Effect of Different Environmental and Nutritional Factors on Biosurfactant Production from *Candida guilliermondii*

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ABSTRACT

Yeast strain S9, which produced a high amount of biosurfactant, was isolated from pickledolive and identified as a strain of *Candida guilliermondii* using VITTEK 2 compact system, this strain was selected out of 13bioemulsifier producing strains. The effects of some environmental and nutritional factors on biosurfactant production were evaluated, the maximum value of E24% was observed at pH 4 which was 70% and the optimal temperature for biosurfactant production was 30 °C, E24% was 75%. Among different edible and heavy oils, the sesame oil and heavy oil 150 were the best carbon sources in production of biosurfactant, E24% was82% and 78% respectively and among different organic and inorganic nitrogen sources, the yeast extract was the best organic nitrogen source for biosurfactants production, E24% was 85%, while $NaNo_3$ was the best inorganic nitrogen source for biosurfactants production, E24% was 80% and at the 7^{th} day the maximum production of the emulsifier, E24% was 75% in shaking incubator at 150 rpm

Keywords:*Candida guilliermondii* ,bioemulsifier, Emulsification index (E24 %)

تاثير مختلف العوامل البيئية و التغذوية على انتاج المستحلبات الحياتية من خميرة *Candida guilliermondii*

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الخلاصة:

عزلة الخميرة (S9) ، و التي تتميز بانتاج عالي من المستحلب الحياتي ، تم عزلها من الزيتون المخلل و تم تشخيصها على انها سلالة تابعة للنوع *Candida guilliermondii* باستخدام نظام VITTEK 2 compact. تم اختيار هذه السلالة من بين ثلاثة عشر عزلة تم اختبار قدرتها على انتاج المستحلب الحياتي باستخدم طريقة قياس النسبة المئوية للاستحلاب(Emulsification Index 24 (E24 %) و تم تقييم تاثير عدد من العوامل البيئية و التغذوية على انتاج المستحلب الحياتي من العزلة اعلاه حيث وجد ان اعلى قيمة لانتاج المستحلب الحياتي كان عند قيمة اس هيدروجيني بلغت 4 حيث كان E 24 % عندها 70% فيما كان زيت السمسم و الزيت الثقيل 150 افضل مصادر الكاربون لانتاج المستحلب و بقيم E24% بلغت 82% و 78% على التوالي. من بين عدد من مصادر النيتروجين العضوية و اللاعضوية ، كان $NaNo_3$ افضل مصدر نيتروجين لاعضوي حيث بلغت قيمة(E24%) 80% و من بين مصادر النيتروجين العضوية كانت

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مستخلص الخميره yeast extract الافضل و بقيمة E24% بلغت 85%. درجة الحرارة المثلى لانتاج المستحلب كانت 30 درجة مئوية و بلغ عندها الاستحلاب 75% في حين كانت افضل فترة حضانه لانتاج المستحلب سبعة ايام و بلغ عندها الاستحلاب 76% وبسرعة دوران بلغت 150 دورهادقيقة.

Introduction

Biosurfactants are surface-active compounds from biological sources, usually extracellular, produced by bacteria, yeast or fungi. Researches on biological surfactant production has grown significantly due to their advantages they preferred over synthetic compounds for their biodegradability, low toxicity, diversity of applications and functionality under extreme conditions. Although the majority of microbial surfactants have been reported in bacteria, the pathogenic nature of some producers restricts the wide application of these compounds. A growing number of aspects related to the production of biosurfactants from yeasts have been the topic of research during the last decade [1].

Surfactants are amphiphilic compounds possessing both hydrophilic and hydrophobic moieties. They can reduce surface and interfacial tensions by accumulating at the interface between two immiscible fluids, thus stabilizing emulsions, or increasing the solubility of hydrophobic or insoluble organic compounds in aqueous media. They can be of synthetic or biological origin and the market for these compounds is on expansion [2]

Due to their interesting properties such as lower toxicity, higher biodegradability, higher foaming capacity and higher activity at extreme temperatures, pH levels and salinity, biosurfactants have been increasingly attracting the attention of the scientific community as promising candidates for the replacement of a number of synthetic surfactants [3]. These compounds are biological molecules with noticeable surfactant properties similar to the well-known synthetic surfactants and they also include microbial compounds with surfactant properties [4].

Materials and Methods

Microorganism

Yeast strain was isolated by using successive culturing on potato dextrose agar from pickled olive till pure and isolated colonies of the strain was obtained and then was identified by using yeast card reader in VITTEK 2 compact system.

Biosurfactant production

Cultures were grown on a minimal basal medium (MB) [5] which composed of the following components in distilled water 1 g/l K2HPO4, 1g/l KH2PO4, 0.2 g/l MgSO4,7H_2O, 1 g/l NH_4No_3, 0.05 g/l $FeCl_3$, 10 g/l glucose; 10 ml of olive oil was added as carbon source then sterilized by autoclaving at 121°C for 15 min. This medium was used for production the bioemulsifier from yeast isolate; also the media was prepared at different pH values (2-7).

Estimation of Emulsification index (E24%)

To determine the emulsification index, Batista *et al.*, method was applied [6]. Yeast cultures were centrifuged at 10,000rpm to separate biosurfactant from microorganism cell. A mixture of biosurfactant and crude oil (v:v) was vortexed for about 2 min andleft for 24 h.Emulsification index (E24%) determined bymeasuring the column height of emulsified oilagainst the total height multiplied by 100.

Determination of factors affecting biosurfactant production

In all the experiments yeast was cultured atbasal salt medium in different environmental conditions to determine the optimum nutritional and environmental factors for biosurfactant production which included the following factors: pH, different oil source, nitrogen source, temperature and incubationperiod. At the end of experiment, cultures broths were centrifuged at 10,000 rpm for 5 min. to separate biosurfactant from microorganism cells. After that (E24%) was estimated.

Effect of pH:

Twenty five ml of sterilized mineral salts broth containing 0.5ml olive oil in (100 ml flasks) each flask was prepared at certain pH ranged from 2 to 7 and inoculated with 1 ml of 24 hr. fresh yeast culture inoculated in potato dextrose broth, then and incubated at 30ºC with shaking for 10 days at 150 rpm, after incubation period the cultures were centrifuged and the emulsification index (E24%) was estimated for the supernatant.

Effect of temperature:

Twenty five ml of sterilized mineral salts broth containing 0.5ml of olive oil was inoculated with 1 ml of yeast culture broth and incubated at different temperatures (30ºC, 40ºC and 50ºC) in shaker incubator for 10 days at 150 rpm, after incubation period E24% was estimated for the supernatant.

Effect of different nitrogen sources:

Twenty five ml of sterilized mineral salts broth containing different nitrogen sources ($NaNo_3$, NH_4No_3, NH_4Cl, urea, peptone and yeast extract)was inoculated with 1 ml of yeast culture broth and incubated at 30ºC with shaking for 10 days at 150 rpm. After incubation period E24% was estimated for the supernatant.

Effect of different kinds of oils (vegetable oils and heavy oils):

Twenty five ml of sterilized mineral salts broth containing different kinds of oils(sun flower, corn, sesame, olive, oil 40, oil 60 and oil 150) wasinoculated with 1 ml of yeast culture broth, and incubated at 30ºC with shaking for 10 days, after incubation period E24% was estimated for the supernatant.

Effect of incubation period:

Twenty five ml of sterilized mineral salts broth was inoculated with 1 ml of activated yeast culture broth and incubated at 30ºC with shaking for (1–10) days, after each incubation period (day) (E24%) was estimated for the supernatant.

Extraction of bioemulsifier:

Tow extraction methods were employed as follows:

Method 1: Extraction with diethyl ether: equal volumes of cell free supernatant and diethyl ether were mixed in (250ml) separating funnel very well by shaking and allowed to stand, the aqueous layer was removed and the emulsifier layer was collected in a sterilized and weighed glass Petri dishes and dried in oven (40 – 45) ºC, the amount of dried emulsifier was measured [7].

Method 2: Extraction with chloroform – methanol (1:1):

Bioemulsifier in the supernatant was extracted twice with an equal volumes of chloroform – methanol (1:1), and mixed in separation funnel, the aqueous layer at the bottom of the separation funnel was removed and the emulsifier layer was collected in a glass petri dish and left in oven at (40 – 45) ºC till dryness, the emulsifier was collected by scrubbing and preserved in a clean screwed glass vials as dried powder [8].

Results and discussion

Isolation and Identification of yeast isolates

Twenty five samples were obtained from different source which include soil (10) and foods (15). Fungi mainly *Aspergillus* sp. were dominated and the yeast were absent in soil samples. Only (13) yeast isolates were able to produce bioemulsifier.

Nine isolates belong to *Candida* sp., two isolates belong to *Rhodotorula* sp. and two isolates were belonging to *Cryptococcus* sp. The isolates were identified by VITEK 2 compact Yeast card reader with the software version V2C 03.01 in Ibn Al-Balady Hospital, Baghdad.

All yeast isolates were screened for bioemulsifier production. *Candida guilliermondii* (isolate no. 1 and 9) were the best in bioemulsifier production of (figure-1) depending on the value of emulsification index E24% which was done to each isolate.

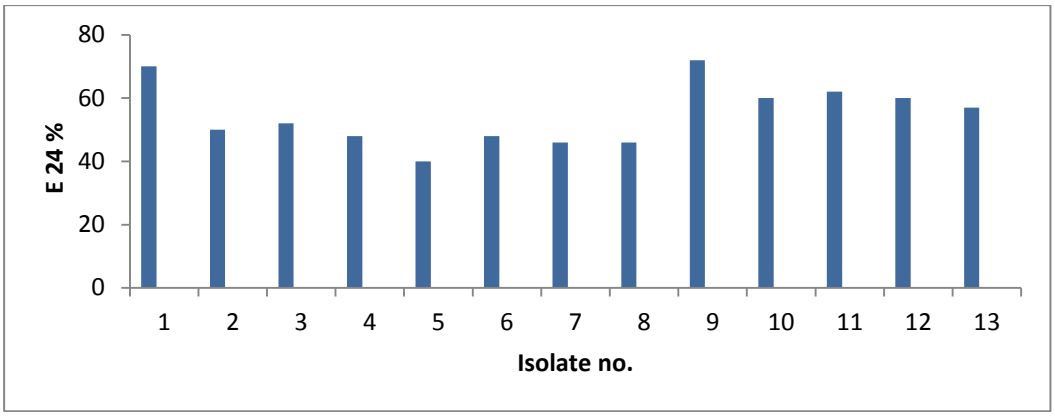

Figure 1- Emulsification index (E24%) for *yeast* isolates grown in media containing olive oil incubated at 30˚C for 8 day.

Candida is belong to family Saccharomycetaceae .Many species are harmless commensals or endosymbiosis of hosts including humans, but other species, or harmless species can cause disease. *Candida albicans* can cause infections (candidiasis or thrush) in humans and other animals,especially in immune compromised patients [9].

Among *Candida* species, *C. albicans*, which is a normal constituent of the human flora, a commensal of the skin, gastrointestinal and genitourinary tracts, are responsible for the majority of *Candida* bloodstream infections (candidemia). Other medically important *Candida* species include *C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis* [10]. Other *Candida* species, such as *C. oleophila* have been used as biological control agents in fruit [11].

The effects of culture media conditions on bioemulsifier production:

Effects of pH

It was observed that pH 4 gave the maximum production for bioemulsifier from *Candida guilliermondii S9*E24% was (72%) (figure- 2).

*C. guilliermondii*S9 produce the bioemulsifier in all pH values used in the study but the maximum yield appeared at pH 4. This character is useful in many applications since there is no need to keep the pH value at certain value which need addition of acid or bases and hence it reduce the coast and efforts.

The effect of pH on the production of bio emulsifier form *Yarrowia lipolytica* was studied and the best pH value was at pH 8 which is the same pH of see water [12]. Bednarski *et al*. found that without controlling pH, the bioemulsifier synthesis decreased [13]. The production of bioemulsifier by *Rhodotorula glutinis* is significantly affected by pH where the optimum value was 4 [14]. It was also found that the maximum production of bioemulsifier from *C. rugosa* and *R. muciliginosa* was obtained in a pH value of 7.5 [15].

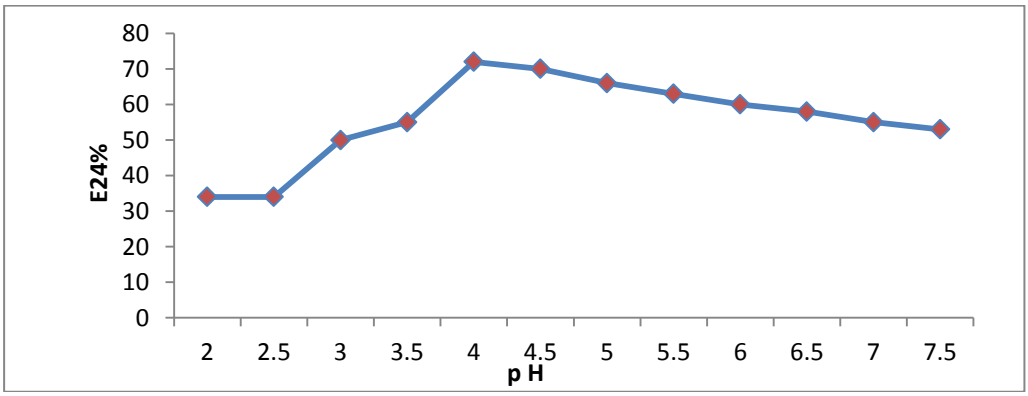

Figure 2- Effect of pH on biosurfactantproduction by *Candida guilliermondii*S9 Effect of Temperature.

The highest production of bioemulsifierby *C. guilliermondii* S9 was observed at 30ºC depending on the results of E24% (77%) (figure -3). It was important to know that *C.guilliermondii* S9 produced bioemulsifier in a wide range of temperatures (25-50) ºC. Production of bioemulsifier in relatively high temperature (40ºC) indicates that this type of yeast can grow at this temperature, and produce bioemulsifier and this is useful in industrial application because it reduce the cost of cooling.
At (40ºC) the bioemulsifier activity E24% was 40%. It's known that the temperature affect cellular enzymes mainly which means that it can affect the enzymes that control pathways responsible for the production of bioemulsifier. The bioemulsifier obtained from *C. bombicola* give similar results at 25˚C and 30˚C [16]. Desphande and Daniels observed that the growth of *C. bombicola* reached maximum value at 30˚C while 27˚C was the best temperature for the production of bioemulsifier [17].

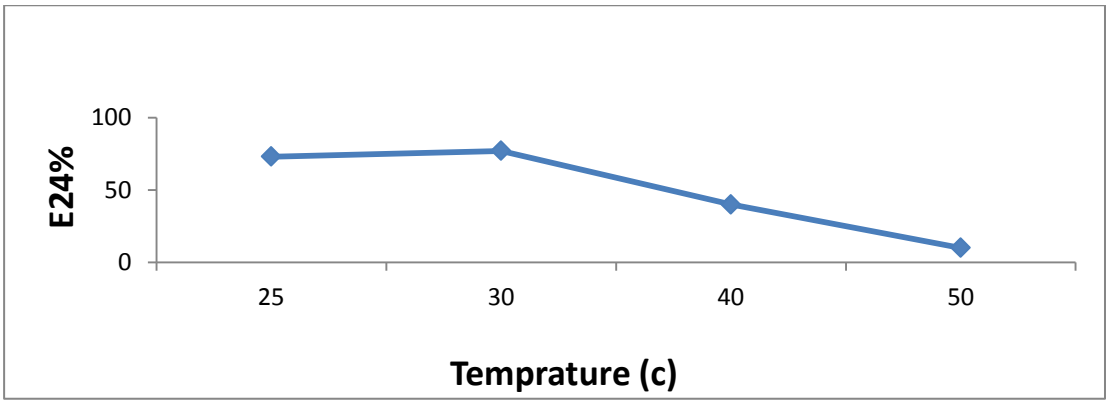

Figure 3- Effect of temperature on bioemulsifier production from *Candida guilliermondii S9*.Effect of nitrogen source**:**

Fine balance is required between carbon and nitrogen sources in order to produce bioemulsifier from yeast. One of the important factors that affect the growth and production of bioemulsifier from yeast is the nitrogen source, because the yeast requires nitrogen to complete its metabolic pathways. In this study, $NaNO_3$ proved to be the best nitrogen source for production of *Candida guilliermondii* bioemulsifier among different organic and inorganic nitrogen sources, the bioemulsifier activity E24% was 80% (figure -.4).
This salt is inorganic nitrogen source, very soluble and utilized easily as nitrogen source for cell metabolism and growth enhancing and may play important role in the pathways of emulsifier biosynthesis and/or extracellular secretion. Another important aspect about this salt is that it is considered as toxic to most organisms, so the ability of this yeast to utilize it means the ability to reduce its concentrations.
Cooper and Paddok studied the effect of the nitrogen source, using sodium nitrate, ammonium chloride, ammonium nitrate, urea and yeast extract on the bioemulsifier production by *Torulopsis bombicola*in agitated flasks. They observed that nitrate was not a good nitrogen source since it affected the biomass growth while yeast extract promoted a higher surfactant production. When the yeast extract was substituted by peptone, the bioemulsifier concentration obtained was reduced to half and a very low concentration was obtained with urea [18].
Johnson *et al.* reported the influence of the nitrogen source in the production of a bioemulsifier by the yeast *Rhodotorula glutinis* IIP-30. The author revealed that potassium nitrate presented the best result in comparison to other nitrogen sources (ammonium sulphate and urea) [14].
The production of surface-active compounds often occurs when the nitrogen source is depleted in the culture medium, during the stationary phase of cell growth [19]. Kitamoto *et al.* studied the cell growth of *Candida antarctica* and its bioemulsifier production in a culture medium containing ammonium ion and peptone as nitrogen sources. They noticed that the production of glycolipids starts when the nitrogen source is exhausted after 50 hours of fermentation, reaching a concentration value of 38 g l^{-1} after 200 hours of fermentation [20].

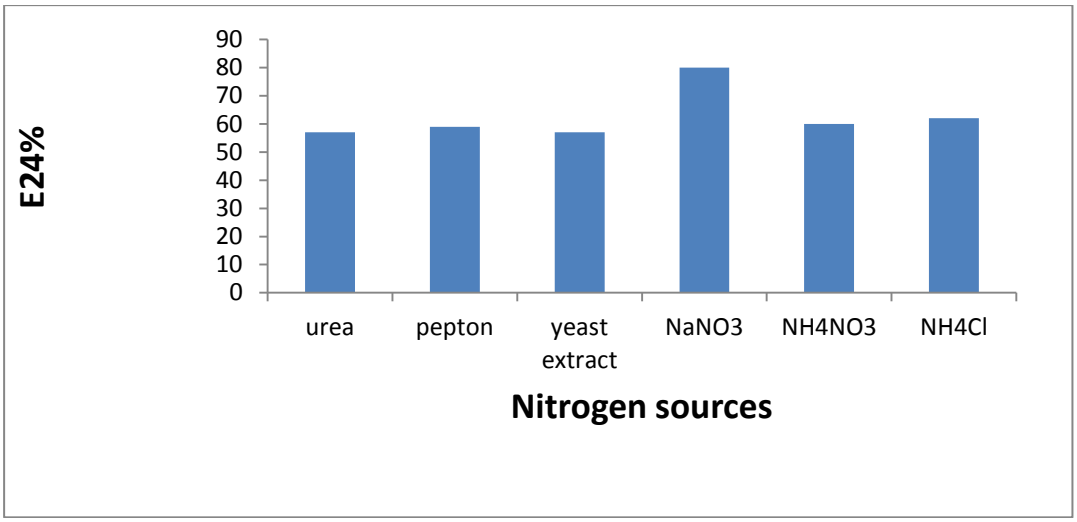

Figure 4- Effect of different nitrogen sources (organic and inorganic) on bioemulsifier production from *Candida guilliermondii* S9.

Type of oil:

The type, quality and quantity of bioemulsifier are influenced by the nature of carbon substrate. Media containing edible oils and heavy oils were used for production of bioemulsifier from *Candida guilliermondii* S9. Both edible oils (olive oil, sesame oil, corn oil and sun flower oil) and heavy oils (oil 40, oil 60 and oil 150) were suitable for bioemulsifier production. Among different edible oils, sesame oil was the best carbon source in production of the bioemulsifier, the E24% was 82%, and among heavy oils , oil 150 was the best in production of bioemulsifier E24% was 78%(figure -5).

In this study we add glucose to the mineral salt media as well as the edible and heavy oil, this because glucose is a simple carbon source and can enhance growth to enhance production of higher amount of the emulsifier.

Zinjarde and Pant demonstrated that the surfactant biosynthesis by *Yarrowia_lipolytica* NCIM 3589 using soluble substrates such as glucose, glycerol, sodium acetate or alcohol was not viable. They identified the presence of a bioemulsifier in culture media containing crude oil and alkanes (C10-C18) [12]. Different carbon sources such as hexadecane, paraffin, soybean oil, olive oil, corn oil and cottonseed oil were used to produce bioemulsifier from *Yarrowia_lipolytica*, hexadecane was identified as the best one [21]. Casas and Ochoa studied the effect of medium composition on bioemulsifier production by *Candida bombicola*. The carbon sources promoting the best biosurfactant production were glucose (100 g l^{-1}) and sunflower oil (100 g l^{-1}) used simultaneously, resulting in a bioemulsifier concentration of 120 g l^{-1} after 144 hours of fermentation [16].

The hydrophilic substrates are initially metabolized by the microorganism for its energetic requirements and afterwards microorganism also uses such substrates in the synthesis of the polar portion of the biosurfactant molecule. On the other hand, hydrophobic substrates are exclusively used for the production of the apolar moiety of the biosurfactant. The *Candida* species seem to be capable to incorporate fatty acids directly into the production of biosurfactants [22].

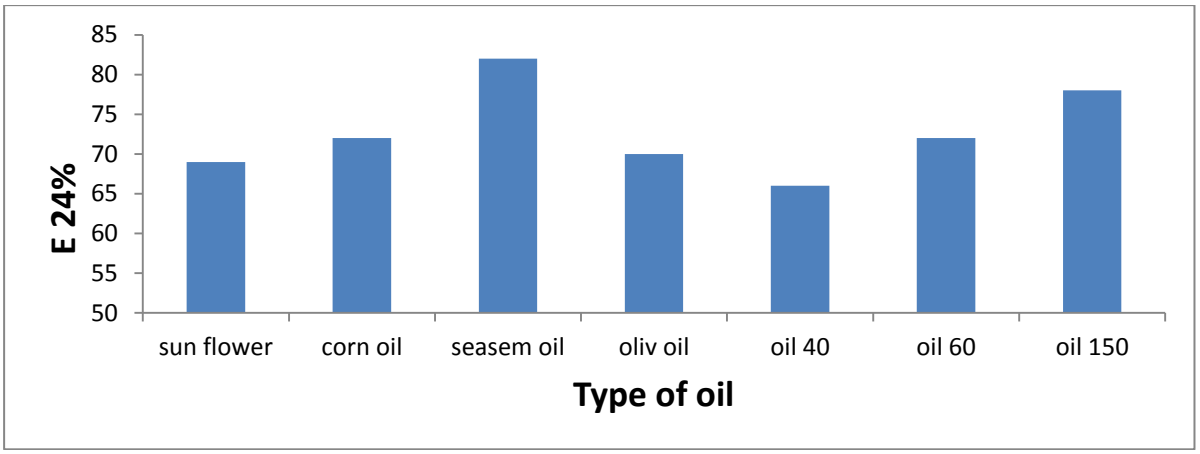

Figure 5- Effect of different oils on bioemulsifier production from *Candidaguilliermondii S9.*

Incubation period:

Culture of *C. guilliermondii (S9)* was incubated for 10 days in mineral salts medium supplemented with olive oil (1%) to determine the best incubation period for production of bioemulsifier and samples were taken daily, the results showed low production of bioemulsifier in the first 5 days in general, but it reached the maximum values after 6-7 days. The maximum value of E24% for *Candida guilliermondii* was 76% at the 7th day (figure -6).

This long bioemulsifier production indicates that the production does not take place in the primary metabolism (log phase) but it take place in the secondary metabolism and hence the bioemulsifier itself is a secondary metabolite and its production is related to the absence of simple carbon sources such as sugars and the presence of more complex ones such as lipids. Cletus *et al*. found that the maximum bioemulsifier production from *C. bombicola* NRRL Y-17069 occurred at the 6th day while the maximum bioemulsifier production from *Candida* sp. NRRLY-27208 occurred at the 7th day [23].

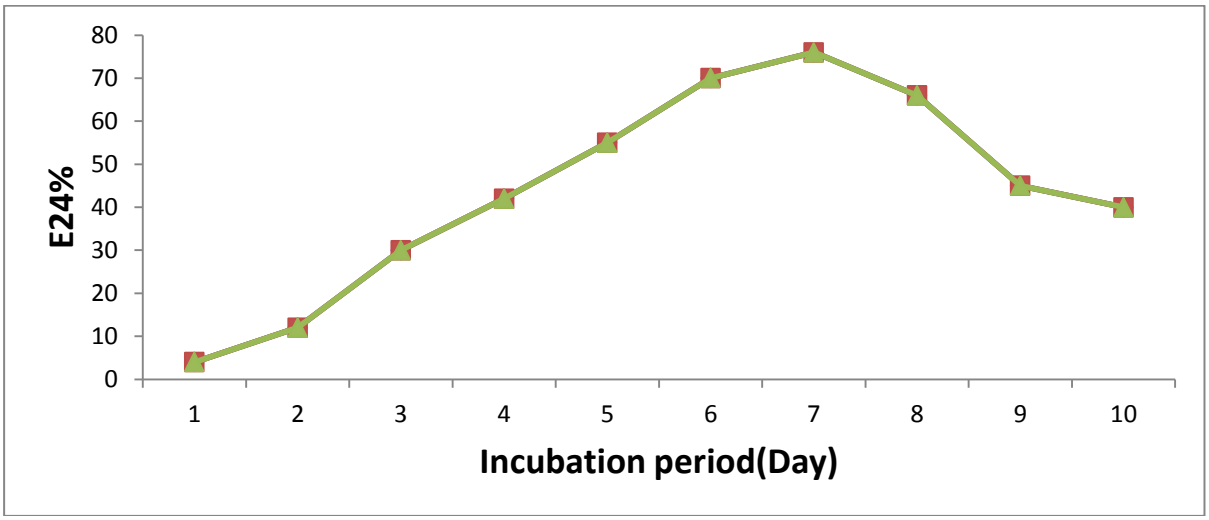

Figure 6- The effect of incubation period on production of bioemulsifier by *Candida guilliermondii S9.*

Candida guilliermondii S9 isolated from pickled olive produces high amount of biosurfactant, this strain is able to utilize different carbon and nitrogen sources for biosurfactant production, biosurfactant production by *C. guilliermondii* S9 is influenced by environmentalcondition and media composition. It favors acid medium and can grow and produce bioemulsifier at wide range of temperature (25- 50 C).

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