Biological Journal of Microorganism
7th Year, Vol. 7, No. 28, Winter 2019
Received: July 2, 2017/ **Accepted:** November 15, 2017. **Page:** 1- 10

Statistical Approach for Production of Lipid from a Newly-isolated *Wickerhamomyces Siamensis* SAKSG Strain from Trout Fish

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Abstract

Introduction: Oleaginous yeasts could produce lipid with high contents of poly unsaturated fatty acids (PUFA). Isolation of *oleaginous* species with a strong ability to produce lipid and long-chain polyunsaturated fatty acid (LCPUFA) could be considered as an effective step to the commercialization of LCPUFA production.

Materials and methods: Having isolated an oleaginous yeast species from fish, the species were identified by the molecular method. One factor at time and response surface methods was used for optimization of lipid and oleic acid production. Fermentation was being conducted in five days.

Results: The yeast specie which isolated from the gills of trout fish was *Wickerhamomyces Siamensis SAKSG*. Response Surface Methodology (RSM) indicated that optimal conditions for lipid production (27% of dry biomass) was obtained at the middle levels of glucose (42 g/L) and minimum amount of soy bean (5 g/L). The optimal amount of oleic acid (66.5% of lipid) was obtained at high levels of glucose (80 g/L) and soybean (15 g/L). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) were also produced by this new isolated yeast.

Discussion and conclusion: *Wickerhamomyces SiamensisSAKSG* as oleaginous yeast had great ability to produce lipid with a reasonable amount of LCPUFA. Chemical condition of media highly impact on LCPUFA production. More to the point, protein had positive effect to LCPUFA production via stimulation of the yeast growth.

Key words: Oleaginous Yeast, Optimization, Poly-unsaturated Fatty Acids, Response Surface Methodology, *Wickerhamomyces Siamensis*

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Introduction

Nowadays, many people and researchers are paying more attention to the use of functional foods as a diet due to the healthy effect of them. PUFAs as functional food have an important effect in human health. These essential fatty acids such as α-linolenic acid (ALA), arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) cannot be produced on human body so scientists have paid high attention to using them in the daily diets (1).The Food and Drug Administration has stated that it is essential for human to consume0.3-0.5 g of EPA and DHA mixture. Because of the importance and shortage of PUFAs, scientists are searching the appropriate sources to industrially produce them (2,3). Plants and animals are unable to produce LCPUFAs. The main sources of LCPUFAs are the fish lipid, but it requires several difficult and complicated extraction and purification steps. Numerous researches showed that the lipids of some oleaginous species are a rich source of LCPUFAs and so, the commercial exploitation of oleaginous species began to engage for human consumption (1,4,5). Low growth rate and sensitivity of marine bacteria to mechanical stresses are the main problems that are hindering their application for industrial production of LCPUFAs (3). Other important sources of LCPUFAs are algal species including *Nitzschia*, *Nannochloropsis*, *Navicula*, *Phaeodactylum*, and Porphyridiu. Many kinds of the problems like high production cost, sensitivity to shear stress, and high oxygen demand can get in the way to use the algal species for industrial purposes (2). Alternatively, fungi and yeasts have been considered for PUFAs production because of rapid growth rate and high tolerance to a wide pH range (6). The effect of physical and chemical conditions of fermentation medium in LCPUFAs production has been studied in extensive researches. Several studies have indicated that incubation temperature (7), carbon source, and the ratio of carbon to nitrogen (8) affect the yield of lipid and LCPUFA production in oleaginous species. According to the previous studies, the isolation of oleaginous species that have good ability to produce LCPUFAs and lipid is an effective step to industrialize a wider range of micro-organisms for commercial proposes. Thus in this study, an oleaginous yeast species was isolated from fish and then was identified by using the molecular method. Medium conditions were screened by the one factor at a time method and then optimized by using response surface method.

Materials and Methods

Choosing the Appropriate Fish Species: In the present study, 5 fish species including Common Carp, Mullet, Zander, Trout, and Caspian Kutum fish were chosen. The surface of fish body was washed with alcohol 70%. Then, 1 g of fish gills was removed by a sterile knife. The samples were incubated at 25ºC in a YGC. The single-yeast-colony was isolated, after the growth in PDA, and was kept in the refrigerator at 4ºC temperature (9).

Seed and Fermentation Media: Seed culture (100 ml) containing glucose mono-hydrate (30 g/liter) and soybean (10 g/liter) was prepared in a 500 ml Erlenmeyer and then sterilized at 121ºC for 15 min. Afterward, the yeasts were inoculated at 26ºC temperature and 170 rpm for 72 h. 3-10% of seed cultures were added to the fermentation medium. $(NH_4)_2SO_4$ (2 g/liter), KH_2Po_4 (3 g/liter), and $MgSO_4$(1.5 g/liter) were used as mineral elements in fermentation media (10); moreover, carbon and nitrogen sources as well as pH were used as variables. In order to produce lipid and fatty acid profile, the fermentation medium was incubated at 28ºC and 180 rpm for 5 days.

Analytical Methods: The dry weight of biomass, total lipids and profiles of fatty acids were examined by using methods that were published in previous researches (10,11,12,13).

Species Identification: In order to identify the yeast species which produce the maximum amount of lipid, firstly the yeast sample was incubated in seed culture medium for 48 h; and then, the DNA was extracted using the DNA extraction kit (K721, Thermo, USA). The D1/D2 26S rDNA was amplified using the forward (5'-GCATATCAATAAGCGGAGG AAAAG -3) and reverse primer (5'GGTCCGTGTTTCAAGACGG-3') in the PCR (14). The PCR product (680 base pairs (bp) length nucleotides) was extracted from the gel and sent to the Takapu-Zist Company to determine the nucleotide sequence. After sequence determination, they were blasted using NCBI website and the phylogenetic tree was drawn by using the Mega5 software.

Results

Oleaginous Species Isolation from Fish: The results showed that three isolated yeast species produced more than 20% w/w lipid and so, they were considered as oleaginous species. Because of the highest ability of the yeast species isolated from the trout fish to produce lipid (Figure 1) these species were selected for more analysis.

Li et al (9) reported that glycerol as a carbon source had a significant effect on the lipid production in some yeast species. Therefore, glucose was replaced by glycerol (30%). The results indicated that glycerol had a negligible effect on the biomass production, while glycerol supported better condition for the production of lipid in isolated yeast from *Common Carp* and *Zander*. However, due to the lowest biomass production, glucose, instead of glycerol, was used as the carbon source for lipid production (Figures 1 and 2).

The results showed that yeast which was isolated from *zandar* species produced higher amount of biomass than glucose (Figure 2).

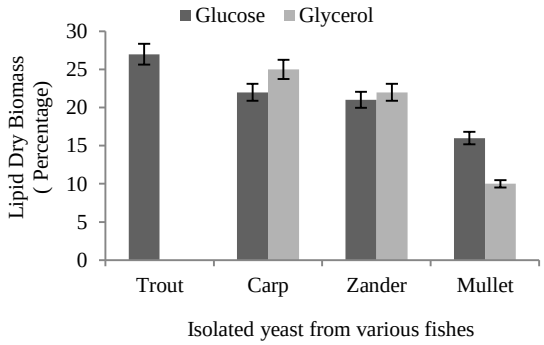

Fig. 1- The effect of the glycerol and glucose carbon source on the lipid percentage of yeast samples isolated from fish gills.

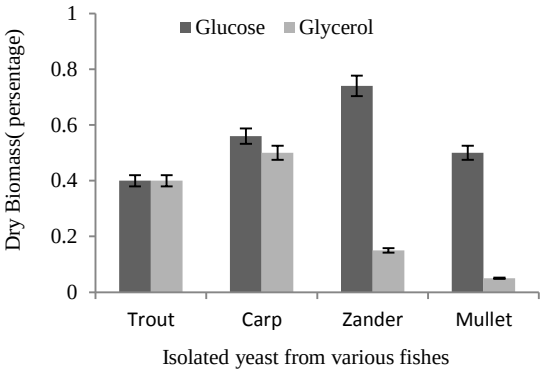

Fig. 2- The effect of the glycerol and glucose carbon source on the dry weight biomass of yeast isolated from fish gills.

Molecular Identification of the Microbial Species: In this step, the similarity of Non Transcribed Spacer 2 sequence of the isolated yeast was compared with the ribosomal sequence of other similar species obtaining from NCBI website. The phylogenetic tree that was drawn with mega5 software (Figure 3) verified that the isolated species was very similar to *Wickerhamomy sp* and therefore it was named *Wickerhamomyces Siamensis* SAKSG (Figure 3) which has not been reported as oleaginous species yet. *Wickerhamomyces Siamensis* which was isolated form phylloplane was so closest to *Wickerhamomyces ciferrii* (15).

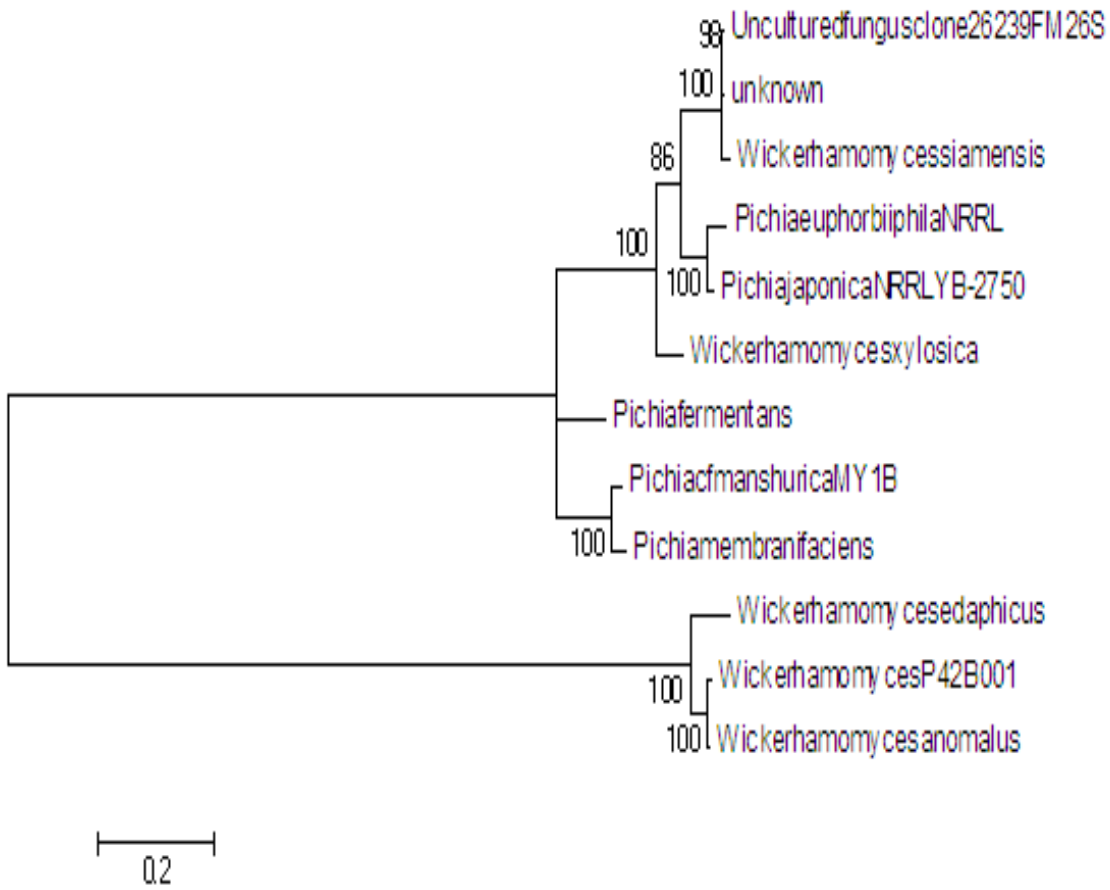

Fig 3- The phylogenetic of the isolated yeast strainunknown with 11 similar species based on the Nearest neighbor interchange analysis of the Non Transcribed Spacer 2 region (The bootstrap level: 1000 pseudo-replications)

The "One Factor at a Time" Method: The "one factor at a time" method was used to screen important variables by changing one factor while keeping other variables constant.

pH: The effect of pH on lipid and biomass production by*Wickerhamomyces Siamensis*SAKSG showed that pH= 6 among the others, it had the greatest impact on biomass and lipid production (data not shown). Thus, pH = 6 was chosen for subsequent experiments.

Carbon Source: Three carbon sources such as glucose, xylose, and sucrose were selected for investigating their effects on lipid and biomass production. The results showed that xylose and glucose had the highest and the lowest effects on the biomass production (Figure 4), respectively; but regarding to the lipid production, it was vice versa (Figure 5).

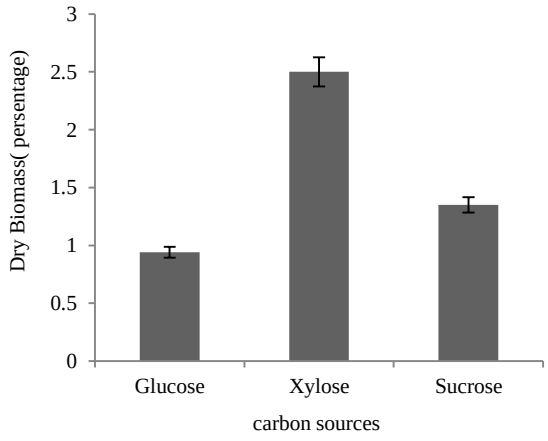

Fig 4- The effect of Glucose, xylose and Sucrose on dry weight biomass of Wickerhamomyces Siamensis SAKSG

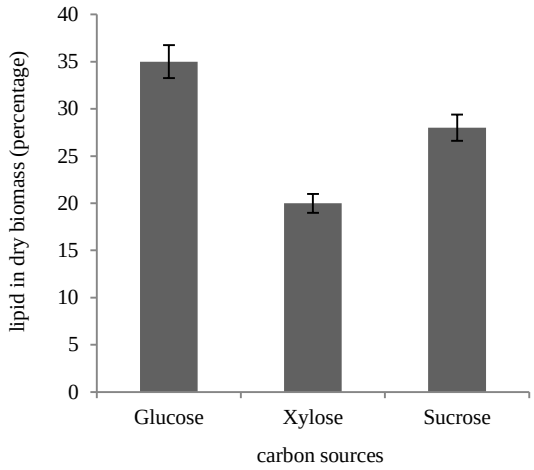

Fig 5- The effect of Glucose, xylose and Sucrose on the lipid of *Wickerhamomyces Siamensis SAKSG*

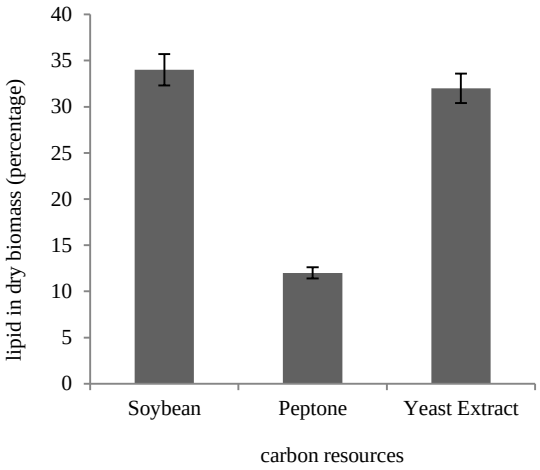

Fig 7- The effect of Soybean, Peptone, Yeast Extract and NH_4NO_3 on the lipid

It was concluded that glucose, among the others, was the best carbon source for lipid production by these species (Figure 4). In other words, glucose greatly converted into lipid and little amount of it were remained to convert into the biomass

Nitrogen Source: The results showed that ammonium nitrate wasn't suitable substrate from lipid and biomass production, while the organic nitrogen sources have a significant effect on lipid and biomass production (Figures 6, 7). As can be seen in Figure 7, soybean had the highest effect on lipid production, and consequently it was selected for further analysis.

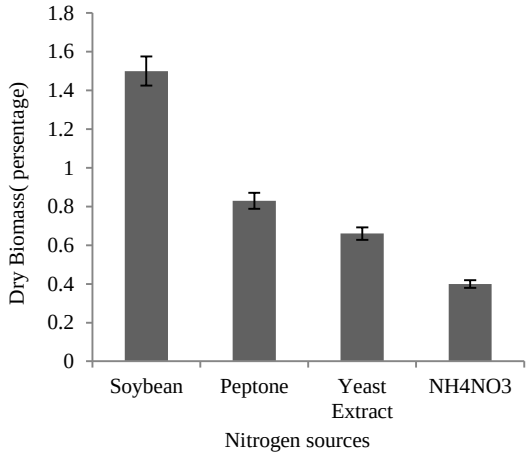

Fig 6- The effect of Soybean, Peptone, Yeast Extract and NH4NO3 on dry weight biomass

According to the results of "one factor at a time method", at the initial pH of 6, glucose and soybean as the carbon and nitrogen sources respectively, were significantly influenced in lipid production *by Wickerhamomyces Siamensis* SAKSG. Subsequently, response surface methodology (RSM) was used to optimize the amount of glucose and soybean toward lipid and oleic acid production.

Optimization by Response Surface Method (RSM): A face centered central composite design (FCCCD) of RSM was applied to optimize the level of glucose and soybean on the lipid and oleic acid production by *Wickerhamomyces Siamensi s*SAKSG. Table 1 indicates the actual and coded substrate content used in the FCCCD.

The results calculated by FCCCD were then examined by analysis of variance (ANOVA) and the results were applied to get a second order polynomial equation. The results either predictions or observations are observed in Table 1. The following equation indicated the effect of glucose (A) and soybean (B) on lipid (Y1) and oleic acid (Y2) production

$$Y1=21.35+0.46\times A-0.89\times B+2.0E\text{-}003\times A\times B-5.55E\text{-}003\times A^2+0.01\times B^2$$

$$Y2=75.14+0.13\times A-6.54\times B+0.08\times A\times B-4.17143E\text{-}003\times A^2$$

In hence 'Prob> F'of model is less than 0.05 it were considered significant. Linear effects of soybean and glucose on the lipid and oleic acid production were significant ($p < 0.05$). The interaction effect of glucose-soybean on the amount of oleic acid production was also significant, but it was insignificant on the lipid production ($p < 0.05$). Moreover, the quadratic effect of glucose on the oleic acid production was not significant ($p < 0.05$). ANOVA indicated that R^2–values for oleic acid and lipid production were 0.95 and 0.98, respectively (data not shown). Observed and predicted results were so close together that it indicated optimization method was done in appropriate style (Table 1).

The model predicted that the maximum oleic acid (66.5%) and lipid production (27.1%) could be obtained by using (g/L) glucose 80, soybean 15 and glucose 42, soybean 5, respectively. The actual and predicted responses are shown in Table 1. As can be seen in Table 1, increasing in the soybean at the middle and constant levels of glucose had negative effect on oleic acid and lipid production (runs6 and 8), on the other hand, with increasing of soybean at the lowest levels of glucose, lipid was slightly reduced while oleic acid greatly declined (runs3 and 5).At the constant level of soybean with increasing in glucose level lipid production decreased while this condition caused oleic acid extensively to increase (runs 9, 10).

Lipid and Oleic Acid Results: Figure 8 shows effect of different levels of glucose and soybean on the amount of lipid production. The response surfaces indicated that similar to other oleaginous species, lipid and the type of fatty acids were affected by medium conditions. The lipid of *Wickerhamomyces Siamensis* SAKSG was increased in middle amount of glucose level while increasing in the glucose up to the highest content had negative effect on lipid production.

Table1- The central composite design of the response surface method together with real and predicted data

			Oleic Acid (%)		Lipid (%)	
Sample	soybean (g/l)	Glucose (g/l)	Observed	Predicted	Observed	Predicted
1	10 (0)	19.64 (-1.4)	23	28	22.5	21.3
2	5 (-1)	80 (1)	67	61.05	20	21.7
3	5	30 (-1)	63	66.51	25	26.31
4	10	55 (0)	52	52.78	23	23.5
5	15 (1)	30	19	16.83	21	21.2
6	17.07(1.4)	55	41	40.76	20	19.1
7	15	80	67	66.51	17	15.06
8	2.92(-1.4)	55	58	64.81	28	27.5
9	10	90.35 (1.4)	64.5	67.14	10	11.84
10	10	55	52.5	52.78	24	23.5

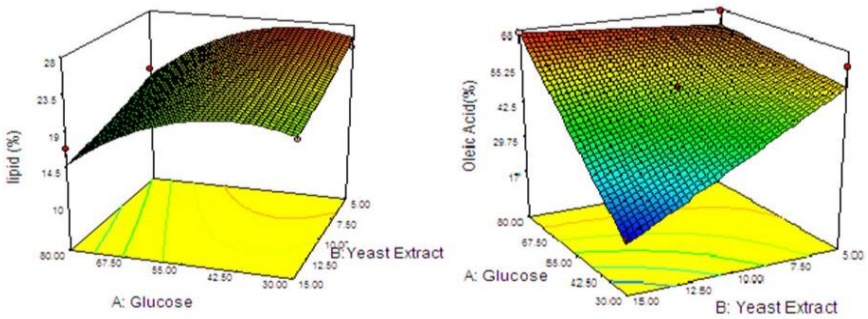

Fig 8- RSM curve for produced lipid and Oleic acid by *Wickerhamomyces Siamensis SAKSG*

Soybean as a nitrogen source has significantly negative effect on lipid production like other microbial oleaginous species (16,17). Figure 5 shows that increasing in soybean level up to 15 g/L at fixed level of glucose caused the oleic acid production to reduce.

Verification of optimum conditions: Oleic acid and lipid production were tested under the predicted optimal culture condition, namely medium containing (g/L): glucose 80, soybean 15 for oleic acid production and glucose 42, soybean 5, for lipid production. The results indicated that the highest oleic acid and lipid production were obtained after 5th day of the fermentation. Oleic acid and lipid production were 68% and 30% respectively, which were so similar to predicted values (66.5% for oleic acid and 27% for lipid production). Regarding these results, errors were 0.7% and 2% for the optimal media of oleic acid and lipid production, respectively.

Arachidonic Acid (ARA), Eicosa-PentaenoicAcid (EPA), and Docosa-Hexanoic Acid (DHA) production: Production of these essential fatty acids by *Wickerhamomyces Siamensis* SAKSG was highly affected by the carbon and nitrogen contents of the medium. The highest amount of DHA (7% w/w of the lipid) and ARA (3% w/wof the lipid) were obtained at 20 g/L glucose and 10 g/L soybean. Increasing in glucose and soybean up to 30 and 15 g/L respectively led DHA to reduce in 6% of lipid. In both above mentioned culture media, EPA was produced at 5% of lipid, just as important; in other treatments these three essential fatty acids were not produced.

Discussion and conclusion

In recent years, lipid and PUFAs production from oleaginous species have been attracted great attention by scientist. In this research at the first time new isolated oleaginous marine yeast called *Wickerhamomyces Siamensis* SAKSG was first identified by molecular method from gills of trout fish. Similar to other oleaginous yeast species, in *Wickerhamomyces Siamensis* SAKSG the maximum amount of lipid was obtained at the lowest amount of soybean and the middle levels of glucose. According to Ho (18); osmotic pressure and the inhibitory effect of high amounts of glucose could be considered as major reason for this result. Increasing in protein content had negative effect on lipid production so in this condition carbon is entered the citric acid cycle instead of fat acid biosynthesis pathway based on Jin et al(16) report. Olstorpe et al (4) reported that in *Wickerhamomy cesanomalus* C_{16} and C_{18} fatty acids were dominant; and linolenic acid (3%) was also observed. Increasing in fermentation temperature from 15 to 30ºC led to a decrease in C_{16} and C_{18} fatty acids.

At the low level of carbon, lipid synthesis was halted in this circumstances, oleic acid production was stopped. In this condition lipid instead of glucose has been consumed to provide the energy that was needed in starvation condition. In this circumstance, syntheses of poly unsaturatet fatty acid such as PUFA were induced. Eroshin et al (19) reported that three steps for lipid production in oleaginous microorganisms were observed. In the beginning, carbon resources were used for biomass production subsequently with reduction in biomass production lipid synthesis considerably induces in the second phase. At the end, poly unsaturated fatty acids productions start growing. In this condition, growth was halted and autolysis begins so that accumulated lipid as nutritional source will be consumed by the microorganisms. All fatty acids except LCPUFA were produced in all treatments while LCPUFA was only produced in some condition that carbon reduced to lower content and protein sources raised to the highest level. Dyal and Narine (7) reported

that in oleaginous species, the medium conditions greatly affected on EPA production.

With respect to the samadlouie et al (17) study, it is worth mentioning that the increase in glucose leads to increase in malic enzyme activity as main enzyme involved in lipid and NADPH production in this condition oxygen as critical factor for enzyme activity contribution in pathways of PUFA production considerably reduces in cell. Thus, desaturase and elongase enzymes are strongly decreased by the low oxygen level (20) so enzymes become less active and the oleic acid was accumulated in lipid. Thus, the highest level of long-chain PUFAs may be seen at the lowest amount of accumulated lipid. There are many reports which verify these results. For example, Jareonkitmongkol et al (21) showed that low glucose level (1%) had an important effect on EPA production. Xue et al (22) stated that 2% of glucose greatly affects the EPA increment in oleaginous species. With respect to these studies, there is always a reverse relationship between lipid accumulation and EPA production. Also, there are similar reports about other long-chain unsaturated fatty acids in other oleaginous species (23,24,25). Long-chain unsaturated fatty acids were produced in micro-organisms during a couple of steps, of which the first step is acetate to oleic acid transformation and the second one is the transformation of oleic acid to the long-chain unsaturated by desaturating and elongating enzymes (26, 27). Furthermore, (28) showed that EPA production increment in *Mortierella* fungal species is due to the increase of desaturating enzymes. It can be concluded that the reduction of glucose and lipid accumulation in *Wickerhamomyces Siamensis* SAKSG were the main factors for EPA accumulation. It is notable that nitrogen is a crucial substrate for micro-organisms growth(6). Moreover, (29) reported that the substrates rich in nutrients play an important role in EPA accumulation. Also Jang et al (30) stated that conditions which lead to induction growth rate such as addition of nitrogen in the middle of fermentation have a considerable effect on EPA production. It could be concluded that high nitrogen level that make growth rate increase, had great positive effect on EPA production. Marine bacteria(31)and some of the more primitive fungi such as the *Oomycetes*, including the pythiaceous species (*Pythium* and *Phyto-phthora*) (32), and the *Zygomycete* genus like *Mortierella*(33) generally produced more EPA than DHA, while *Wickerhamomyces Siamensis* SAKSG was produced DHA as twice as EPA in the optimal conditions.

Oleaginous marine yeast that isolated from gills of trout fish had great ability to produce lipid and LCPUFA that named *Wickerhamomyces Siamensis* SAKSG. Results indicated that the gills of fish are appropriate sources of oleaginous yeasts that could be used in industrial purposes. With regard to the fatty acids profile this species may provide appropriate sources of elongase and desaturating genes for applications in the genetic engineering.

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بهینه سازی تولید روغن از گونه Wickerhamomyces Siamensis SAKSG جدا شده از ماهی قزل آلا

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چکیده

مقدمه: گونه‌های روغنی توانایی تولید روغن با مختوی بالای اسیدهای چرب غیر اشباع را دارند. جداسازی گونه‌ای روغنی که توانایی بالایی در تولید اسیدهای چرب غیراشباع بلند زنجیره (LCPUFA) دارد کمک شایانی در جهت تجاری‌سازی آن برای تولید اسیدهای چرب غیراشباع بلند زنجیره است.

مواد و روش‌ها: پس از جداسازی گونه مخمری به روش مولکولی گونه جداسازی شده شناسایی شد. از روش آماری سطح پاسخ برای یهنه سازی تولید روغن و اولئیک اسید استفاده شد.

نتایج: گونه مخمر روغنی جداشده از آبشش ماهی قزل آلا *Wickerhamomyces Siamensis SAKSG* بود. نتایج سطح پاسخ نشان داد که که این گونه در سطوح حدواسط گلوکز (g/l ۴۲) و حداقلی پودر سویا (g/l ۵) بیشتر میزان روغن (۲۷ درصد وزن خشک توده زیستی) را تولید می‌کند. بیشترین میزان اولئیک اسید (۶۶/۵ درصد روغن) در سطوح حداکثری گلوکز (g/l ۸۰) و پودر سویا (g/l ۱۵) تولید می کند. نتایج نشان داد که این گونه میکروبی توانایی تولید دوکوزاهگزاانوئیک اسید (DHA)، ایکوزاپنتانوئیک اسید (EPA) و آراشیدونیک اسید (ARA) را نیز دارد

بحث و نتیجه‌گیری: گونه *Wickerhamomyces Siamensis SAKSG* به عنوان مخمر روغنی توانایی خوبی در تولید روغن و LCPUFA دارد. ویژگی‌های شیمایی محیط کشت از موثرترین عوامل در تولید LCPUFA است. در بین این عوامل میزان پروتین تاثیر زیادی در تولید این اسیدهای چرب دارد. محتوی بالای اولیک در روغن *Wickerhamomyces Siamensis SAKSG* باعث شده این گونه روغنی منبع مناسبی برای تولید بیودیزل در نظر گرفته شود.

واژه‌های کلیدی: مخمر روغنی، بهینه سازی، اسیدهای چرب غیراشباع بلند زنجیره، روش سطح پاسخ، *Wickerhamomyces Siamensis*

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* نویسنده مسؤول مکاتبات
تاریخ دریافت: ۱۳۹۶/۰۴/۱۱- تاریخ پذیرش: ۱۳۹۶/۰۸/۲۴