea 16.54 U/mg were the less inducer for enzyme production Figure (4).

It could be concluded that the organic nitrogen compounds enhancing inulinase production more than inorganic compounds, this may be because organic sources support the growth and biosynthesis of protein, nucleic acid and many other cell constituents by providing cell with nitrogen, carbon and energy, while inorganic compounds need to assimilate into organic molecules to involve in biosynthesis [13].

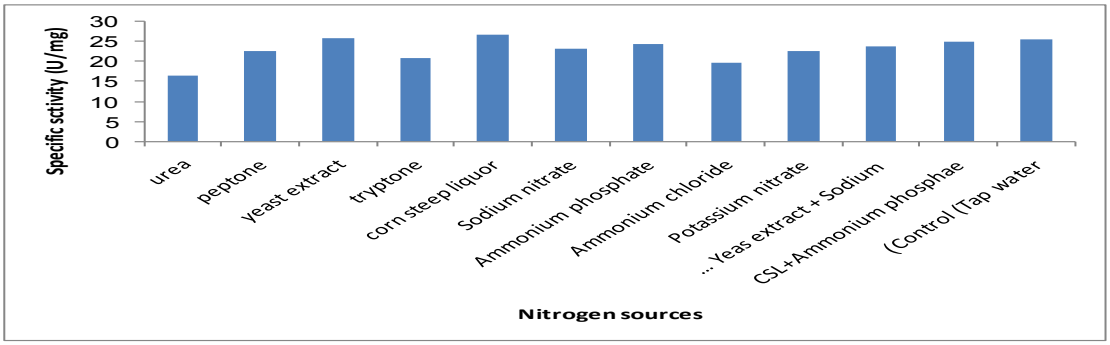

Figure (4) : Effect of different nitrogen sources on inulinase production from *R. oryzae*RC-2. Moisture ratio 3:1 (V:W), J.a.t+S.c.b. media (1:1), inoculum size 0.5 ml contains 2.5x10^4 spore / ml, incubated at 3°c for 4 days.

All compounds with ammonical nitrogen used in this work showed inhibitory effect on enzyme production in comparison with control, this result agree with [12] who found that nitrogen source which contains ammonia showed inhibitory effect on inulinase production from *A. fumigates*.

Corn steep liquor provides proteins, all amino acids, minerals (magnesium, phosphorus, potassium, calcium, chlorine, sodium and sulfur), essential trace elements (iron, boron, manganese, copper and zinc), vitamins, reducing sugars, organic acids (lactic acid) and nitrogen compounds. All of these constituents provide natural nutrition for normal cell metabolism. Corn steep liquor, no ecological, mammalian or human toxicity would be expected from these natural nutritive materials [13].

The results in this study are in agreement with those obtained by [14] whose found that corn steep liquor which used as nitrogen source was the best source for the biosynthesis of inulinase by *A. tamari* [18] found that corn steep liquor was the best for maximum inulinase production from *A. niger* AUP19.

5. Effect of different concentration of corn steep liquor on inulinase production:

Production of inulinase by *R. oryzae*Rc-2 using different concentrations of corn steep liquor was studied. Results obtained showed that enzyme specific activity increases gradually with the increase of corn steep liquor up to 2.5% which represent (28.83 U/mg) and then decrease (Figure 5).

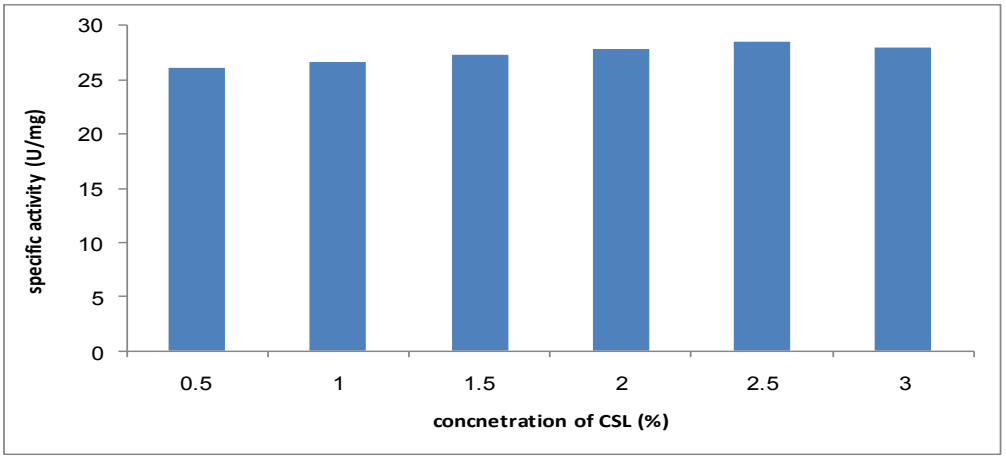

Fig. (5): Effect of different concentrations of CSL on inulinase production from *R. oryzae*RC-2. in J.a.t. + S.c.b. medium(1:1), inoculum size was 0.5 ml contains 2.5 x 10^4 spore /ml incubated at 30c for 4 days.

The higher concentration of corn steep liquor had inhibitory effect in inulinase synthesis. This could be due to the complex nature of this nitrogen source and some of its constituents at higher concentration might have a toxic effect on inulinase production. These results are in consistent with that obtained by Ongen-Baysal [15] who reported that inulinase activity increased with increase in concentration of corn steep liquor and thereafter decreased.

6. Effect of inoculum rate

It was noticed that inulinase production increases gradually with increasing of inoculum size (number of spores per ml). The higher specific activity for inulinase was 28.91, when the solid substrate was inoculated with 2.5 x 10^5 spore / ml Figure (6).

Low enzyme activity was recorded above and below of inoculum size, at high inoculum size the viscosity of fermentation medium might increase due to the tremendous growth of fungi, resulting in nutritional imbalance in the medium or may be using up the nutrients before they are physiologically ready to start enzyme production. Low inulinase production below the optimum inoculum size may be due to insufficient fungal biomass [17].

Inoculum size plays an important role in fermentation process; in a suitable inoculum size, sufficient amount of nutrient and oxygen will be accessible for growth, the need to use an appropriate number of spores so that can grow and cover most of the particles of solid substrate without the emergency of a state of competition for nutrients available in a limited.

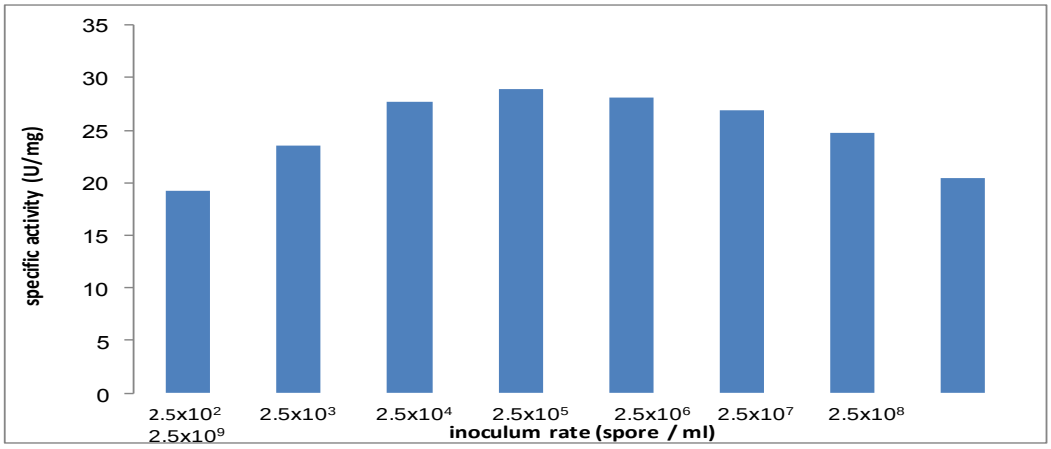

Fig. (6): Effect of inoculum rate on inulinase production from *R. oryzae*RC-2 in J.a.t. + S.c.b medium (1:1), Moisture ratio 3:1 (V:w), inoculum rate 0.5 ml contains 2.5 x 10^4 spore / ml, incubated at 30°C for 4 days.

Different optimum inoculum rates have been reported for inulinase production, 2% for *A. niger* [18], 1% for *A. niveus* and 5% for *A. tamari* [14]. Schipper [11] found that the best inoculum size was 4.9% for inulinase production by SSF using *Kluyveromyces* strain S120. Inoculum size for the maximum inulinase production in SSF by the marine yeast *Pichiaguillie rmondii* was found to be 2.5% [1]. High inulinase production by the marine yeast strain *Cryptococcus aureus* G7a in SSF was obtained 2.75% [13].

Berg [14] found the most suitable inoculum size for inulinase and biomass production using SSF, was 6% for10 days old cultures of *A. niger*. Maximum inulinase activity achieved was 250 U/g in sugarcane bagasse medium using inoculum size about 1x10^{10} cell/ml of *K. marxianus* [18]. Ongen - Baysal [15] found that the optimum inoculum size for inulinase production from *A. niger*, was 1.5 x 10^6 spore/ml of medium.

7. Optimum temperature for inulinase production

Temperature is one of the factors that affect enzyme production. The optimum temperature for inulinase production by *Rhizopusoryzae* RC-2was found to be 30°C (29.44 U/mg). However, the decrease or increase in the incubation temperature lead to decrease enzyme production as it was illustrated in figure (7).

Temperature is affected in all vital events in the cell directly through influence in the genetic material and enzymes and lipids in the cell membrane and lead to influence in the quantity and speed of growth. When temperature increase to more than the optimum degree, this will lead to a rapid decline in the velocity of growth due to denaturation of the enzyme, this is because the rupture of weak bonds in the secondary and tertiary of enzyme construction, and this change is very significant [16].

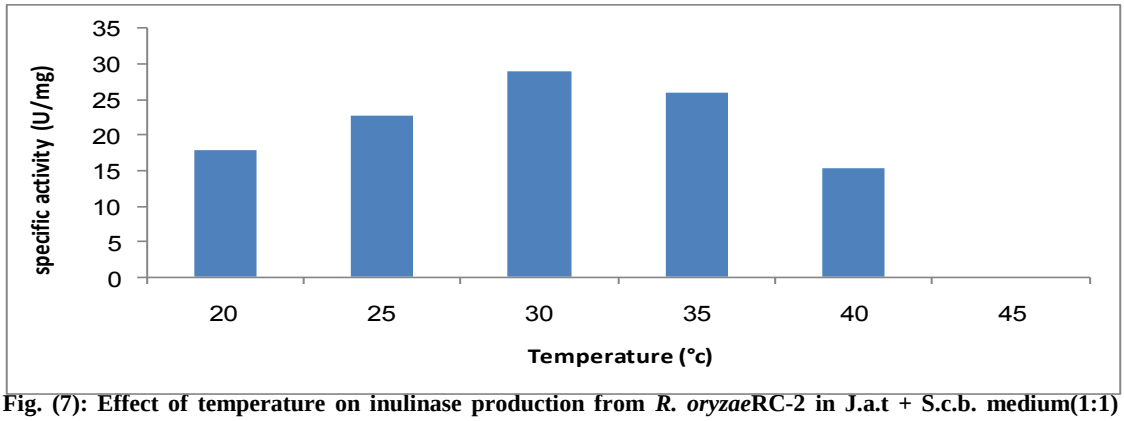

Fig. (7): Effect of temperature on inulinase production from *R. oryzae*RC-2 in J.a.t + S.c.b. medium(1:1) (w:w), moisture ratio 3:1 v/w, inoculum size 0.5 ml contains 2.5x10^4 spore / ml, incubated at 30°c for 4 days

In the case of low temperature to less than optimal temperature, this will lead to slow down the crossing of the solutes through the cytoplasmic membrane in the cell and this lead to the slow of enzyme action [14].

Roses [18] reported "high temperature effects on fungal germination, metabolites formation and sporulation. The fungal activity declined exponentially when optimum temperature for growth reached above maximum".

Fungal growth and secondary metabolite production in SSF are greatly influenced by temperature. During SSF large amount of heat is generated which is proportional to the metabolic activities of the microorganism. However, fungus can grow over a wide range of temperatures (20-55) °C. Nevertheless optimum temperature for fungal growth could be different from that required for product formation [16].

8. Optimum incubation period for inulinase production

Inulinase production by *Rhizopusoryzae*RC-2 was observed during 1 to 8 days. Results revealed that maximum specific activity of inulinase 29.52 U/mg was achieved after 4 days; however it decreased down to (25.64 U/mg) at the 5th day.

Results in figure (8) clearly shows pronounced inulinase production with the increasing of fermentation period up to 96h (4 days) and then decreased. These results found that inulinase synthesis reached to the maximum activity (20.15 U/ml) after 4 days; however, it decreased down to (17.09 U/ml) at the 5th day.

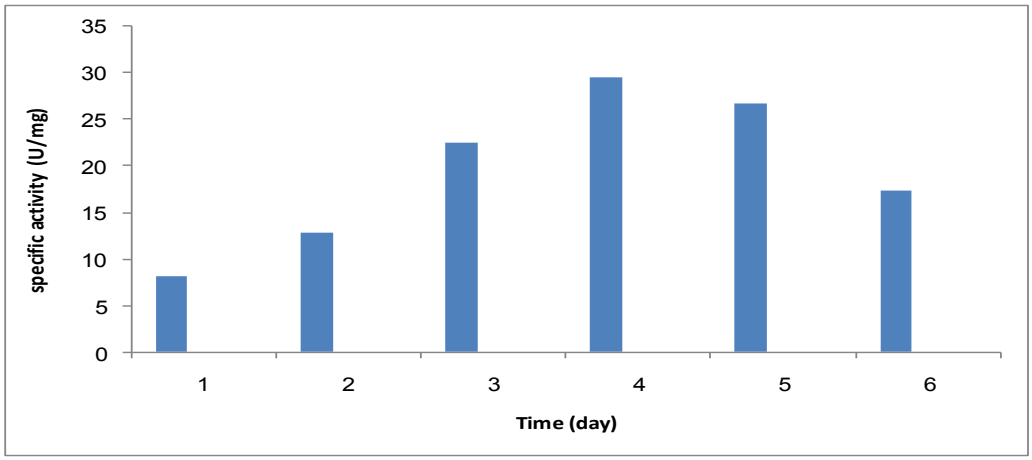

Fig. (8): Effect of incubation period of inulinase production from *R. oryzae*RC-2 in J.a.t + S.c.b. medium (1:1) (w:w), moisture ratio 3:1 (v:w), inoculum rate 0.5 ml contains 2.5x10^4 spore / ml, incubated at 30°c for 4 days.

The time course reported for a maximum production of inulinase by *Penicillium*sp. under aeration conditions was 72 hr [12]. As well as, a maximum inulinase yield 80 U/ml was obtained after 60h by shaking growth of *A. niger* [11]. Ongen - Baysal [15] reported maximum yield of inulinase with *A. versicolor*after 15 day of growth.

It has been reported that inulinase synthesis from *A. niveus* Blochwitz 4128 URM is growth associated and reaches in the optimal near the stationary phase [4]. Whereas [2] found hatinulinase production by *A. tamarii*with the increasing of fermentation period up to 72h and then decreased, and the maximum activities of inulinase (23.63 U/ml) were gained at the end of logarithmic phase.

The decline in enzyme activity after 5th day of fermentation may be due to the secretion of proteolytic enzymes which are known to cause the denaturation of inulinase [15]. This may also be attributed to decrease in nutrient availability in the medium at the end of the cultivation process or catabolic repression of enzyme [12].

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