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Optimization of Oil Production Fermentation and Analysis of Fermentation Process for Chemical induction of Fungus Penicillium Citrinum Asc2-4 associated with Pyrosomella verticilliata

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Abstract. Increasing the biomass and oil production of the Pyrosomella verticilliata associated fungus Penicillium citrinum Asc2-4 using inorganic salt chemical induction. The results revealed that KH2PO4, MgSO4, and FeCl3 were significant in promoting oil concentration, and orthogonal optimization revealed that adding these three inorganic salts at 4 g/L, 1.5 g/L, and 1.0×10-7 g/L could increase amount of biomass and oil concentration to 14.52 g/L and 4.73 g/L, respectively, which were 29.64% and 89.20% higher than before fermentation optimization. Gas chromatography analysis of the fatty acid content and the fraction of the bacterial oil revealed that the oil contained as much as 94.08% C16 and C18 fatty acids, and the bacterium has the potential to being employed as a raw material for biodiesel. The fourth day of fermentation with filamentous fungal form proved to be beneficial for the fungus to collect oil, with the sugar-oil conversion rate reaching 15.30%, making it a suitable time point for sugar supplementation fermentation. The fourth and sixth days had the greatest values for biomass and oil concentration, respectively. This research can serve as a theoretical foundation for the advancement of industrial oil production by Penicillium citrinum Asc2-4.

Keywords：microbial oil; substrate utility; inorganic salt; fermentation optimization；fermentation process

1. Introduction

Microbial oils and fats can be used as a further source for biodiesel production and have received keen interest for their sustainability and regenerative properties[1]. The common oil-producing bacteria are mainly algae, bacteria, and fungi, while few studies on Pyrosomella verticilliata associated microorganisms as oil-producing fungi have been reported so far. Oil-producing fungi have large biomass to provide adequate intracellular space to accumulate more oil, fast growth rate to shorten the fermentation cycle effectively, and can judge the growth according to morphological characteristics during the fermentation process.

The current industrial oil production by microorganisms still suffers from low substrate utilization efficiency[2]. Improving the substrate utilization of oil-producing fungi through proven low-cost strategies to enhance the production of fungus and oil is supposed to be widely utilized in the future microbial oil production industry. Of interest is the benefit of non-nutrient inorganic salt induction studies of the medium to improve substrate consumption rate and nutrient utilization. Wei[3]et al. initial addition of 4 g/L (NH4)2SO4 and 8 mM NaCl at 32 h increased the sucrose conversion of Sphingomonas sp. by 137.50%. Trace elements are critical to the fermentation process and can become catalysts or even inhibit the fermentation process when they are not present.

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Therefore, it is necessary in order to understand the processes of bacteriophage growth, product production, and substrate consumption. In this paper, shake flask fermentation was used to initially investigate the effects of nine inorganic salts on the growth and oil accumulation of Penicillium citrinum Asc2-4, and the fermentation process was analyzed by optimizing Penicillium citrinum Asc2-4 in the optimized fermentation medium. This study lays the foundation for the application of oil production technology for the Ascosphaerella symbiotic microorganism Penicillium citrinum Asc2-4.

2. Materials and Methods

2.1 Strain and culture medium

Experimental strain: Penicillium citrinum Asc2-4 is an excellent oil-producing fungus isolated and identified by the group, screened from the sea squirt of the shrimp pond in Leizhou City, Guangdong Province, and stored in the Guangdong Province Microbial Strain Conservation Center (conservation number: GDMCC 60059)

Seed medium formulation: Potato leaching Powder 8 g/L, glucose 20 g/L, sea essential salt 15 g/L, Ammonium sulfate 10 g/L.

Fermentation medium formula: fresh potato 200 g/L, glucose 100 g/L, peptone 0.5 g/L, yeast paste 0.5 g/L, sea salt 15 g/L, sodium citrate 0.1 g/L.

2.2 Experimental steps

Take a Penicillium citrinum Asc2-4 spore from the slant medium, inoculate it in the seed medium and incubate it in a shaker at 28 ℃ and 140 rpm for 2 days, and then incubate it in the fermentation condition of "28 ℃, 5% inoculum, 100 mL/250 mL filling volume, 140 rpm" for 7 days.

After fermentation, extracted mycelium in Petri dishes, dried in an oven at 109° C, weighed in constant weight, and calculated. The total oil was extracted by adding 4.00 mol/L hydrochloric acid 6.00 mL per 1.00 g of dry mycelium, adding 2 times the volume of chloroform-methanol (2:1, v/v) mixture to extract the oil, and then centrifuged at 2000 r/min for 15 minutes, and evaporated to remove the chloroform-methanol to get the oil. Measure the optical density value (OD value) at 540 nm, draw a standard curve according to the relationship between glucose concentration and optical density value, and repeat three parallels for each test tube to obtain the standard curve equation: y=8.2072x-0.0731 (R=0.9996).

2.3 Calculation formula

Amount of biomass：The extracted mycelium was dried at 109℃ in a Petri dish, weighed at a constant weight, and the biomass was expressed as △X, as showed in equation (1).

Oil concentration and percent oil content: Add 6.00 ml hydrochloric acids with a concentration of 4.00 mol per 1.00 g of dry cells, and treat them in a boiling water bath at 100 ℃and freezing at -20 ℃ for 10 min, and repeat 2-3 times. The liquid was extracted with a chloroform/methanol mixture in a volume ratio of 2:1 (v:v), the upper organic phase was taken after centrifugation, and the organic phase was removed by nitrogen blowing to obtain the oil and grease, and the concentration of oil and grease was expressed as △L [equation (2)]; through △X of equation (1), the percentage of oil and grease △S can be calculated[equation (3)].

Residual sugar and sugar-oil conversion ratio：The amount of residual sugar △Y is calculated as shown in equation (4), X is the glucose content in the fermentation broth (mg/mL); “C” is the glucose content in the dilution solution (mg/mL) ; 250 mL is the volume of dilution solution. The sugar-oil conversion rate △Z is shown in equation (5), where n indicates the number of fermentation days.

$$\triangle X = \frac{\text{Petri dishes with dried bacteria (g)} - \text{Net weight of Petri dish (g)}}{\text{Volume of fermentation broth (L)}} \quad (1)$$

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$$\triangle L = \frac{\text{Mass of lipid per gram of dry bacterium(g/g)}\times\text{Mass of total dry bacteria (g)}}{\text{Volume of fermentation broth (L)}} \quad (2)$$

$$\triangle S = \frac{\triangle L}{\triangle X} \times 100\% \quad (3)$$

$$\triangle Y = \frac{C\times 250\times 4}{0.5} \quad (4)$$

$$\triangle Z = \frac{\triangle L_n - \triangle L_{n-1}}{\triangle S_n - \triangle S_{n-1}} \times 100\% \quad (5)$$

2.4 Data Analysis

Single-factor trials using SPSS 22.0 for data analysis, using the One-Way ANOVA procedure for ANOVA of the data, and Duncan's multiple analysis was used to compare the validation data. $P < 0.05$ was considered a significant difference.

3. Result

3.1 Single factor optimization

The biomass, oil concentration, and percent oil content of nine inorganic salts MgSO4, CaCl2, KH2PO4, NaH2PO4, K2HPO4, FeCl3, ZnSO4, MnSO4, and CoCl2 is illustrated in Figure 1. The concentrations of three inorganic salts, MgSO4, KH2PO4 and FeCl3, significantly promoted the oil concentration of Penicillium citrinum Asc2-4 of 30.11%, 28.14% and 33.22%, respectively, was opposed to the medium without the addition of inorganic salts. The appropriate concentrations of NaH2PO4 and CoCl2 promoted the growth of the bacterium but slightly increased the oil concentration by 0.35% and 4.62%, respectively. CaCl2 increased the biomass but seemed to inhibit the oil production of the bacterium. K2HPO4 neither promoted nor inhibited the growth and oil production of the bacterium. ZnSO4 inhibited the oil production of the bacterium and had no significant effect on the growth of the bacterium.Abbreviations and Acronyms.

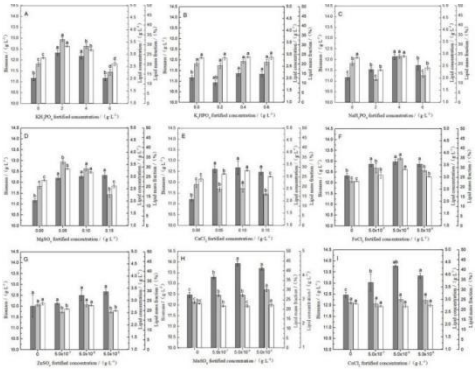

Figure 1. Effect of nine inorganic salts on the growth and oil production of Penicillium citrinum Asc2-4

3.2 Orthogonal optimization

According to the above single-factor experimental results, select KH2PO4, MgSO4, and FeCl3 to affect the concentration of oil and grease significant factors using a "three-factor three-level" scheme for experiments. The specific orthogonal experimental design scheme is given in Table 1.The fermentation medium was designed according to Table 1, and the results of the orthogonal experiments got is given in Table 2. The effects of each factor on the oil concentration were ranked from highest to lowest as KH2PO4, FeCl3, and MgSO4. The optimal combination based on the orthogonal results is A2B3C1, i.e. KH2PO4 4 g/L, MgSO4 1.5 g/L, FeCl3 1.0×10-7 g/L. After fermentation verification, the final oil concentration of 4.73 g/L was 89.20% higher than that of 2.50 g/L before optimization, while the biomass increased from 11.20 g/L to 14.52 g/L, an increase of 29.64%. It can be seen that the effect of inorganic salt orthogonal optimization on oil

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accumulation of Penicillium citrinum Asc2-4 was significantly greater than that of stimulating mycelial growth.

Table 1 Orthogonal experimental design scheme

Level	Factors		
	A：KH2PO4 (g/L)	B：MgSO4 (g/L)	C：FeCl3 (g/L)
1	2	0.5	1.0×10-7
2	4	1.0	1.0×10-6
3	6	1.5	1.0×10-5

Table 2 Inorganic salt orthogonal test results

Test /No.	A：KH2PO4	B：MgSO4	C：FeCl3	ρoil /(g/L)
1	1	1	1	3.65±0.08
2	1	2	2	3.24±0.06
3	1	3	3	3.98±0.17
4	2	1	2	4.23±0.13
5	2	2	3	3.27±0.09
6	2	3	1	4.73±0.16
7	3	1	3	3.53±0.07
8	3	2	1	3.86±0.15
9	3	3	2	3.05±0.21
K1	10.87	11.41	12.24	
K2	12.23	10.37	10.52	
K3	10.44	11.76	10.78	
k1	3.62	3.80	4.08	
k2	4.08	3.46	3.51	
k3	3.48	3.92	3.59	
R	1.79	1.39	1.72	
Order of priority	A＞C＞B			

3.3 Fatty acid determination

As can be seen from Table 3, the bacteria can produce advanced fatty acids (C15~C24), except for 1.26% of tetracosanoic acid, the rest of the lesser content of fatty acids is less than 1%, while the relative content of C16 and C18 fatty acids reached 94.08%. The major fatty acids are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2). Fermented oil has 7 saturated fatty acids (47.05%) and 6 unsaturated fatty acids (52.95%). The highest fatty acid content is C18:2 with 27.02%, and the other three fatty acids with higher content are 25.25% oleic acid (C18:1), 15.30% palmitic acid (C16:0), and 26.23% stearic acid (C18:0).

3.4 Growth, oil production and morphological pattern of mycelium of Penicillium citrinum Asc2-4

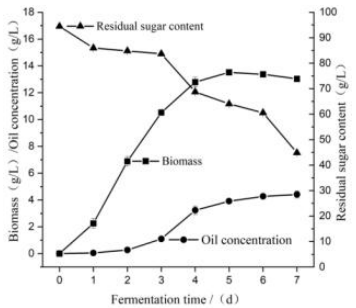

Figure 2. Biomass and oil concentration of Penicillium citrinum

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Asc2-4 at different fermentation times

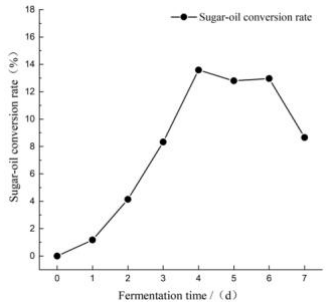

Figure 3. Sugar-oil conversion of Penicillium citrinum Asc2-4 in 7 day

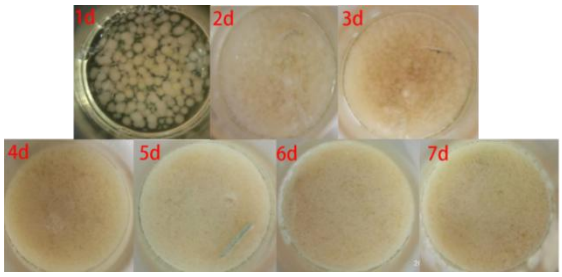

Figure 4. Mycelial sphere morphology of Penicillium citrinum Asc2-4 at 7 days

From Figure 2, it can be observed that the bacterium entered the logarithmic growth period after 1 day of fermentation culture, and entered the stabilization period after day 3. The oil concentration decreased on day 6, and the bacterium biomass decreased after day 5. Among them, the biomass reached a maximum of 13.70 g/L on day 5 and the maximum oil concentration was 4.40 g/L on day 6. Therefore, 6 days is the best fermentation day for oil production and reaching the maximum oil concentration.

By monitoring the sugar consumption of Penicillium citrinum Asc2-4 for 7 days, it was found that the higher sugar consumption was on days 3 to 4. It may be linked to the consumption of more sugar for the synthesis of lipids. The sugar consumption increased from 6 to 7 days with a little increase in oil concentration, and the bacterium may synthesize other substances through sugar consumption. From Figure 3, it can be seen that the strongest sugar-oil conversion was achieved on day 4, reaching 15.30%. This time point is the most suitable sugar supplementation point for Penicillium citrinum Asc2-4.

Relationship between oil production and mycelial sphere morphology： from Figure 4, it can be seen that the mycelium morphology was spherical on the first day and divided into filamentous shapes and the diameter of mycelium balls decreased from the second day of culture. From day 2 to day 7, the mycelium was filamentous. The diameter of mycelium balls increased on the 3rd day and decreased again from the 4th day. In the process of experiment, it can be seen that when the diameter of the mycelium ball is 0.3 ~ 0.4 cm. It is favorable for the accumulation of the biomass of the fungus. And when the diameter of the mycelium ball is 0.1 ~ 0.2 cm, it is favorable for the accumulation of oil in the fungus. It is a known fact that Penicillium citrinum Asc2-4 is similar to other oil-producing fungi in that the filamentous mycelium balls of 0.1~0.2 cm in diameter have the property of accumulating oil better.

By monitoring the sugar consumption of Penicillium citrinum Asc2-4 for 7 days, it was found that the higher sugar consumption was on days 3 to 4. It may be related to the consumption of more sugar for the synthesis of lipids. The sugar consumption was reduced from 6 to 7 days with a slight increase in oil concentration, and the bacterium may synthesize other substances through sugar consumption. From Fig. 3, it can be seen that the strongest sugar-oil conversion was achieved on day 4, reaching 15.30%. This time point is the most suitable sugar supplementation point for Penicillium citrinum Asc2-4.

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4. Conclusion

In this study, we mainly analyzed the effects of different inorganic salts on the biomass, oil concentration, and percent oil content of Penicillium citrinum Asc2-4 through the growth and oil production of the bacterium, and determined the suitable types of inorganic salts for oil production and their concentrations. Finally, to examine the growth pattern of the bacterium in the new medium, the sugar-oil conversion rate, and the correlation between mycelial sphere morphology and oil production.

The types of inorganic salts screened by inorganic salts that significantly increased the concentration of oils: KH2PO4 4 g/L, MgSO4 1.5 g/L, FeCl3 1.0×10-7 g/L. The produced oil had an saturated fatty acid content of 47.05% and an unsaturated fatty acid content of 52.95%. It was established that that the commonly held view[5] of the catalytic effect of trace elements on the products is right. It has been reported[6] that the ideal biodiesel should have long-chain fatty acid methyl ester (C14~C22) components, and its biodiesel indexes such as iodine value, CN value (cetane number) and low temperature performance are closely related to the fatty acid carbon chain and unsaturation degree. Based on the above, it can be seen that the fatty acid fraction and content of the oil of Penicillium citrinum Asc2-4 is similar to that of Di et al[7] who studied Lipomyces starkeyi (36.7% palmitic acid, 4.5% palmitoleic acid, 6.1% stearic acid, 47.5% oleic acid and 5.2 % linoleic acid). However, the C16 and C18 fatty acids of the oil produced by this experimental strain accounted for 94.08% of the total fatty acid content, which was high and relatively homogeneous. In comparison, it has more potential for a biodiesel feeds.

It is well established that the study of the shaking bed fermentation process can provide a reference value for batch replenishment fermentation[8]. In this study, the bacterium was monitored for 7 days for growth, lipid accumulation, sugar consumption, and mycelial ball morphology and size. The results showed the bacteria had the highest biomass (13.70 g/L) on day 5, the highest oil concentration (4.40 g/L) on day 6, and the strongest oil conversion efficiency (15.30%) on day 4. It is thus known that day 5 is the end of logarithmic growth, the optimal fermentation time is day 6, and day 4 is the best replenishment point. The mycelial sphere morphology was spherical from day 1 and split into a filamentous form on day 2, and the best mycelial sphere diameter was about 0.18 cm on day 6 of oil production. The results of this study can apply flexibly to the actual fermentation of Penicillium citrinum Asc2-4.

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References

[1] Mondal, M., Khan, A.A., & Halder, G. (2019). Estimation of biodiesel properties based on fatty acid profiles of Chlamydomonas sp. BTA 9032 and Chlorella sp. BTA 9031 obtained under mixotrophic cultivation conditions. Biofuels, 12, 1175 - 1181.

[2] Capus, A., Monnerat, M.M., Ribeiro, L.C., de Souza, W., Martins, J.L., & Sant’ Anna, C. (2016). Application of high-content image analysis for quantitatively estimating lipid accumulation in oleaginous yeasts with potential for use in biodiesel production. Bioresource technology, 203, 309-17 .

[3] Wei, L., Mao, Y., Liu, H., Ke, C., Liu, X., & Li, S. (2022). Effect of an inorganic nitrogen source (NH4)2SO4 on the production of welan gum from Sphingomonas sp. mutant obtained through UV-ARTP compound mutagenesis. International journal of biological macromolecules.

[4] López i Losada, R., Owsianiak, M., Ögmundarson, Ó., & Fantke, P. (2020). Metal residues in macroalgae feedstock and implications for microbial fermentation. Biomass & Bioenergy, 142, 105812.

[5] Qiang, H., Niu, Q., Chi, Y., & Li, Y. (2013). Trace metals requirements for continuous thermophilic methane fermentation of high-solid food waste. Chemical Engineering Journal, 222, 330-336.

ISSN:2790-1688 DOI: 10.56028/aetr.3.1.659
[6] Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L., & Pérez, Á.G. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. Bioresource technology, 100 1, 261-8 .

[7] Di Fidio, N., Dragoni, F., Antonetti, C., de Bari, I., Raspolli Galletti, A.M., & Ragaglini, G. (2020). From paper mill waste to single cell oil: Enzymatic hydrolysis to sugars and their fermentation into microbial oil by the yeast Lipomyces starkeyi. Bioresource technology, 315, 123790 .

[8] Teworte, S., Malcı, K., Walls, L.E., Halim, M., & Rios-Solis, L. (2022). Recent advances in fed-batch microscale bioreactor design. Biotechnology Advances .