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Research Article

Conditional Optimization of Single Cell Protein Production from Crude Oil by *Pseudomonas aeruginosa*

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Abstract

The study was carried out to investigate the possible biodegradation of crude oil as a carbon by the bacterium *Pseudomonas aeruginosa* isolated from marine environment (Ras El-Menkar- Benghazi- Libya) using basal yeast extract protease peptone-3 (BYP) enriched medium. The isolated bacterium was identified and characterized according to its cultural condition and microbial biochemical properties. Different experiments were developed throughout this study to stimulate bacterial growth and production of single cell protein (SCP). The results show that the optimal concentration of crude oil as a carbon source for the highest bacterial growth (1.14g/l), and production of SCP (0.65g/l; 57.02% of the biomass dry weight) was 1%. This was required to utilize up to 50.6% of oil as a carbon source. As to the nitrogen source, the optimal concentration of ammonium chloride was 0.1%, in which the bacterial growth and SCP production increased to 1.23 g/l and 0.67 g/l respectively. The stimulating effects of organic and inorganic factors on the bacterial growth and SCP production was also tested. Addition of inorganic nutrients such as potassium phosphate (0.05%), magnesium sulphate (0.01%), and organic nutrient in the form of yeast extract (0.1%) to the fermentation medium slightly promoted the bacterial growth which reflected positively on SCP production and the percentage of the consumed crude oil, (>57%) at final pH value of 8.0. The obtained results indicated that the isolated *Pseudomonas aeruginosa* posses the ability to utilize the crude oil and use it as a carbon for bacterial growth and production of SCP.

Keywords

Crude Oil, *Pseudomonas aeruginosa*, SCP, Nitrogen Source, Inorganic and Organic Factors

1. Introduction

In the past years, researchers have been focusing on finding alternatives to tackles the issues associated with food shortages, especially protein at global and national levels. It was estimated that the global reserve of protein is around 211 million tons annually [1]. It is also estimated that the annual shortage of protein is about 30-40 million tons. Due to the high shortage and the growing demands of protein around the globe, new methods for production of proteins have been emerged through the past years, including those produced from oily seeds, soya and single cell protein (SCP) microbial biomass, [2, 3].

SCP refers to the dry cells in microorganisms such as al single cells. Recently, the use of crude oi gae, bacteria, acti-

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nomycetes, molds and yeasts that consist of l for the production of single cell protein has drawn a significant attention. Hamer and Hamdan [4] have utilized hydrocarbons derived from crude oil as a main source for production of single cell protein in Middle East and Africa. As a matter of fact, crude oils are rich in hydrocarbons which are important sources of nitrogen and carbon making them favorable media for the growth of multitude of microorganisms and production of energy. Microbial degradation of crude oil helps to provide nutritional supply and energy that are essential for the growth of microorganisms, [5].

Yosuri [6] has explained the production of single cell protein from hydrocarbons as beneficial due to the low cost of production, availability of crude oil and highly efficient productivity of the protein. The study has also indicated that the nutritional value of the single cell protein is high and free from toxic and unwanted wastes and can be used as a proper nutrition for animals.

Concentration of cultural medium components is critical to determine the favorable condition for the growth of microorganisms. High concentrations may lead to inhibition of microorganism growth, while low concentrations may be too low to accommodate the needs of the microorganism. Nitrogen plays a major role in the growth of microorganisms as it is an essential component in amino acids and protein structure of microorganism. Nitrogen is also an important co-enzyme and accounts for about 14% of dry biomass of microorganisms, [7, 8]. Harris [9] has shown that addition of nitrogen source increased biodegradation of crude oil by microorganism compared to medium free from nitrogen source. Moreover, Ławniczak et al., [10] have shown that balanced C:N:P ratio have significant effect on the initial stage of hydrocarbon biodegradation by the microorganisms and production of SCP.

The elemental phosphorus and magnesium are essential components for cultural media of microbial growth. Phosphorus is a main element in the composition of fatty acids, and phospholipids in cell membranes. It is also incorporated into cell walls of bacteria, and act as a co-enzyme for some metabolic processes and is involved in energy production in the form of ATP [11]. Phosphorus acts as buffering agent for pH of cultural media [12]. Magnesium act as a catalyst for hydrolysis of hydrocarbon by microorganisms and in the synthesis of fatty acids. It also regulates the cellular level of ionization and acts as a co-factor for multiple enzymes that are involved in the production of ATP and ADP. Magnesium is involved in the structure of cellular walls and membranes of bacteria, as well as protein synthesis through transcription of ribosomes. On the other hand, potassium is imperative element in energy production for all living organisms. It actively contributes to protein synthesis and act as a catalyst for multiple metabolisms. Therefore, the present of this element in the fermentation media of hydrocarbon biodegradation at an appropriate concentration increase the ability of microorganism to utilize crude oil which is used as a carbon source for the growth and production of energy [13]. Moreover, the acceleration of crude oil biodegradation requires the presence of oxygen since the initial step of hydrocarbon biodegradation involves the oxidation of the substrate by oxygenating enzymes for which molecular oxygen is needed [14]. *Pseudomonas aeruginosa* has been widely used in hydrocarbon biodegradation. This is due to the production of biosurfactant by the bacterium which facilitates the breakdown the atomic bonds of hydrocarbon and the byproduct is used as a carbon for bacterial growth and production of SCP [15-17].

The growth of microorganisms and production of SCP using crude oil as a sole source of carbon can decrease the pollution of the environment, meanwhile increases the supply of protein with protein supplements or animal feed [3]. Moreover, the production of SCP by *Pseudomonas aeruginosa* is of high nutritional value owing to the fact that SCP has a high content of essential and non-essential amino acids [18].

The present study was designed to determine the ability of the isolated bacterium *Pseudomonas aeruginosa* to produce SCP using crude oil as a single source of carbon for its growth and production of energy. Also, the aim of the study was to increase the potentiality of the bacterium for degradation of crude oil and production of high amount of SCP by optimizing the cultural condition used for bacterial growth.

2. Materials and Methods

2.1. Diagnosis of the Isolated Bacterium

The bacterium *Pseudomonas aeruginosa* was isolated from the contaminated soil of Ras Al-Manqar beach, Benghazi, Libya. It was identified and characterized according to the methods by Dawood et al., [19] with the expiation that crude oil used instead of motor oil. It was used for the utilization of crude oil as a single carbon source for growth and production of SCP.

2.2. Strain Maintenance Condition and Activation

The marine bacterial isolate was preserved at 4 ℃ on the marine agar slant medium and activated every three weeks.

2.3. The Used Media

Basal yeast extract Proteose peptone-3 (BYP) medium consists of (g/l): sodium sulfate, 2.0; potassium nitrate, 2.0; ammonium chloride, 1.0; potassium phosphate, 0.5; aqueous magnesium sulfate, 0.1; yeast extract, 1.0; proton-peptone-3, 1.0; calcium chloride, 0.001; anhydrous ferrous sulfate (trace) was used to determine the ability of the bacterial isolate to utilize the crude oil as a carbon source for the growth and SCP production [20].

2.4. Cultural Condition and Inoculation

Conical flasks, of 500 ml size were used in which 50 ml of BYP medium distributed at three replicates per treatment, plugged and autoclaved at 121 ℃ and atmospheric pressure 15 lbs / inch ^{3}for 15 minutes. After sterilization, the flasks were left to cool to a temperature of 50 ℃. Afterwards, the required concentration of filter sterilized (silver filters 0.2 µ m pore size, spring House, USA,) crude oil (Albury field oil - Libya) was added. Afterwards, the cold flasks were inoculated with 24-hour age bacterial inoculum at a concentration of 5% at pH (7.3), and incubated in a rotary incubator (Incubators, GP, UK) at a temperature of 30 ± 1 ℃ for 48 hours at a rotation speed of 150 RPM.

2.5. Experimental Design

I. Identification and characterization of the bacterial isolate.

II. Determination the effect of some factors on the growth of bacterial isolate and SCP production using BYP medium as a basal medium.

1. Carbon source: different concentrations (0.025, 0.05, 0.1, 0.15 and 0.2%) of crude oil were used as a single carbon source.

2. Ammonium chloride: different concentration of ammonium chloride (0.06, 0.08, 0.10, 0.12, 0.14 and 0.16%) as a nitrogen source were used.

3. Magnesium sulphate: Different concentration of magnesium sulphate (0.006, 0.008, 0.010, 0.012, 0.014%) as a growth factor.

4. Yeast extract: different concentrations (0.06, 0.08, 0.10, 0.12 and 0.14%) of yeast extract as a promoting growth factor were used.

5. Potassium phosphate: different concentration (0.01, 0.03, 0.05, 0.07 and 0.09%) added as a growth factor.

2.6. Analysis

2.6.1. Separation of Residual Crude Oil

At the end of the incubation period, the remaining crude oil from the bacterial culture was separated using diethyl ether as a solvent (T-Baker Lab Chem. India), and left in a dry weight-known beaker for 24 hours until the solvent evaporates.

2.6.2. Determination of Residual and Consumed Crude Oil

The residual crude oil was determined by taking the variation between the beaker containing the crude oil and the weight of beaker alone, while the weight of consumed crude oil was determined by the difference in the weight between the added primary crude oil and the remaining crude oil.

2.6.3. Biomass Determination

The biomass of the bacterial isolate was separated from the food fermentation medium by centrifugation (Series, 1020D, UK) at 10,000 rpm for 10 minutes. Subsequently, the produced biomass was rinsed with sterile distilled water and suspended for two hours, and precipitated at 6,000 rpm for 20 minutes. Next, the precipitated cells were dried in an oven at 65 ℃ for 24 hours. The actual weight of the biomass dry weight was determined using sensitive balance (Mettler P165 Gallenkamp, Switzerland).

2.6.4. Protein Content Determination

The concentration of protein in a single sample was determined following the method by Legett-Bailey [21] using a spectrophotometer (Jenway 6300, UK) at 330 nm wavelength and bovine albumin was used as a standard.

2.7. Statistical Analysis

All data presented as mean ± SD (Tables 3, 4, 5, 6 and 7). Graph Pad 5.05 was used to investigate the statistical correlation between the production of SCP and the cultural conditions. ($P<0.05$).

3. Results

1. Identification and characterization of the bacterium:

The bacterium *Pseudomonas aeruginosa* was identified according to the cultural characteristic, physiological properties and biochemical test as described in table 1 & table 2.

Table 1. *Microbial characterization of Pseudomonas aeruginosa.*

Tests	Results
Catalase	+ve
Oxidase	+ve
Urease	+ve
Citrate	+ve
Indol	+ve
Motality	+ve
Methyl red	-ve
Gelatin decomposition	+ve
Growth on MacConky	+ve
Growth on Citramide	+ve

Table 2. *Biochemical test of the bacterium Pseudomonas aeruginosa.*

Characters	results
Colonies shape	Circular
Colonies edge	Irregular
Cells shape	Strenuous
Colonies color	Creamy
Colonies high	High
Gram staining	Negative
Stain production	Green stain

2. The effect of different concentrations of crude oil on bacterial growth, crude oil consumption and SCP production:

This experiment was aimed to demonstrate the effect of different concentrations of added crude oil on the ability of bacteria to consume crude oil, bacterial growth and SCP production. It is clear that crude oil consumption, biomass dry weight and SCP production was directly proportion to the amount of the added crude oil to the fermentation media (Table 3). The highest biomass dry weight obtained was 1.14g/l, with the consumption of 50.60% of the used crude oil, and the highest SCP production was 0.65g/l. addition of crude oil more than 1% have negative effect on growth of the bacterium and even the production of SCP.

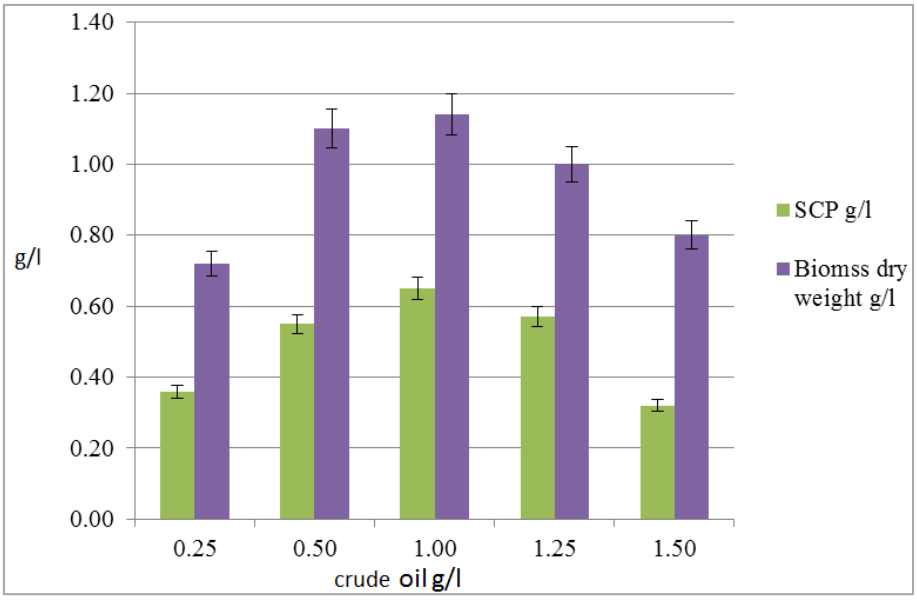

Figure 1. *The relation between bacterial growth and production of SCP using different concentration of crude oil.*

Table 3. *Effect of different concentrations of crude oil consumption on bacterial growth, and SCP production.*

crude oil%	consumed crude oil%	SCP g/l	Biomass dry weight g/l	SCP%	final PH
0.25	35.2	0.36 ±0.045	0.72 ±0.004	49.44	8
0.5	49.4	0.55 ±0.009	1.10 ±0.003	50.09	8.24
1	50.6	0.65 ±0.036	1.14 ±0.015	57.02	8.18
1.25	42.4	0.57 ±0.071	1.00 ±0.053	56.9	8.14
1.5	24.82	0.32 ±0.052	0.80 ±0.073	40	8.32

3. Effect of different concentrations of ammonium chloride on crude oil consumption, bacterial strain growth, and SCP production:

Six different concentrations of ammonium chloride as a nitrogen source were used to show the best concentration that stimulate bacterial growth, SCP production and enhanced its ability for consumption of high amount of crude oil. The results shown in table 4 clearly demonstrated that the fermentation medium containing 0.1% stimulated bacterial growth up to 1.23g/l, SCP production to 0.67g/l that is

equivalent to 54.95% of the biomass dry weight. Moreover, the consumed crude oil by the bacterial isolates was also increased reaching 58.35% in the same medium.

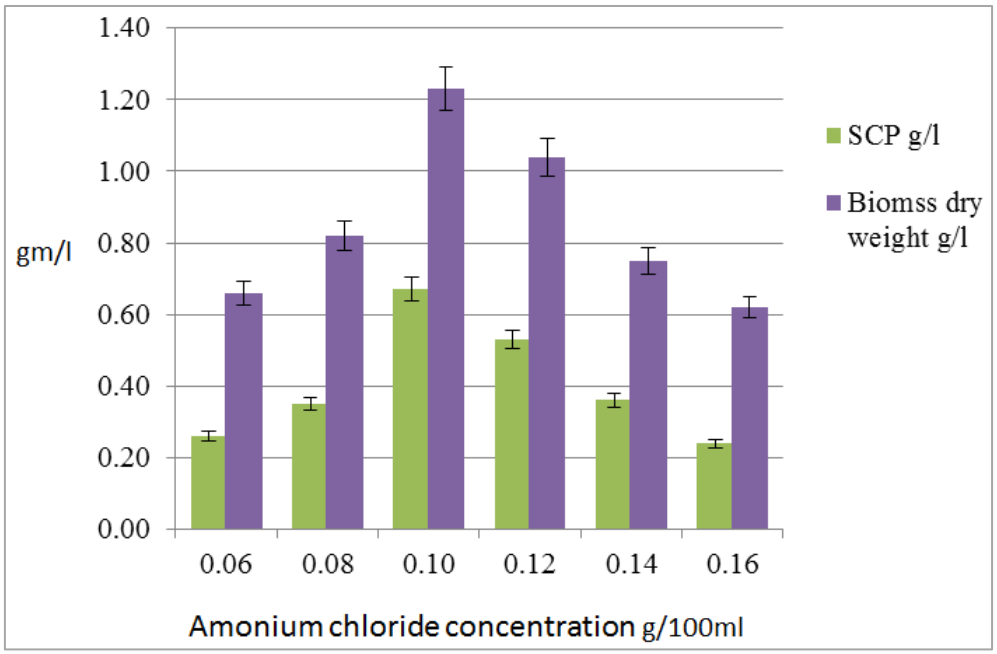

Figure 2. *The relation between bacterial growth and production of SCP using different concentration of ammonium sulphate.*

Table 4. *Effect of ammonium chloride concentration on consumption of crude oil, bacterial growth and SCP production.*

Ammonium chloride gm/100 ml	consumed crude oil%	SCP g/l	Biomass dry weight g/l	SCP%	final pH
0.06	15.2	0.26 ±0.007	0.66 ±0.041	34.54	8.12
0.08	37.2	0.35 ±0.025	0.82 ±0.025	43.75	8.12
0.1	58.35	0.67 ±0.006	1.23 ±0.37	54.95	8.24
0.12	40.64	0.53 ±0.004	1.04 ±0.001	51.01	8.1
0.14	28.41	0.36 ±0.072	0.75 ±0.006	48.31	8.08
0.16	18.53	0.24 ±0.008	0.62 ±0.006	38.87	8.13

4. Effect of different concentrations of aqueous magnesium sulfate on crude oil consumption, bacterial growth and SCP production:

Given the fact that crude oil consists only of carbon and hydrogen, it is essential to add an inorganic source to improve the activity of the used bacteria. Different concentrations of magnesium chloride were added to the fermentation medium to find out the optimal concentration that support the highest crude oil biodegradation and production of SCP. As shown in table 5, the consumption of crude oil and production of SCP as well as bacterial growth were increasing with increasing concentration of magnesium sulphate. The optimal concentration that showed the highest yield was in a medium containing 0.01%. Increasing the amount of magnesium sulphate in the fermentation medium has led to poor crude oil consumption, weak growth and low biosynthesis of SCP.

Table 5. *Effect of magnesium sulphate concentration on the consumption of crude oil, bacterial growth and of SCP production.*

Magnesium sulphate gm/100 ml	consumed crude oil %	SCP g/l	Biomass dry weight g/l	SCP %	final PH
0.006	25.33	0.30 ±0.046	0.77 ±0.008	39.14	7.77
0.008	40.1	0.42 ±0.006	0.91 ±0.008	45.83	7.73
0.01	53.01	0.71 ±0.051	1.27 ±0.052	55.9	8.11
0.012	38.22	0.39 ±0.008	0.88 ±0.053	44.56	7.89
0.014	15.6	0.23 ±0.038	0.65 ±0.009	35.87	7.91

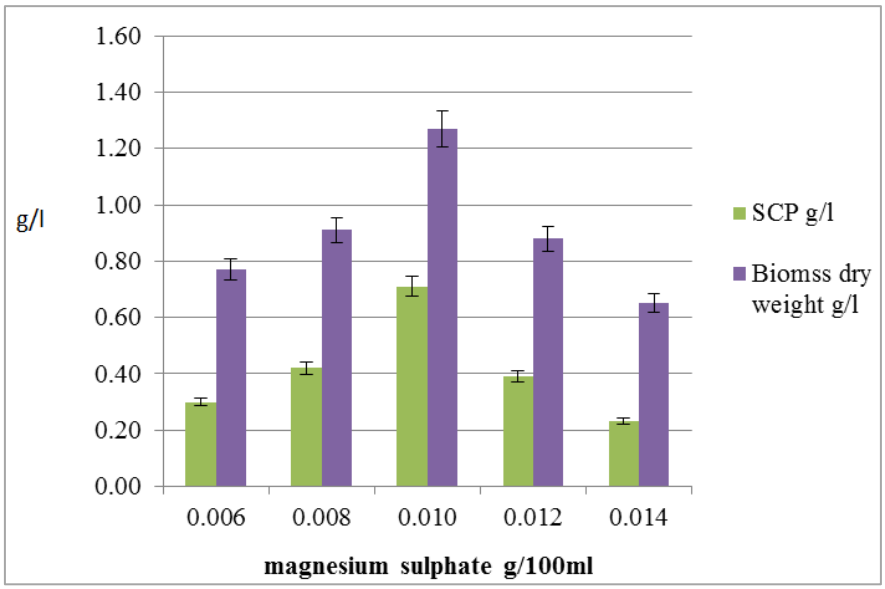

Figure 3. *The relation between bacterial growth and production of SCP using different concentrations of magnesium sulphate.*

5. Effect of different concentrations of yeast extract on bacterial growth, crude oil consumption and single cell protein production:

The presence of yeast extract in the fermentation media further stimulated the activity of the bacterial isolate for higher crude oil biodegradation. It also improved the bacterial growth and increased the production of SCP. As shown in table 6, the best concentration of the added yeast extract was at concentration (0.10%) in which the percentage of consumed crude oil reached 57.36%, which resulted in higher biomass dry weight (1.29g/l) and more SCP production (0.74g/l, 57.01%).

Table 6. *Effect of different yeast extract concentration on crude oil consumption, bacterial growth and SCP production.*

Yeast Extract	consumed crude oil%	SCP g/l	Biomass dry weight g/l	SCP%	final PH
0.06	46.55	0.42 ±0.038	0.86 ±0.036	48.7	7.87
0.08	48.41	0.57 ±0.042	0.98 ±0.009	53.78	7.82
0.1	57.36	0.74 ±0.051	1.29 ±0.053	57.01	8.24
0.12	41.94	0.43 ±0.059	0.94 ±0.062	45.38	7.9
0.14	19.22	0.21 ±0.63	0.55 ±0.066	38.09	7.78

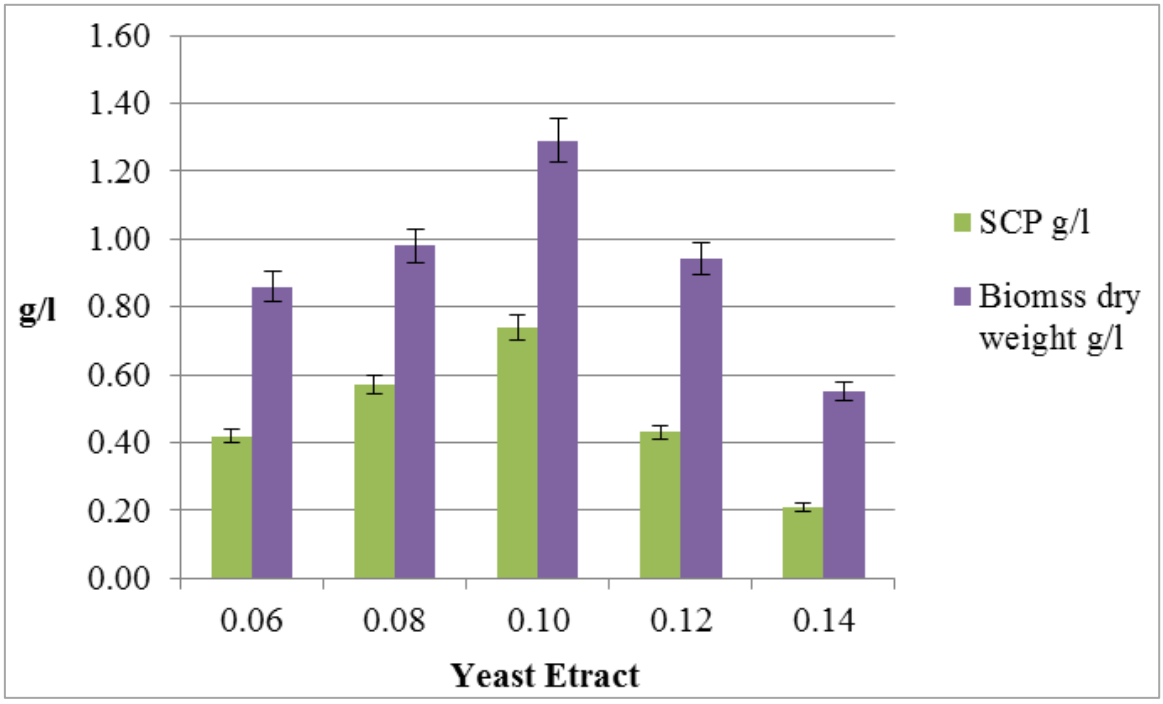

Figure 4. *The relation between bacterial growth and production of SCP using different concentrations of yeast extract.*

6. Effect of different concentrations of potassium phosphate on bacterial isolate growth, oil consumption and SCP production:

As shown in table 7 there was stimulation in biodegradation of crude oil and bacterial growth and production of SCP by the addition of potassium phosphate to the fermentation medium. The best result was in the cultural medium containing 0.05% potassium phosphate in which the percentage of crude oil consumption was 57.75% and the highest bacterial growth was 1.24g/l. Additionally, the highest biosynthesis of SCP was 0.67g/l. This amount is equivalent to 57.04% of the total biomass dry weight.

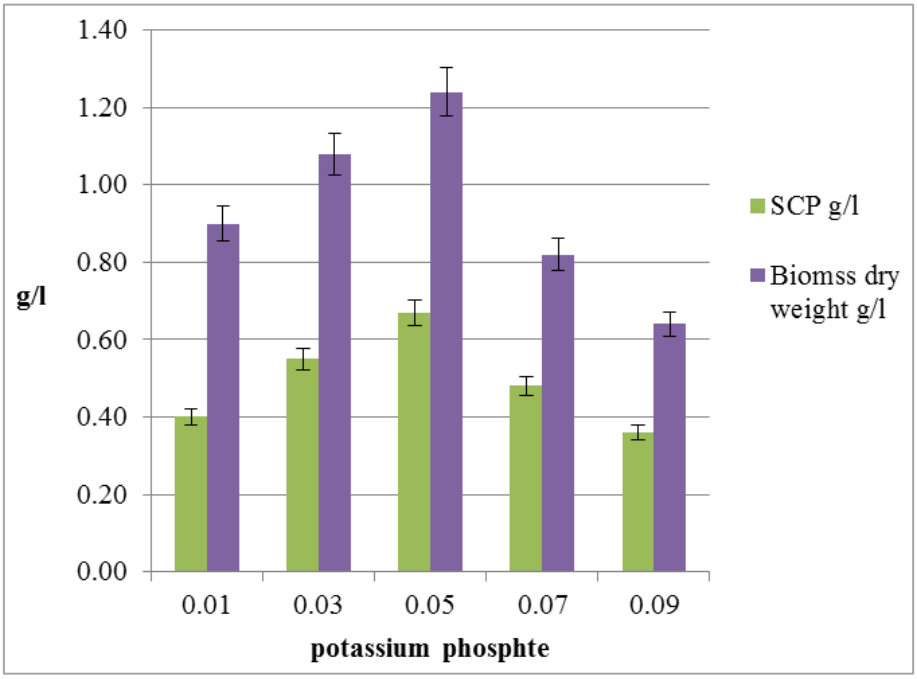

Figure 5. *The relation between bacterial growth and production of SCP using different concentration of potassium phosphate.*

Table 7. Effect of different concentrations of potassium phosphate on bacterial isolate growth, oil consumption and SCP production.

Potassium phosphate %	consumed crude oil%	SCP g/l	Biomass dry weight g/l	SCP%	final pH
0.01	33.64	0.40 ±0.56	0.90 ±0.063	44.21	7.43
0.03	50.52	0.55 ±0.071	1.08 ±0.007	51.04	7.67
0.05	57.75	0.67 ±0.045	1.24 ±0.018	54.03	8.0
0.07	36.66	0.48 ±0.072	0.82 ±0.015	58.22	7.32
0.09	9.22	0.36 ±0.062	0.64 ±0.053	56.54	7.21

4. Discussion

It has been postulated that degradation of crude oil and production of SCP by bacteria were affected by the physiological nature of the fermentation media. Crude oil is rich in carbon making it a highly favorable for the bacteria to produce sufficient energy that is necessary for both bacterial growth and metabolic activity that is involved in production of SCP. {Harris, 2003 [9]; Shen et al., 2014 [22]; Hinchion et. al., 2017) [1]. There for, different experiment were carried out to determine the best physiological condition of the fermentation medium for high crude oil biodegradation, best growth of bacteria and more SCP production by the bacterium *Pseudomonas aeruginosa*. The results of the effect of different concentration of crude show that the presence of 0.1% of crude oil in the fermentation media was the best for more crude oil biodegradation, best growth and high SCP production It is noteworthy to highlight that crude oil above 1% showed to have suppressing effect on oil degradation. This is consistent with many previous studies [23, 24, 25]. Moreover, the obtained data are in agreement to that obtained by Hamamura et al., [26] and Sathishkumar et al., [27]. On the other hand, Varjani, et al., [11] and Varjani [28] postulated that the best consumption of crude oil was 3% using two types of bacteria. In addition, Christova et al [19] found that up to 93% of aliphatic crude oil was degraded by *Bacillus cereus* after 48 hours of incubation. Our results also indicated that the crude oil utilization and production of SCP was effected by the concentration of nitrogen in the form of ammonium chloride and most suitable concentration was 0.1%. Similar results were obtained by Mathew, et al., [29] who determined that the biodegradation of crude oil and the activity of the dehydrogenase enzyme which is involved in the pathway of oil biodegradation increased in fermentation medium containing 0.1% ammonium chloride and 0.2% as a nitrogen source. Besides, Wrenn et al., [30] and Wrenn et al., [31] demonstrated the stimulating effect of ammonium chloride on the biodegradation of crude oil bacterial growth and production of SCP. On the other hand, a study by Christova, et al., [19] showed that ammonium sulphate was used for biodegradation of crude oil by *Bacillus cereus*.

Given the fact that crude oil consists only of carbon and hydrogen, it is essential to add an inorganic source to improve the activity of the used bacteria. Different concentrations of magnesium sulphate were added to the fermentation medium to find out the optimal concentration that support the highest crude oil biodegradation and production of SCP. The optimal concentration that showed the highest yield was in a medium containing 0.01%. Magnesium ion catalyzes several anabolic reactions that are involved in energy production such as ATP [32]. Increasing the amount of magnesium sulphate in the fermentation medium has led to poor crude oil consumption, weak growth and low biosynthesis of SCP. This is because of the inhibitory effect of magnesium on the activity of the enzymes and the metabolic activity of the bacterium [33]. Likewise, a study explained by Guo-Liang [34] showed that the presences of magnesium sulfate at a concentration of 0.02% significantly stimulated the consumption of crude oil by the bacterium *Pesudomonus aeruginosa*. MacLeod and Daugulis [34] and Fida et al, [35] have found that the best concentration of magnesium sulfate for high biodegradation of polycyclic aromatic hydrocarbons by bacteria is 0.05 g / l. This is slightly different from our results. This is may be due to the nature of used hydrocarbon or the used fermentation media and/or the used bacterial strains.

The presence of yeast extract in the fermentation media further stimulated the activity of the bacterial isolate for higher crude oil biodegradation. It also improved the bacterial growth and increased the production of SCP. This is due to the availability of carbon source, which results from the breakdown of long chain hydrocarbon by activated dehydrogenase enzyme, which can easily be used for building up of energy [33]. The produced energy used for the metabolic activity for bacterial growth and SCP production. The stimulating effect of yeast extract on crude oil degradation and metabolic activity probably due to its chemical composition, which contains organic acid, vitamins and nitrogen sources. These findings are consistent with those by Foght et al., [36] and Christova et al., [19] who explained that the addition of 0.1% of the extract yeast to the fermentation media yielded the highest degradation of crude oil by bacteria. Moreover, Ijah [35] has used higher concentration of yeast extract (0.7%) to stimulate crude oil consumption by bacteria. Fida, et al., [36] have used

0.05% yeast extract for the same purposes.

Our results also indicated that the addition of potassium phosphate to the fermentation media at concentration of 0.05% also promote crude oil biodegradation and bacterial growth. The stimulating effect of potassium phosphate was due to its composition, which is considered as a best source for building up of bacterial nucleic acid, cytoplasmic membrane and other vital cell constituents. Increasing the amount of added potassium phosphate have suppression effect on crude oil degradation and also bacterial growth due to the reduction of the utilized crude oil to simple carbon compound which was used as a source of energy for building cell component and accumulation of SCP. Moreover, the presence of high concentration of potassium phosphate may increase the permeability of bacterial cell membrane causing diffusion of simple carbon molecules and short chain carbon compound outside of the cell or may decrease the pH of the fermentation media which reduces the activity of the bacterial metabolic enzyme responsible for cell growth and biosynthesis of SCP. Similar observation was described by Foght et al., [20]. Also, our finding are in agreement to that of Zucchi et al., [37] who found that 0.06% highly stimulated crude oil biodegradation by the used bacterium. Moreover, Vyas & Dave, [38] and Popoola, and Yusuff, [39] showed that the presence of phosphate and potassium at optimal concentration highly promoted the degradation of crude oil.

5. Conclusion

The isolated bacterium *Pseudomonas aeruginosa* possesses the ability to degrade the crude oil and can be used as a carbon source for bacterial growth and production of SCP. Moreover, changing the physiological properties of bacterial fermentation media promote more crude oil degradation and single cell protein production.

Abbreviations

BYP	Basal Yeast Extract Protease Peptone-3
SCP	Single Cell Protein

Data Availability Statement

All data generated or analysed during this study are included in this published article.

Conflicts of Interest

The authors declare no conflicts of interest.

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