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Bioextraction of Sunflower Seeds Oil via Syrian Actinobacteria Strains in Comparison with Chemical Method

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ABSTRACT

Sunflower is one of the most valuable oil crop, because of its oil content. Generally oil of plant materials was produced by chemical methods which were very dangerous and toxic for health, so enzymatic process has emerged as a promising technique for extraction of oil as environment-friendly method. It was recently discovered that Actinobacteria can produce a diverse mixture of hydrolytic enzymes that can degrade various kinds of organic compounds such as starch, cellulose and hemicelluloses successfully, so they can be used to liberate oil bodies by degrading the cellulosic walls of sunflower seeds and run the reaction to produce oil as bio-procedure instead of chemical one. After 10 strains of Actinobacteria (2 *Micromonospora* and 8 *Streptomyces*) were isolated from water samples, and characterized, their cellulytic activity in vitro were tested by culturing pure strains on CMC medium, and usage of Congo-Red as an indicator for cellulose degradation, oil from Sunflower seeds was bioextracted by 6 strains (S4, S5, S6, S7, S8, S10) that revealed high ability in cellulose degradation, and compared with the oil extracted by hexane. As a result, the percentage of oil extracted by selected Actinobacteria strains was ranging between 26.79% in S6 and 94.64% in S8 over 4 weeks, whereas the percentage of oil extracted by hexane was 35.2% and it took about 4 hours as reaction time. So the final results demonstrated that the oil bioextraction from sunflower seeds by Actinobacteria would result in an increase in oil production valued at more than 60% compared with chemical method. Furthermore the bio-produced oil was consistent with international standards after comparing with virgin oil.

Introduction

Sunflower (*Helianthus annuus*) is a valuable oil crop from the economic and ornamental viewpoint because of their seeds [1]. Seeds are the fruits (achenes), they are 10-15 mm long and 4 mm broad, cylindrical or drop-shaped. The seed consists of a hard hull (pericarp) and a kernel, which is the actual seed [2]. The seed is commonly used as a vegetable oil in many parts of the world, they are also edible and can be used as snacks and as ingredient for bakery specialties. They may be sprouted and put into salads, and they are valued for their oil content, which accounts for about 50% of the seed dry weight. Regular sunflower oil is characterized by a high concentration of linoleic acid (62-75%), followed by oleic acid (16-27%). Saturated fatty acids do not amount for more than 15% of the fatty acid content. The rest of the dry matter consists of protein (14-20%) and fiber (lignin 5-8%). Sunflower protein is less rich in lysine (about 4% protein) than soybean protein but has relatively high amounts of sulfur amino acids (1.9 and 2.2% protein for cystine and methionine, respectively) [3].

The energy value of sunflower seeds has been little studied, most researches give values of 17.9 and 19.7 MJ/kg respectively [3-5]. Sunflower is found all over the world, from Russia to South America. Russia, Ukraine and Argentina produce more than 50% of the world's sunflower seeds [2,6].

Enzymatic process for oil extraction has emerged as a promising technique for extraction of oil from plant materials [7]. The main advantages of enzymatic process are that it is environmental friendly and does not produce volatile organic compounds as atmospheric pollutants [8]. One disadvantage associated with this kind of extraction is the long process time which is necessary for enzymes to liberate oil bodies. Another factor (sometime neglected) is the use of enzymes which are not commercially available [1].

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Actinobacteria, which are gram-positive, filamentous bacteria capable of secondary metabolite production such as enzymes, have generally been the target of researcher due to their ability to produce a diverse array of bioactive compounds [9-11]. Actinobacteria species commonly occurs as saprophytic forms in soil, water, marine environments, humus, and sediments [12].

Most Actinobacteria produce a diverse mixture of hydrolytic enzymes that permit the utilization of various kinds of organic compounds such as starch, cellulose and hemicelluloses. Actinobacteria, especially *Streptomyces*, have been most widely studied for the versatility and diversity of useful metabolites that they can produce [13]. Thus, most studies on Actinobacteria were focused on their potential in producing biological compounds [14,15] rather than their function in any specific environment [16, 17].

Sunflower seeds might also result in increasing lag time of degradation in the rumen and in methane emissions reduction [18,19], and their seeds improve rumen bacterial activity: they reduce the rumen protozoa - rumen bacteria predators – and thus increase total bacteria and subsequent bacterial amino acid flow [20].

Sunflower seeds oil is one of the most consumed oil in world [21]. It is generally produced by a process containing an extraction by an organic solvent: hexane. This solvent is very dangerous and toxic for health and environment. Besides, the residues of hexane may persist in oil and in the consumer's food. Thus, the present work was focused on describing the process of oil extraction from sunflower seeds by using Actinobacteria strains as bio-procedure instead of hexane, and finally to obtain at least a rate of oil comparable to the one obtained by the classic procedure. The other objective of this research is to get with such a procedure a quality of oil [22] similar to the one of olive oil (virgin oil).

Materials and Methods

Chemicals

Glucose, malt extract, yeast extract, agar-agar, peptone, ferric citrate ammoniacal, sodium thiosulfate, K_2HPO_4, glycerol, L-tyrosine, L-asparagine, $MgSO_4.7H_2O$, NaCl, $FeSO_4.7H_2O$, standard saline solution, $CaCl_2$, KH_2PO_4, $NaNO_3$ and $FeCl_3$ were purchased from Sigma -Aldrich. All these chemicals were of analytical grade.

Sampling and culturing

Water samples were collected from Barada river. A clean sampling scoop was used to take samples from 5 to 20 cm depths; the samples were diluted with sterile water down to 10^{-4}. Mounts of 1 ml of each of the pretreated 10^{-3} and 10^{-4} dilutions were spread in triplicate over the surface of ISP4 (International *Streptomyces* Project) agar (glucose 0.4 %, malt extract 1 %, yeast extract 0.4 %, Distilled water 1 L and Agar 1,8%) [23]. The initial pH of the medium was maintained at 7.2 - 7.4. All plates were incubated at 28°C for 3 to 4 weeks [24-26].

Identification and selection of strains

The strains were characterized according to their morphological and microscopic properties. Adequate phenotypical tests were used for the identification of strains including the color of colony and the presence of aerial and substrate mycelia, the branching of mycelia, sporophores shape, and pigment production. Also physiological criteria included: the ability of the strains to utilize different carbon resources, and degradation of casein were performed at 28°C on ISP6 agar (peptone 2%; ferric citrate ammoniacal 0.05 %; sodium thiosulfate 0.008 %; yeast extract 0.1%; K_2HPO_4 0.1%; Agar 1.8% and distilled water 1 L, pH 7.2) [23]. The production of melanoides pigments was carried out on ISP7 agar (glycerol 1,5 %; L-tyrosin 0.05 %; L-asparagine 0.1 %; K_2HPO_4 0.05 %; $MgSO_4.7H_2O$ 0.05 %; NaCl 0.05 %; $FeSO_4.7H_2O$ 0.001 %; standard saline solution 0.1 %; Agar 1,8 % and Distilled water 1L, pH 7.2) [23, 27].

Strains of Actinobacteria were examined for their cellulose degrading ability by culturing pure strains on Carboxy Methyl Cellulose (CMC) medium (0.5 %, yeast extract 0.1 %, K_2HPO_4 0.07 %, KH_2PO_4 0.3 %, $MgSO_4.7H_2O$ 0.05 %, $FeSO_4.7H_2O$ 0.001 %; $ZnSO_4$ 0.0001%, Distilled water 1 L and Agar 1.8 %), and incubated them at 28°C for 7 days. The most bioactive strains in cellulose degradation were obtained by the use of Congo Red. The usage of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies [28], and only these were taken for further study. Cellulose-degrading potential of the positive strains was also qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of diameter of clearing zone and colony [29, 30].

HC = halo diameter – colony diameter/dish diameter * 100

Characterization of sunflower seeds

Origin of seeds

The local collected seeds were a mixture of hybrid varieties, and were oven-dried at 40°C for 4 h in order to calculate the humidity rate [31].

Pericarps ratio in seeds

Seventy grams of whole seeds were used. The kernels of seeds were separated from pericarps, and 53.55g of kernels were recuperated.

Oil extraction rate from whole seeds by hexane [33]

Seventy grams of whole grounded sunflower seeds were placed in the cartridge of Soxhlet which was covered by layer of wool. In the same time, 200 ml of hexane as solvent were added to the flask of a Soxhlet of 250 ml (m_i=102,6 g). During distillation, boiled hexane was condensed and dropped on the cartridge of Soxhlet, entraining the fats. After 1½ h, the extraction was stopped. Then the solution of extraction was evaporated at reduced pressure using Rotary evaporator until the solvent was totally removed. Finally, the flask was weighted (m_f=131,3 g), and the % of recuperated oil was calculated as $\mathbf{m_s}$ according to this equation:

$$m_s = (m_f - m_i) * 100/\text{weight of seeds}$$

m_s = yield of oil extraction of whole seeds wt %

Oil extraction of pericarps by hexane [33]

Sixteen grams of dried pericarps (value equivalent to previously calculated ratio of pericarps in seeds) were placed in the Soxhlet using hexane as extraction solvent. Finally, the percentage of recuperated oil after the extraction by hexane, was calculated as $\mathbf{m_p}$ according to this equation:

$$m_p = (m_f - m_i) * 100/\text{weight of seeds}$$

m_p = yield of oil extraction of pericarps wt %.

and the percentage of oil in the kernels (m_k) was calculated according to this equation:

$$m_k = m_s - m_p$$

Oil bioextraction of sunflower seeds

Seventy grams of Sunflower seeds were cracked, the pericarps were carefully removed, and the obtained kernels were used for oil extraction. The dried kernels were grounded using a laboratory blender for 45s. Then, 500 ml of distilled water were added to the grounded kernels. After the decantation, the wetted kernels were grounded for 2 min. Then, 500 ml of distilled water were added again to the frothy obtained solution. The obtained suspension was boiled in water bath at 90°C for 2 min in order to eliminate the lipase enzymes. After, the temperature of the suspension was lowered to one where the activity of Actinobacteria strains is optimum. Then $MgSO_4$ (0.02 %), $CaCl_2$ (0.02 %), KH_2PO_4 (0.1 %), K_2HPO_4 (0.1 %), $NaNO_3$ (0.1 %), and $FeCl_3$ (0.001 %) were added to the medium which was completed to 1000 ml by distilled water. Some drops of NaOH were added to the medium for adjusting pH to 7.2, then the reaction was run in three 100 ml flasks by adding 80 ml of suspension as a substrate and inculcating with 10^7 cfu of selected Actinobacteria starter culture, then incubated at 28°C with constant shaking at 100 rpm over four weeks. The upper oil phase was collected after centrifugation at 10,000 rpm for 30 min. The amounts of oil recovered were calculated as percentages of total oil present in sunflower seed kernels.

All of these stages were carried out in triplicates and by using the suspension without strain as standard to get correct results [33].

Comparison between the quality of extracted oil and the norm CODEX

In order to characterize the obtained oils, their acidity and density were measured. These data were compared with ones of the norm CODEX (collection of internationally recognized standard). Density was calculated by weighting a known volume of oil. Acidity is represented by the percentage of free fatty acids, present naturally in the oil before the saponification. It is estimated by the indication of acidity, which is the number of mg of KOH, necessary to neutralize the free acidity of 1g of oil. Then, 2g of the oil sample were completely dissolved in 50ml of ethanol ethylic ether (50 %). Then, this solution was titered by alcoholic KOH (N/10) in the presence of phenolphthalein. According to the experimental data, the indication of acidity of oil samples was calculated [22].

Results and Discussion

Characterization of the Actinobacteria strains

After morphological and biochemical tests, 10 strains of Actinobacteria were obtained, most of the strains belonged to the genus *Streptomyces* (S2, S3, S4, S5, S6, S7, S8, S9 and S10), and just two of them were characterized as *Micromonospora* (M1 and M2). Strains appeared with different colors on solid medium like black, orange and white, although they took about 3 weeks to reach the sporulation stage whereas they took just 3 days to grow during the screening stages, and most of them were not able to produce melanoid pigments on ISP7. In addition, strains were tiny and appeared with granular or cutaneous texture, and the substrate mycelium was present in most of the isolated strains. Although most of isolated Actinobacteria strains revealed ability to use fructose and glucose as carbon sources, their ability to use other carbon sources were different (**Table 1**).

Selection of strains

Strains M1, M2, S4, S5, S6, S7, S8 and S10 revealed high ability of cellulose degradation *in vitro* by measuring the transparent halos diameter around cultivated colonies and calculating HC. The following strains had the greatest ability in cellulose degradation after 48h incubation on CMC agar: S4, S5, S6, S7, S8, S10 (**Fig. 1**). These 6 strains were used later to degrade cellulosic walls of sunflower seeds to run the reaction and produce oil.

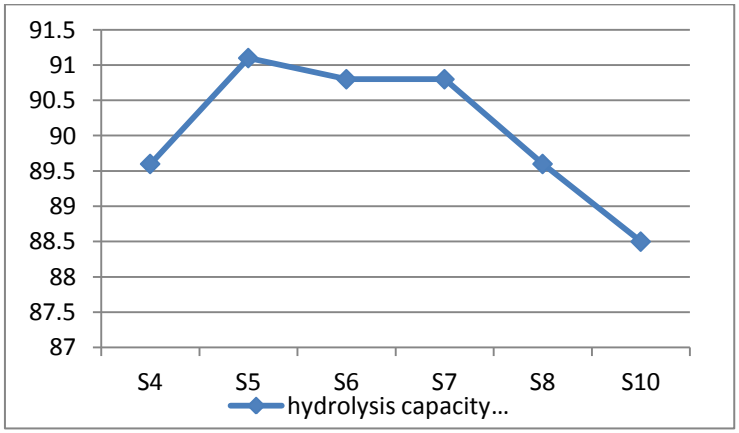

Figure 1. Cellulose degradation capacity of selected Actinobacteria strains

Table 1: Growth characteristics of Actinobacteria strains.

Carbon Source	Actinobacteria Strains									
	M1	M2	S3	S4	S5	S6	S7	S8	S9	S10
Raffinose	+	+	-	-	-	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	-	+	-
Galactose	+	+	+	-	+	-	+	+	+	-
Glucose	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	-	+	+	+	+	-	+	-
Mannitol	+	+	-	-	-	+	-	+	+	+
Aspargen	+	+	-	+	+	-	+	-	+	-
Phenyl alanine	+	+	+	-	+	-	+	+	+	-
Glutamine	+	-	+	+	-	+	+	-	+	+
Lysine	+	+	-	-	+	+	+	-	+	-
Histidine	+	+	-	+	-	+	-	+	+	+
Treptophan	+	+	-	+	+	-	+	-	+	-
Casein	+	+	+	-	+	+	+	+	+	-
Cysteine	+	-	+	+	-	+	+	-	+	+
Alanine	+	+	+	-	+	+	+	-	+	-

Characterization of sunflower seeds

Seeds humidity rate

The calculated humidity rate allowed to conclude that the rate of humidity is similar to the one indicated by supplier and literature, and nearly to 10%.

Pericarps ratio in seeds

After kernels of seeds were separated from pericarps, the kernels were 53.55 g. Thus, the pericarps ratio was 23% of the whole seed weight.

Yield of oil extracted by Actinobacteria comparing with oil extracted by hexane

The yield of extracted oil of whole seed (m_s) by hexane was 41%, while it was in pericarps (m_p) 5.8%, so the percentages of oil in the kernels (m_k) was 35.2%. Therefore, most of the extracted oil was concentrated in kernels, so oil production in subsequent stages using bio-procedure will be focused on the kernels.

After pericarps were separated from kernels to extract oil by selected Actinobacteria strains (S4, S5, S6, S7, S8, S10), the amounts of bioextracted oil (M_{bio}) were measured over 4 weeks in triplicates, and as a result the S5 and S8 strains revealed the highest ability in oil production comparing with others (**Fig. 2**), In addition, the highest amount of bioextracted oil was mentioned in the fourth week in all strains.

The percentage of bioextracted oil in kernels ($M_{bio\%}$) was calculated by the following equation:

$$M_{bio\%} = 100 * M_{bio} / 5.6$$

$M_{bio\%}$=the percentage of extracted oil in kernels

M_{bio}= the amounts of extracted oil in each strain over 4 weeks (S4, S5, S6, S7, S8, S10).

5.6= kernels amount in 80ml of suspension, (**Table 2**).

The results showed the importance of oil bioextracted by Actinobacteria comparingwith the oil extracted by hexane after measuring the amount of extracted oil and reaction time (**Table3**).The percentage of oil extracted by selected Actinobacteria strains from seeds was ranging between 26.79% in S6 and 94.64% in S8 over 4 weeks, whereas the percentage of oil extracted by hexane was 35.2% and it took about 4 hours (reaction time). Therefore, if the amount of oil extracted by hexane considered as optimal quantity via chemical method, the bioextraction by Actinobacteria would give an increase in oil production valued at more than 60%.

Comparison between quality of extracted oil and virgin oil

Bioextracted oil was consistent with international standards after comparing with virgin oil and Chem-extracted one. The oil was revealed yellowish in color (compatible with natural color of the conventional oil) with 0.85 of density and 1.65 of acidity indication.

Conclusion

The bioextraction method by Actinobacteria was ideal in term of quantity and quality comparing with chemical methods, so it can be used to produce oil on a wide range as an eco-friendly method, but as recommendation quantity and quality of some components in bioextracted oil must be studied in future comparison with oil extracted by traditional methods to illustrate the importance of bio-method in oil production and quality.

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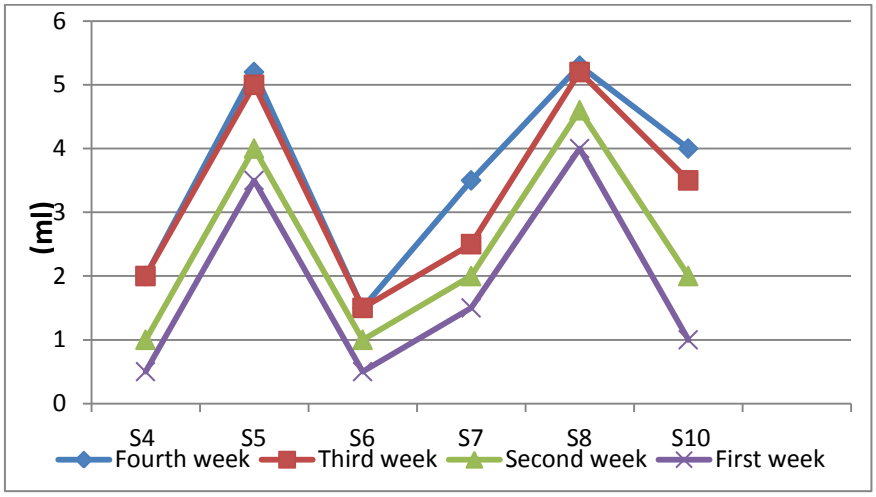

Figure 2. Comparison between Actinobacteria strains' ability to extract oil over 4 weeks.

Table 2: The percentage of bioextracted oil (Mbio%).

Strain	1st week Mbio%	2ed week Mbio%	3ed week Mbio%	4th week Mbio%
S4	8.929	17.86	35.71	35.71
S5	62.5	71.43	89.29	92.86
S6	8.929	17.86	26.79	26.79
S7	26.79	35.71	44.64	62.5
S8	71.43	82.14	92.86	94.64
S10	17.86	35.71	62.5	71.43

Table 3: Comparison between the amount of extracted oil by Actinobacteria and hexane.

Selected Strain	The amount of oil extracted by hexane, %	The amount of oil extracted by Actinobacteria (4 weeks)%
S4	35.2	35.71
S5	35.2	92.86
S6	35.2	26.79
S7	35.2	62.5
S8	35.2	94.64
S10	35.2	71.43

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