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SINGLE CELL PROTEIN PRODUCTION BY ISOLATED AND IDENTIFIED BACTERIA USING CRUDE OIL AS A CARBON SOURCE

KHOSHEE **Q M**OHAMMEDSALIH and **M**USTAFA **M H**AIDER
Dept. of Biology, Collage Of Science, University of Duhok, Kurdistan Region–Iraq

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ABSTRACT

In this study, we isolated and identified the bacteria from contaminated soil with crude oil, from different oil reservoirs of Duhok city, according to their morphological characterization and biochemical activity. The identification of bacterial isolates showed five different strains of bacteria, namely, *Enterobacter cloacae, Serratia marcescens, Cronobacter sakazakii, Burkholderia cepacian, Raoultella ornithinolytica* that are capable of degrading crude oil and have potential to grow on Bushnell-Haas Media (BHM). We also investigated the efficiency of the identified bacteria for consumption of crude oil as a single source of carbon and energy, and for production of single cell protein (SCP). The optimal conditions for bacterial growth, decomposition of crude oil and production of SCP were also determined. Our results showed that 48 hours of incubation and 0.75% concentration of crude oil were the most optimal for the biodegradation of crude oil, bacterial growth and for production of SCP. Additionally, it was noted the increase in the final pH of fermentation media of the five isolated bacteria owing to their metabolic activity leading to the producing substances which increase the final pH value more than the initial pH of the used media.

***KEYWORDS*:** Bacterial isolates, Biodegradation, Crude oil, SCP, Factors

INTRODUCTION

Petroleumphydrocarbons (PHs), also known as crude oil, and its derivatives are one of the most commonly used raw materials in industry, and they are considered as the greatest source of energy for many countries (Shen et al. 2015). However, there is a risk of leak of oil from underground storing containers, or during oil exploration and refinery activities, which may lead to alteration of the soil's physicochemical characteristics and disturb the balance of living things (Vlaev et al. 2011; Kadri et al. 2018).

In contrast to higher organisms' species, certain microbes have the catabolic capacity to use petroleumihydrocarbon as a source of carbon and energy. Several literatures and, studies have highlighted the fact that microorganisms play a significant role in treating the environmental pollution by oil (Ismail, 2015; Dashti *et al*. 2019). One of the safest ways to remove hydrocarbons from the environment is through a process known as biodegradation, which employs microorganisms such as bacteria, archaea, and fungi to break down oil wastes and its derivatives (Medina-Bellver, 2005; Ayangbenro and Babalola, 2017).

The process of biodegradation depends mainly on the genomic characteristics of the microorganism and the enzymes it secretes (Peeples, 2014), and it is one of the applications of biotechnology (Raddadi et al. 2015; Ramos et al. 2011). Additionally, catabolic genes carrying on plasmid and chromosomal DNA (nahAC, PAHRHD, Alkb, Alma, and C230) identified by molecular typing (Ehis-Eriakha et.al. 2020) play a significant role in the breakdown of the complex components of crude oil (Jahangeer, 2013; Raddadi et al. 2015).

The use of microorganism in oil biodegradation is environmentally friendly and cost effectively approach (Alegbeleye et al. 2017). As a matter of fact, low concentration of microorganisms can perform highly efficiently in removing hydrocarbons from the environment compared to chemical treatment or washing and combustion, which are associated with production of toxic compounds (Alegbeleye et al. 2017).

In the past years, efforts have been directed at different parts of the world, whether at the regional or international level, to find solutions

to tackles the obstacles associated with the food shortages,especially protein. The world's protein requirements for the 7.3 billion peoples of the world are approximately 202 million tones globally (Henchion et al. 2017). The severe deficiency of natural protein sources has paved way for production of protein that were unknown earlier, such as oilseed protein, soybean protein and single cell protein (SCP) (Ramadass et al. 2018).

Based on the information from the literature, the percentage of protein in cells of microorganisms ranges 50-80% of cell dry weight, which is higher than any other food source (Abduljabbar et al. 2009). In addition, it contains the vital amino acids. lipids, minerals and some vitamins particularly vitamins that humans and animals require for their growth (Abduljabbar et al. 2009; Boze et al. 2001; Chama, 2019).

Furthermore, such protein can be manufactured, handled and stored with ease. The usage of crude oil for the manufacture of SCP can decrease the production budget and waste treatment decreases the negative environmental influence of these residues (Spalvins et al. 2018).

Aim of the study

The aim of this study was to isolate and identify the indigenous bacterial strains in various Duhok oil fields with respect to their capacity to decompose crude oil. In addition, the study was tailored to evaluate the effectiveness of the identified bacteria in breaking down cru de oil. The optimal conditions for microbial decomposition of crude oil and production of SCP were also determined.

MATERIALS AND METHODS

Preparation of Bushnell Haas Media (BHM)

Yeast extract 1, proteose peptone 1, ($MgSo_4$) 0.2, (KH_2PO_4) 1, (K_2HPO_4) 1, ($NaNo_3$) 1, ($Cacl_2$) trace 0.02, ($Fecl_2$) 0.02 , agar 15 and crude oil were dissolved in one liter of distilled water. This medium used for determination of the ability of bacterial isolates to consume hydrocarbon (Malatova, 2005).

Isolation and Diagnosis of Bacteria

Contaminated soil samples were screened for bacteria. The screening was carried out by diluting 1gram of soil with diluted with a sterilized distill water of 10^{-4}. One 1 ml of the diluted sample was cultured in BHM medium containing 0.5ml crude oil as a carbon source. Second screening and purification were carried out to obtain bacterial isolates that were effective in the utilization of crude oil. Based on cultural characteristics and results of biochemical testing these bacteria were identified (Al-Mehmdi and Al-Rawi, 2019).

Identification and characterization of bacterial isolates by Vitek®2

Vitek is an identification system that can identify bacteria and fungi. This test uses biochemical reactions and nutrient usage of the microorganism to make the identification. The test requires that an adequate amount of growth be obtained during a set growth period of 18-70 hrs. For all obtained bacterial isolate species identification was performed by Vitek®2 compact automated system, using GN-ID and gram GP-ID cards for identification of both Gram negative and gram-positive bacteria according to manufacturer's instruction (Pincus, 2006). /

Cultural conditions and inoculation

Conical flasks (250 ml) were used, and 50 ml of BHM was distributed in each of them, with 3 replicates for each isolate and treatment. The flasks were covered with cotton roll before being sterilized using autoclave (DAIHAN LAB TECH CO., Korea) at 121°C for 15 minutes. Subsequently, the flasks were cooled down to temperature 50 °C. Afterwards, 0.25% of filtrate crude oil (Kashe refinery oil -Duhok). At room temperature, the culture media were inoculated with 5% of two days cultured bacterial cells at pH 7 and incubated at 30±1°C in shaking incubator (SPX-150BIII Bio Incubator) at 150 RPM for 48 hours (Mukred et al. 2008).

Experimental design

Identification and characterization of bacterial isolates Were carried out by Vitek®2 system provide an automated platform for microbial identifying by numerous biochemical tests.

Determination of the impact of incubation time and concentration of crude oil on the growth of bacterial isolates, and the utilization of crude by the bacterial isolates for the production of SCP. The biomass dry weight, SCP, residual crude oil and final pH was determined based on the following:

1. Incubation time: The inoculated culture flasks were incubated at 30 ± 1ºC for (24, 48, 72 and 96 hours).
2. Concentration of carbon source: different concentration of crude oil (0.12, 0.25, 0.5 and 0.75%) were added separately to inoculated culture flasks.

Analysis

Separation of residual crude oil

Separation the remaining crude oil from bacterial culture after the incubation using a separating funnel, and diethyl ether as an organic solvent. After which the separated crude oil is left on a dry plate with a known weight until the solvent evaporates (Al-Murghan, 2007).

Determination of consumed and residual crude oil

The remaining weight is determined by weighing the plate containing the crude oil and determine the consumed weight as the difference between the initial weight of crude oil added and the weight of the remaining crude oil (Al-Mehmdi and Al-Rawi, 2019).

Biomass dry weight assay

The biomass of bacterial isolates was separated from the media by centrifugation at 5000 RPM for 30 minutes. Then, the precipitated biomass was placed in dry glass plates of known weight and dried at a temperature of 65 $^{\circ}$C for 24 hours. The plates were left in room temperature and weighed using a sensitive scale (Varjani and Upasani, 2017).

Determination of amount and percentage of single cell protein

After the biomass drying process, 0.1g of dry weight was taken from each bacterial strain separately and grinded as a powder, then 10 ml of NaOH (1N) solution was added to it and left for 24 hours with shaking. Afterwards, the samples were centrifuged at 5000 RPM for 15minutes, then the filtrates were collected, and the precipitate was discharged. Leggett-Bailey (1967) method was used to determine the amount of protein in each sample depending on the standard protein curve prepared using different concentrations (0, 1, 2, 3, 4, 5 and 6) mg/ml of albumin (Gao and Liu, 2012).

Statistical analysis

The data presented as mean ±SD and were analyzed statistically by IBM SPSS Statistics 22 software and Microsoft Excel 2016. The probability value (P-value) less than 0.05 were considered statistically significant using one-way ANNOVA. While ($P < 0.01$) were considering to be high significant.

RESULTS AND DISCUSSION

Identification and characterization of the bacterial isolates

The isolated bacteria were identified following Vitek's identification system. Table (1) shows the morphological characteristics of bacterial isolates. The bacteria were listed according to their color, shape, odor of colony, staining. Table (2) displays the enzymatic activities of the isolated bacteria. All the isolated and identified bacteria were positive to catalase assay. While, *Burkholderia cepacia* was the only isolate that showed oxidase activity.

Table (1): Morphological characteristics of isolated bacterial strains

Morphological characteristics	Isolated strains				
	Enterobacter cloacae	*Raoultella ornithinolytica*	*Serratia marcescens*	*Cronobacter sakazakii*	*Burkholderia cepacia*
Color	Greyish to white	Pale pink to bright red	Yellow-tan	Grayish	Yellow to white
Shape	Circular	Circular	Circular	Circular	Circular
Edge shape	Convex	Circular	Smooth	rough	Smooth
Odor	Foul odor	Smooth	Rancid odor	dirt like odor	Bad odor
Gram stain	Negative	Fishy odor	Negative	Negative	Negative
Cell shape	Rod shape	Negative Rod shape	Rod shape	Rod shape	Rod shape

Table (2): Biochemical reactions of isolated bacteria

Biochemical tests	*Enterobacter cloacae*	*Serratia marcescens*	*Cronobacter sakazakii*	*Burkholderia cepacia*	*Raoultella ornithinolytica*
Catalase	+ve	+ve	+ve	+ve	+ve
Oxidase	-ve	-ve	-ve	+ve	-ve

2. Effect of incubation duration on the growth of bacterial isolates, biodegradation of crude oil and production of SCP

The most optimal time for the highest bacterial growth, production of SCP and consumption of crude oil was at the 2nd day of the incubation period (table 3 and figure. 1 a, b and c)). Moreover, the bacterium *S. marcescens* was superior to the other tested bacterial isolates, in which the biomass dry weight reached the highest (7.86 g/l) as well as the highest SCP yield (4.45 g/l) was obtained. However, the highest amount of crude oil consumption was achieved by *C. sakazakii* strain (0.05 g/l). The minimum biomass dry weight and produced SCP observed in the case of *R. ornithinolytica* strain were 4.62 g/l and 2.45g/l, respectively. There was significance deference ($P < 0.01$) between biomass dry weight and production of single cell protein. Also, there were significant differences in biomass dry weight of all bacteria at different incubation periods, also significant difference was observed for SCP production at all incubation period. The decrease in the growth of bacteria, SCP production, and utilization of crude oil after 48 hours of incubation for all tested bacteria may be attributed to the accumulation of some harmful chemicals resulting from the metabolic reactions of bacteria, the depletion of the nitrogen source, and alteration in the pH of the medium during the incubation. Similar findings have been described earlier (Naveen et al. 2010). In addition, several studies had shown that 48 hours of incubation was the best duration for the growth of bacteria and for production of SCP (Guo-liang et al. 2005; Batista et al. 2006; Al-Murghan, 2007). Nonetheless, Manzano (2003), had indicated that the percentage of decomposition of aromatic polychlorinated compounds reached the highest after 72 hours of incubation.

Table (3): Effect of different incubation periods on dry weight and SCP production

Bacterial isolates	Time	Biomass weight g/l	SCP g/l	SCP %	Residual Sugar g/l	Final pH
Enterobacter cloacae	1st day	3.79 ±0.434	2.11 ±0.085	55.8	0.17 ±0.052	8.5
	2nd day	7.42 ±0.140	4.17 ±0.060	56.2	0.14 ±0.026	8.8
	3rd day	3.42 ±0.393	1.92 ±0.040	56.0	0.12 ±0.049	8.4
	4th day	1.69 ±0.041	0.92 ±0.055	54.2	0.11 ±0.020	8.4
Serratia marcescens	1st day	2.03 ±0.151	1.14 ±0.040	56.2	0.19 ±0.045	8.4
	2nd day	7.86 ±0.081	4.45 ±0.285	56.7	0.16 ±0.035	8.5
	3rd day	7.04 ±0.081	3.97 ±0.090	56.4	0.08 ±0.051	8.5
	4th day	5.25 ±0.165	2.82 ±0.020	53.9	0.05 ±0.015	8.5
Cronobacter sakazakii	1st day	1.21 ±0.041	0.67 ±0.060	55.0	0.20 ±0.020	8.8
	2nd day	5.67 ±0.131	3.24 ±0.065	57.3	0.15 ±0.035	9.1
	3rd day	5.60 ±0.185	3.17 ±0.060	57.0	0.08 ±0.026	9.1
	4th day	4.65 ±0.196	2.48 ±0.030	53.3	0.07 ±0.020	9.3
Burkholderia cepacia	1st day	2.21 ±0.134	1.18 ±0.080	53.3	0.18 ±0.015	8.4
	2nd day	4.63 ±0.145	2.52 ±0.395	54.6	0.13 ±0.046	8.8
	3rd day	4.22 ±0.130	2.31 ±0.295	54.9	0.11 ±0.035	9.0
	4th day	3.37 ±0.115	1.81 ±0.245	53.8	0.10 ±0.020	9.0
Raoultella ornithinolytica	1st day	0.91 ±0.040	0.48 ±0.208	53.1	0.22 ±0.049	8.3
	2nd day	4.62 ±0.152	2.45 ±0.260	53.2	0.15 ±0.036	8.6
	3rd day	4.43 ±0.170	2.35 ±0.282	53.1	0.12 ±0.025	9.0
	4th day	4.60 ±0.095	2.42 ±0.140	52.6	0.09 ±0.032	9.0

Each number represent the mean of triplicates and numbers between brackets represent standard deviation (±S D)

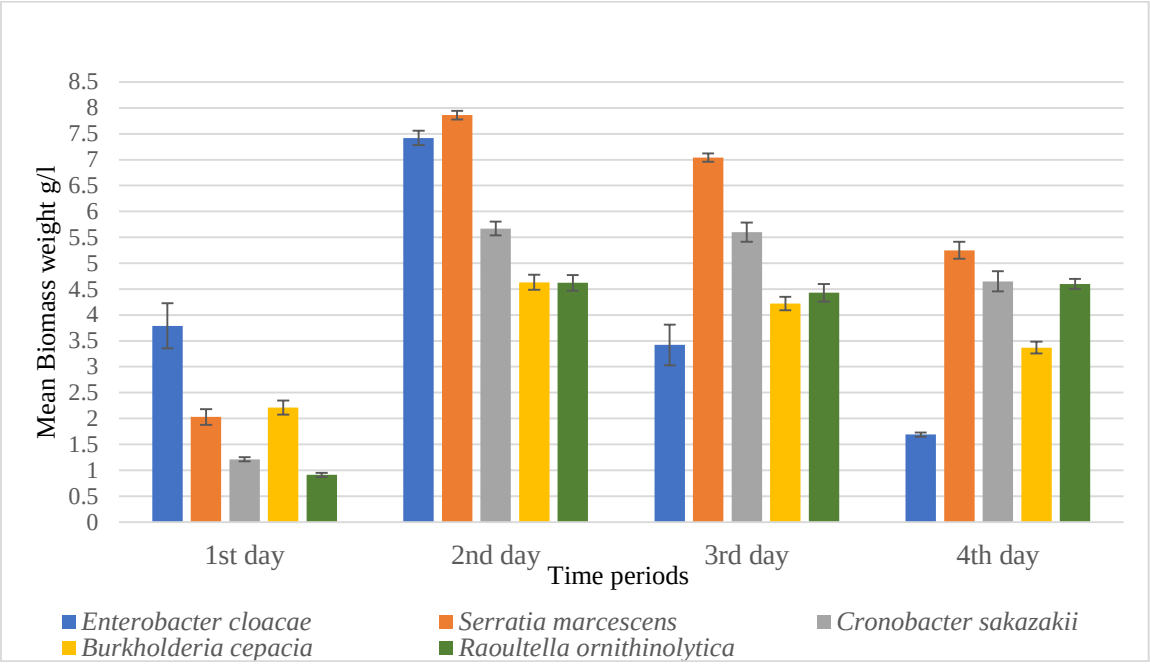

Fig. (1): Effect of different incubation periods on biomass dry weight

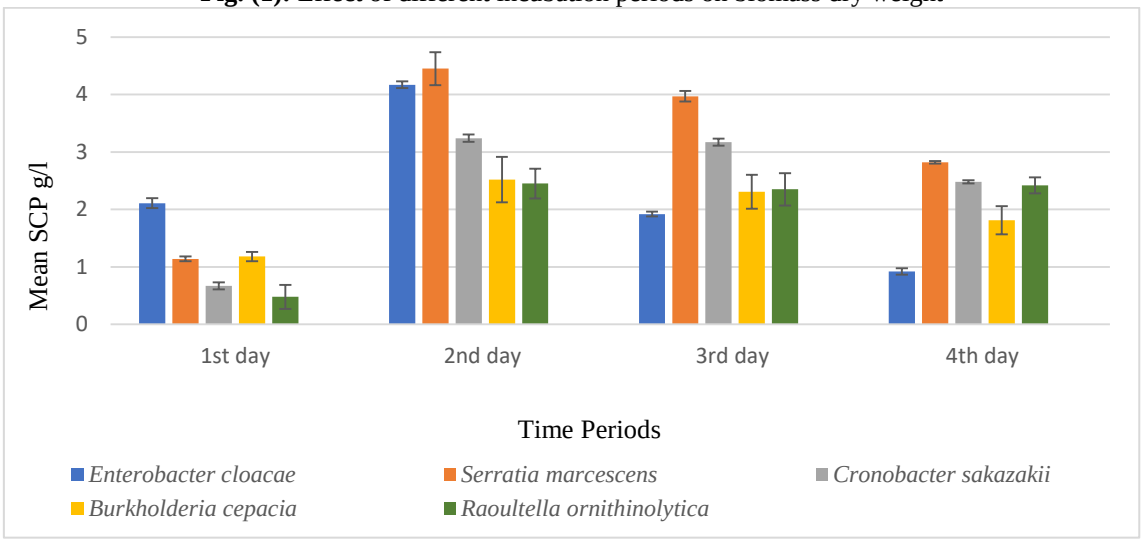

Fig. (1b): Effect of different incubation periods on amount of SCP g/l

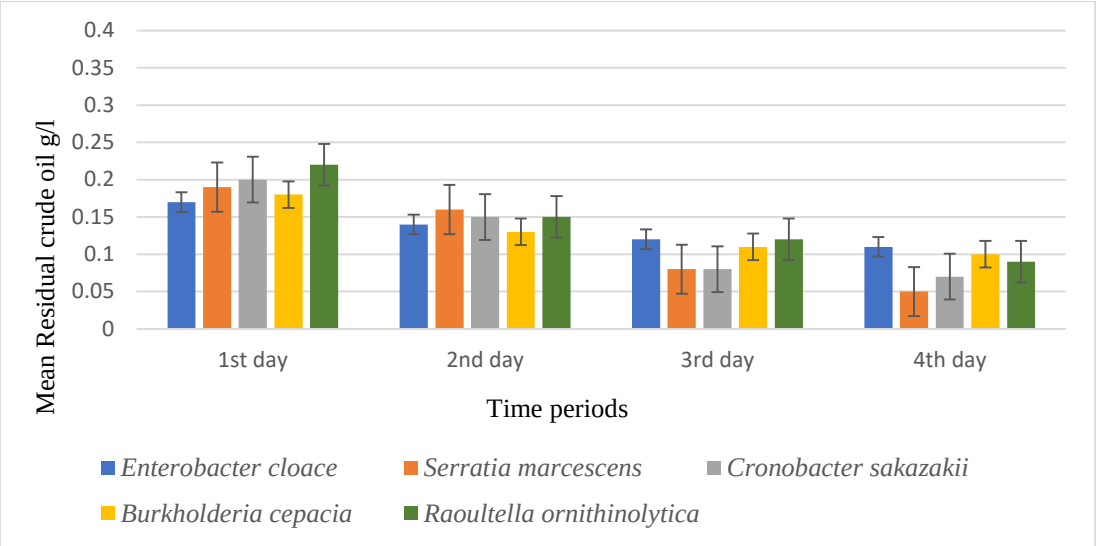

Fig. (1c): Effect of different incubation periods on residual oil g/l

3. Effect of different concentration of crude oil on the growth of bacterial strains, oil biodegradation and production of SCP

It was clear, that an increase in biomass occurs upon increasing the concentration of crude oil in the fermentation medium (tables 4 and figure 2 a, b, c). It was also noted that the increase in consumption of crude oil was directly proportional with the increase in the concentration of crude oil. The bacterium *B. cepacia* recorded the highest consumption of crude oil compared to the other isolates in which the concentration of residual sugar decreased from 0.75% to 0.2%. In addition, the highest biomass dry weight (9.26 g/l) and production of SCP (4.82 g/l) was obtained by the same isolate and fermentation medium. In comparison with the other isolates using the same fermentation medium containing 0.75% crude oil, as a carbon source, we found that the minimum amount of bacterial growth was recorded (6.43 g/l) in *R. ornithinolytica*, lesser amount of SCP seen in Strain *C. sakazakii* (3.60 g/l), Moreover, the minimal amount of crude oil consumption was seen in *R. ornithinolytica* isolate (0.36g/l) using similar fermentation conditions.

Table (4): Effect of different concentration of crude oil on the growth of bacterial strains, oil biodegradation and production of SCP

Bacterial isolates	crude oil concentration %	Biomass weight g/l	SCP g/l	SCP %	Residual Sugar g/l	Final pH
Enterobacter cloacae	0.12	1.27 ±0.141	0.70 ±0.070	55.5	0.05 ±0.026	8.1
	0.25	3.84 ±0.144	2.10 ±0.120	54.8	0.09 ±0.025	8.2
	0.50	4.83 ±0.140	2.7 ±0.088	55.8	0.13 ±0.023	8.2
	0.75	7.29 ±0.252	4.03 ±0.035	55.3	0.31 ±0.036	8.3
Serratia marcescens	0.12	2.83 ±0.190	1.59 ±0.030	56.1	0.05 ±0.035	8.2
	0.25	3.75 ±0.079	2.05 ±0.075	55.6	0.08 ±0.030	8.2
	0.50	7.08 ±0.035	3.96 ±0.050	55.9	0.19 ±0.015	8.4
	0.75	7.41 ±0.090	4.08 ±0.062	55.0	0.28 ±0.020	8.5
Cronobacter sakazakii	0.12	2.17 ±0.160	1.23 ±0.070	56.8	0.03 ±0.025	8.6
	0.25	3.5 ±0.295	1.94 ±0.034	55.3	0.10 ±0.020	8.7

	0.50	6.43 ±0.072	3.61 ±0.051	56.2	0.17 ±0.025	8.9
	0.75	6.45 ±0.060	3.60 ±0.041	55.9	0.21 ±0.025	9.0
Burkholderia cepacia	0.12	1.01 ±0.072	0.55 ±0.035	54.2	0.06 ±0.016	8.7
	0.25	3.88 ±0.140	2.10 ±0.055	54.0	0.09 ±0.026	8.8
	0.50	7.81 ±0.105	4.24 ±0.020	54.3	0.11 ±0.015	8.9
	0.75	9.26 ±0.146	4.82 ±0.058	52.1	0.2 ±0.152	8.9
Raoultella ornithinolytica	0.12	3.53 ±0.167	1.89 ±0.045	53.9	0.04 ±0.026	8.0
	0.25	3.42 ±0.200	1.81 ±0.100	53.1	0.11 ±0.015	8.0
	0.50	5.92 ±0.079	3.15 ±0.049	53.3	0.21 ±0.020	8.2
	0.75	6.43 ±0.045	3.46 ±0.050	53.9	0.36 ±0.015	8.4

Each number represent the mean of triplicates and numbers between brackets represent standard deviation (±S D)

There were statistically significant differences at significance level (a =l0.05) in biomass dry weight of all bacteria at different concentrations of crude oil, also significant difference was observed for SCP production at all concentrations of crude oil.

The present study indicates that as the amount of hydrocarbon increases, the consumption of hydrocarbon by the studied isolates also increases, along with an increase in dry biomass weight. These findings are in line with previous research conducted by (Zaki et al. 1983; Rahman et al. 2002; Diaz-Rambres et al. 2003; Eriksson et al. 2022). Additionally, results of the study are consistent with the findings of Sathishkumar et al. (2010) and Haider et al. (2023). These two studies likely explored similar topics and also observed an increase in hydrocarbon consumption and biomass weight with higher hydrocarbon levels. On the other hand, the studies by Varjani et al. (2015) and Varjani (2017) showed that the optimal crude oil consumption was at 3% for two different species of bacteria. At this concentration, the bacterial cells adapted and utilized the hydrocarbon as a single source of carbon and energy. They also produced emulsifiers to aid in breaking down the fat and consuming it. However, when the concentration of hydrocarbon residues exceeded 3%, it resulted in decreased cell division due to the high toxicity of the higher concentration (Abdelkader et al. 2015).

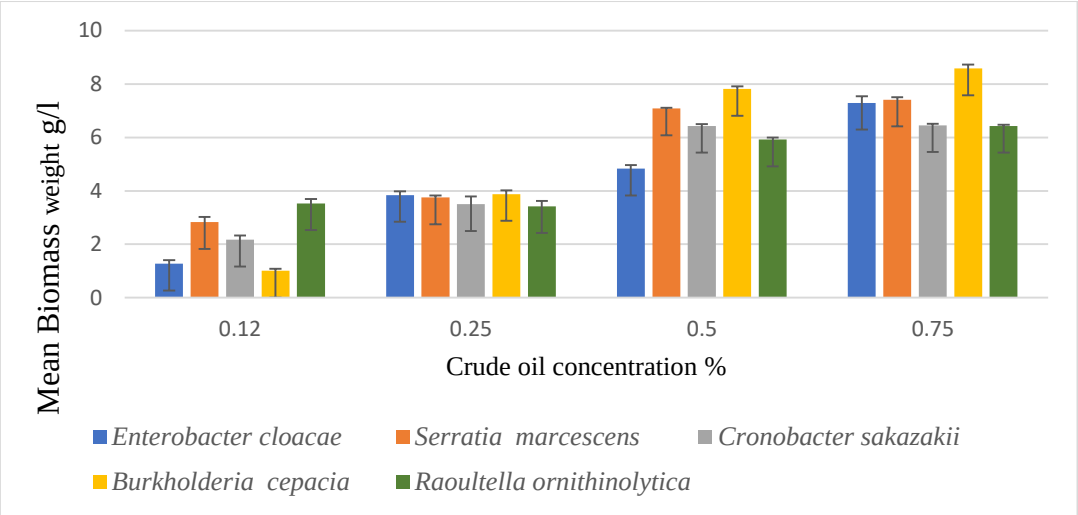

Fig. (2a): Effect of different crude oil concentration on Biomass weight g/l

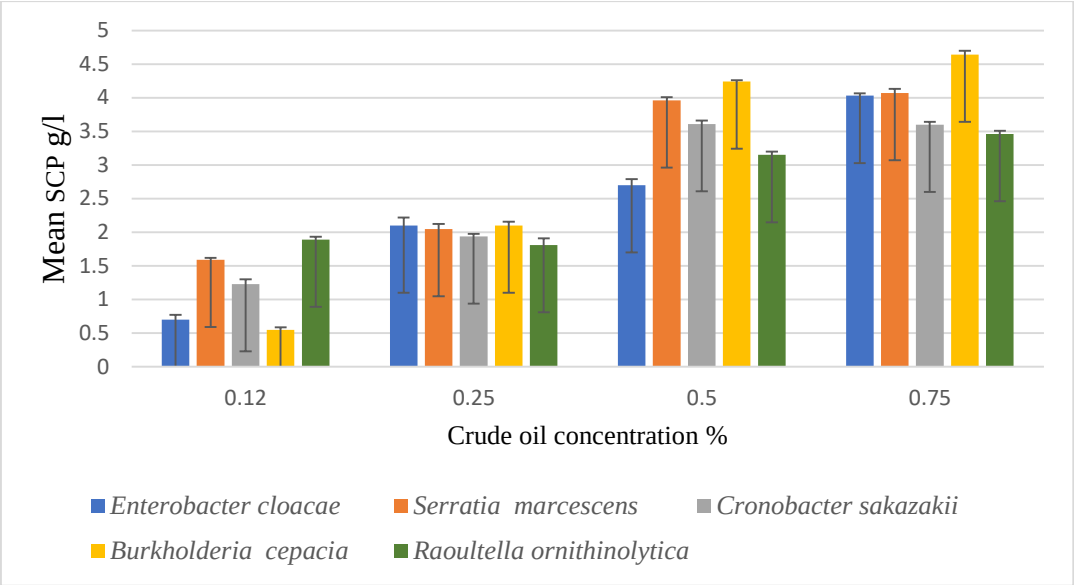

Fig. (2b): Effect of different crude oil concentration on amount of SCP g/l

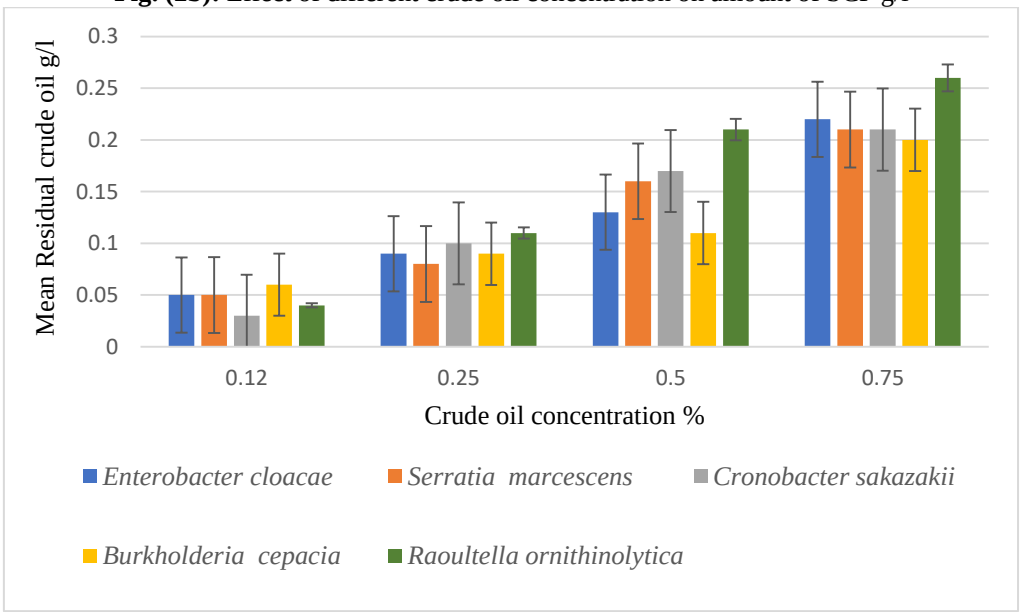

Fig. (2c): Effect of different crude oil concentration on residual oil g/l

CONCLUSION

In present study, it was demonstrated that the five isolated bacteria have the capacity to biodegrade crude oil and utilized as energy and as a sole carbon source. Also, the most optimal incubation period and concentration of crude oil was 48 hours and 0.75%, respectively. These results are of high interest as they help to reduce soil polluted by crude oil using the isolated bacteria. In addition, biodegradation of crude oil by the isolated bacteria help to produce single cell protein which in turn is used as animal feed. This process is highly cost effective and efficient for treatment of polluted soil.

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انتاج البروتين أحادي الخلية بواسطة بكتيريا معزولة و معرفة باستخدام النفط الخام كمصدروحيد للكربون

الخلاصة

في هذه الدراسة, تم عزل و تشخيص البكتيريا من التربة الملوثة بالنفط الخام ، من خزانات المختلفة لمدينة دهوك ، وفقًا لتوصيفها المورفولوجي و الاختبارات البيوكيميائية أظهر التعرف على العزلات البكتيرية من خمس سلالات مختلفة وهي *Enterobacter cloacae* و *Serratia marcescens* و *Cronobacter sakazakii* و *Burkholderia cepacian* و *Raoultella ornithinolytica* القادرة على تحلل الزيت الخام ولديها القدرة على النمو على الوسط الغذائي (Bushnell-Haas Media (BHM . وتبين من الدراسة بان للبكتريا المعزولة الكفاءة على االتحلل الحيوي للنفط الخام واستخدامة كمصدر وحيد للكربون والطاقة لغرض النمو وإنتاج البروتين أحادي الخلية (SCP). وعند تحديد االفترة الزمنية المثلى للحضانة لنمو البكتيريا وإنتاج بروتين احادي أظهرت النتائج أن 48 ساعة من الحضانة وتركيز 0.75٪ من النفط الخام كمصدر للكربون هي الأمثل للتحلل الحيوي للنفط الخام والنمو البكتيري وانتاج بروتين احادي الخلية. فضلا عن ذلك لوحظ ارتفاع في القم الهيدروجيني لأوساط التخمر لجميع العزلات لمستخدمة وهذا يعود للنشاط الايضي لهذه العزلات وانتاج المركبات التي تغير من الرقم الهيدروجيني الاولي المستخدمة لهذه الاوساط.