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响应面法优化异养培养条件提高链带藻Z_8油脂产量的研究

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摘要:【目的】微藻油脂含量过低是阻碍微藻生物柴油工业化的主要障碍之一,研究拟通过单因子优化以及响应面法优化链带藻Z_8(*Desmodesmus intermedius* Z_8)异养培养条件,从而提高其油脂产量。【方法】通过单因素试验探究不同浓度条件下的葡萄糖、硫酸镁、柠檬酸、磷酸氢二钾、硝酸钠及氯化钙等对藻种Z_8生物量和总脂量的影响;在此基础上利用P-B试验从6个考察因素中筛选出显著影响因素,最后通过CCD试验来确定最佳工艺条件。【结果】通过P-B试验筛选出4个显著影响因素,分别为:葡萄糖、硫酸镁、柠檬酸、磷酸氢二钾;通过CCD试验获得了最佳工艺条件,具体如下:硫酸镁质量浓度200 mg/L、磷酸氢二钾质量浓度0.07 g/L、葡萄糖质量浓度18 g/L、柠檬酸质量浓度9 mg/L、硝酸钠质量浓度1.5 g/L及氯化钙质量浓度0.04 g/L。在此条件下藻株Z_8的生物量可达到7.7 g/L,与未优化前的生物量5.47 g/L相比提高了40.77%,总脂量可达3.17 g/L,与优化前的总脂量2.47 g/L相比提高了128%。【结论】经响应面法优化后的链带藻Z_8生物量和总脂量均有较大提高,研究所采用的递进式优化方法可为提高微藻的油脂产量及其它发酵工艺的提升提供有益的参考。

关键词:微藻;总脂量;生物质能源;响应面法
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Enhancement of Lipid Production in *Desmodesmus intermedius* Z_8 by Optimization of Heterotrophic Culture Conditions using Response Surface Method

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Abstract:[**Objective**]With the increasing depletion of fossil energy, biomass energy as an alternative energy source has become more concerned. Biodiesel is considered to be one of the best alternative energy sources to solve the problem of limited fossil fuel reserves due to its extremely high similarity to fossil diesel in terms of energy density and combustion performance. Currently, most biodiesel is produced worldwide from oil crops or animal oil materials. When the edible oil crops were used as raw materials for the production of biodiesel, it would compete with the food market, and then lead to food shortages. Microalgae can grow 10-50 folds faster than terrestrial plants. Nutritional composition such as lipids, carbohydrates and proteins of microalgae is 3 folds more than those derived from plants and fish. They can be cultivated on non-arable land without seasonal limitation. At the same time, the harvesting could be in a daily basis. Therefore, microalgae have been widely regarded as one of the most promising raw materials for biofuels. Nevertheless, one of the main factors hindering the industrialization of microalgae biodiesel is its low production of microalgae lipid. In this study, to enhance the lipid production, the total lipid production of the microalgae strain Z_8 was used as the evaluation index, microalgae heterotrophic medium conditions were further optimized. Specifically, the most significant process parameters such as glucose, magnesium sulfate, sodium nitrate, calcium chloride, dipotassium hydrogen phosphate and citric acid were selected for the optimization through using the method of one-variable-at-a time and response surface methodology.[**Method**]The effects of different ingredient concentrations in the medium, such as glucose, magnesium sulfate, citric acid, dipotassium hydrogen phosphate, sodium nitrate, and calcium chloride, on the biomass and lipid production were investigated by using the method of single factor experiment. After completing the above steps, the main influencing factors were found by the Plackett-Burman(P-B)screening test. Then, the selected significant influencing factors were further optimized through the Central Composite Design (CCD)test, and then the optimal culture conditions for microalgae were established.[**Result**]Based on the results of single factor experiment, the biomass and total lipid were firstly enhanced and then remained unchanged when the concentration of glucose was improved. With the increase of the concentration of magnesium sulfate, sodium nitrate, dipotassium phosphate and citric acid, the phenomenon that the biomass and total lipid both increased firstly and then decreased was observed. However, there was no significant change in biomass and total lipid if the calcium chloride concentration was improved. Four significant factors, such as glucose, magnesium sulfate, citric acid, and dipotassium hydrogen phosphate, were obtained through the P-B screening test. At the same time, these four significant influencing factors were further analyzed through the Central Composite Design test, and the optimal culture conditions were as follows: magnesium sulfate 200 mg/L, dipotassium hydrogen phosphate 0.07 g/L, glucose 18 g/L, citric acid 9 mg/L, sodium nitrate 1.5 g/L and calcium chloride 0.04 g/L. Other conditions were consistent with the heterotrophic BG-11 medium. Under these conditions, the biomass of 7.7 g/L and the lipid production of 3.17 g/L were obtained, respectively, which were 40.77% and 128 % higher than those under unoptimized conditions.[**Conclusion**]The biomass and total lipid of the microalgae strain Z_8 are outstandingly improved by the method of response surface methodology. These results indicate that the step-by-step optimization approach could provide some scientific suggestions for enhancing the lipid production of microalgae or improving other similar fermentation processes, which is beneficial to microalgae biodiesel industrialization and commercialization.

Keywords: microalgae; total lipid; biomass energy; response surface method

【研究意义】生物质能源不仅具有来源广泛,可再生的优点,还能减少温室气体排放,有助于生态平衡的维持和人类生活环境的改善[1]。在众多生物质能源中,生物柴油因具备与化石柴油高度相似的能源密度和燃烧性,且其可再生性的优点而备受人们关注。微藻相对于传统生物柴油原料(大豆、油菜、茶籽),具有油脂含量丰富、形态结构简单、生长周期短等优势,被视为生物柴油的第三代原料[2-3]。微藻的

油脂产量是限制微藻生物柴油工业进程的主要因素[4-5],因此提高微藻油脂产量是微藻生物质能源领域的研究热点之一。【前人研究进展】李双双[6]、张婷[7]和潘孝妍等[6-8]通过单因素试验和正交试验的方法分别优化培养基中的碳、氮、磷、镁、钙等因素浓度将微藻油脂产量提高了30%~41%。微藻培养基组分的优化是一个复杂的过程,传统优化方法的局限性使优化效果无法达到最大化[9-10]。响应面法采用多元二次回归方程拟合因素与响应值之间的函数关系,分析回归方程来寻求最优的工艺参数,是一种考虑因素间相互作用并能得到试验水平外优选值的数学统计方法[11]。Yang等[12]利用响应面法优化*Scenedesmus* sp.培养基的碳、氮、磷源等,从而获得超出实验水平之外的优选值,使其油脂产量提高了54.64%。【本研究切入点】*Desmodesmus intermedius* Z_8是本实验室分离的一株兼性且具有较强产油脂能力的藻种[13]。由于大多数微藻属于光合自养生物,国内外对微藻培养条件的优化工作主要集中在自养培养条件上[14]。同时,采用的方法比较单一且各方法的局限性导致优化效率并不高。【拟解决的关键问题】因此,本文以所在实验室自主筛选的藻株Z_8为试验对象,通过单因素试验、Plackett-Burman因素筛选试验和响应面法对藻株Z_8的异养培养基组分进行递进式优化,以期获得较高油脂产量的最佳培养工艺,为微藻生物柴油产业化发展提供可参考的实践经验。

1 材料与方法

1.1 藻种

藻种:本试验所用的藻种为链带藻Z_8(*Desmodesmus intermedius* Z_8),由本课题组从学校附近水域分离获得[13]。

1.2 试验方法

1.2.1 培养方式 异养培养[15]:以10%的接种量将对数生长期末期的藻液接种至BG-11异养培养基中(用葡萄糖替换Na_2CO_3,使其作为唯一碳源),于120 r/min、温度(27±1)℃条件下避光培养6 d。

1.2.2 单因素试验设计 以藻株Z_8作为试验对象,以改良的异养BG-11培养基异养培养藻株Z_8,研究不同葡萄糖浓度(0,5,10,15,20,25,30 g/L)、硫酸镁浓度(0,25,50,75,150,300,450,600 mg/L)、硝酸钠浓度(0,0.1,0.2,0.3,0.5,1.5,2.5,3 g/L)、氯化钙浓度(0,0.01,0.02,0.04,0.08,0.12,0.25,0.5 g/L)、磷酸氢二钾浓度(0,0.01,0.02,0.04,0.08,0.12,0.25,0.5 g/L)、柠檬酸浓度(0,1,2,4,6,8,10,12 mg/L)对藻株Z_8生长及总脂量的影响,处理组的其它因素与异养BG-11培养基一致的条件下,以藻株Z_8的生物量和总脂量为参考指标,确定响应面试验的中心水平[16]。

1.2.3 响应面实验设计 Plackett-Burman(P-B)因素筛选试验[17]:根据单因素试验确定的各个因素的水平,运用Design expert 8.0设计12次的P-B试验,对硝酸钠、硫酸镁、氯化钙、磷酸氢二钾、葡萄糖、柠檬酸、EDTA等7个因素进行总脂量的显著性考察,每个因素设2个水平,以单因素最优值为中心点上下取值,即高水平(+1)和低水平(-1)。

1.2.4 分析方法 生物量的测定[18]:取10.0 mL稳定期藻液,将其通过0.22 μm的滤膜进行真空抽滤,将抽滤所得藻泥用蒸馏水洗涤2~3次,然后将干净藻泥置于烘箱中于60~80 ℃条件下烘干至恒质量。以抽滤过培养基的滤膜为对照,按以下公式计算生物量。

$$B = 100(M - m) \tag{1}$$

式(1)中:B为微藻生物量(g/L),M为烘干后藻泥质量(g),m为滤膜质量(g)。

总脂量的测定[19]:取冷冻干燥的藻粉1.0 g置于离心管,加入8.0 mL 4 mol/L的HCl,振荡均匀,静置35 min,沸水浴12 min后冷却至室温,然后加入16 mL氯仿-甲醇(*V/V*=1∶1)提取剂,充分振荡,浸提20 min。于4 000 r/min条件下离心20 min,取氯仿层,加入8.0 mL含量为1.5 mg/L NaCl溶液,4 000 r/min离心20 min,取氯仿层于已称量的100 mL锥形瓶中,将锥形瓶放置于烘箱中于60~80 ℃条件下烘至恒质量,两次质量差即为1.0 g藻粉的油脂质量,总脂量的计算公式如下:

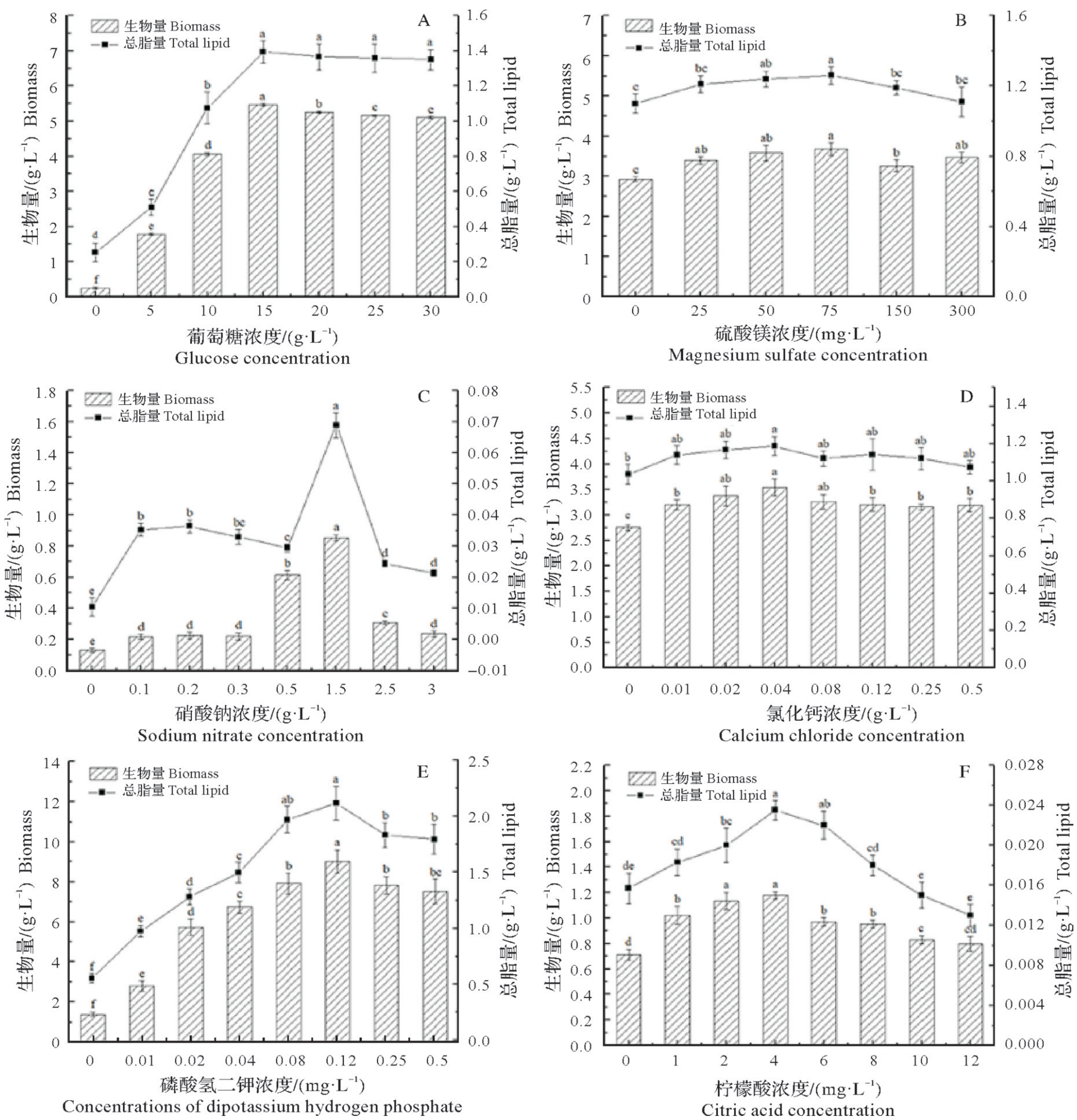
$$P = L/1 \times B \tag{2}$$

式(2)中:P为总脂量(g/L),L为油脂质量(g),B为生物量(g/L)。

2　结果与分析

2.1　单因素影响分析

为获得响应面优化中因素设计的有效阈值,本论文首先对影响藻株Z_8的生物量和总脂量中的葡萄糖、硝酸钠、硫酸镁、氯化钙、磷酸氢二钾及柠檬酸进行单因素试验,其结果如图1所示。



误差棒表示三个平行样品的标准偏差,图中“a,b,c,d,e”表示差异显著性

The error bar represents the standard deviation of three parallel samples.And “a,b,c,d,e,f” in the figure indicates significant.

图1　不同浓度因素处理下的生物量和总脂量

Fig.1　Biomass and total lipids under different concentration factors

从图1可知,随着葡萄糖添加浓度的增加,生物量与总脂量的趋势均先上升后不变;随着硫酸镁、硝酸钠、磷酸氢二钾及柠檬酸的浓度提高,生物量与油脂产量均出现先上升后下降的趋势;然而,伴随着氯化钙添加浓度的增加,生物量与总脂量的趋势没有显著变化。最终从上图可知最优的葡萄糖、硝酸钠、硫酸镁、氯化钙、磷酸氢二钾及柠檬酸的质量浓度分别为:15,1.5,75,0.04,0.12 g/L和4 mg/L。

2.2　响应面试验分析及参数优化

2.2.1　Plackett-Burman(P-B)试验　P-B试验是一种两水平优化试验的方法,其可在较少的试验次数内对众多因素的主效应进行精确估计[20],试验各因素及编码水平见表1。藻株Z_8培养条件优化的P-B试验设

计和结果见表2。采用Design-expert 8.0软件对实验结果进行方差分析,可以得到油脂产量的一阶模型:

$$R=1.25-0.014\times A+0.22\times B-0.030\times C+0.17\times D+0.36\times E+0.19\times F \tag{3}$$

由表3可知,该试验模型显著($P<0.05$),且试验各因素中硫酸镁、磷酸氢二钾、葡萄糖和柠檬酸为显著影响的因素。

表1 P-B试验设计各因素及编码水平

Tab.1 Level of factors in P-B test design

因素 Factors	水平 Levels	
	−1	1
A硝酸钠/($g \cdot L^{-1}$) Sodium nitrate	1	2
B硫酸镁/($mg \cdot L^{-1}$) Magnesium sulfate	50	200
C氯化钙/($g \cdot L^{-1}$) Calcium chloride	0.02	0.06
D磷酸氢二钾/($g \cdot L^{-1}$) Dipotassium hydrogen phosphate	0.03	0.07
E葡萄糖/($g \cdot L^{-1}$) Glucose	10	20
F柠檬酸/($mg \cdot L^{-1}$) Citric acid series	3	9

表2 Plackett-Burman试验设计与结果

Tab.2 Plackett-Burman design and results

试验序号 Test number	A	B	C	D	E	F	油脂产量/($g \cdot L^{-1}$) Lipid production
1	1	1	−1	−1	−1	1	1.05
2	1	1	1	−1	−1	−1	0.70
3	−1	−1	−1	−1	−1	−1	0.36
4	−1	1	1	−1	1	1	2.10
5	1	−1	1	1	1	−1	1.35
6	−1	−1	1	−1	1	1	1.15
7	1	−1	−1	−1	1	−1	1.16
8	−1	1	−1	1	1	−1	1.70
9	−1	1	1	1	−1	−1	1.09
10	1	−1	1	1	−1	1	0.96
11	−1	−1	−1	1	−1	1	1.20
12	1	1	−1	1	1	1	2.23

表中最终值为3次重复试验的均值。

The final value in the table is the mean value of three repeated trials.

表3 Plackett-Burman试验模型及方差分析

Tab.3 lackett-Burman model and variance analysis

方差来源 Source	平方和 Sum of squares	自由度 *df*	均方 Mean square	*F*值 *F* value	*P*值 *P* value
Model	2.99	7	0.43	10.09	0.020 6*
A	0.002 2	1	0.0022	0.052	0.830 6
B	0.60	1	0.60	14.10	0.019 9*
C	0.011	1	0.011	0.26	0.639 1
D	0.34	1	0.34	8.11	0.046 5*
E	1.55	1	1.55	36.70	0.003 7*
F	0.46	1	0.46	10.77	0.030 5*
G	0.028	1	0.028	0.67	0.458 0
残差 Residual	0.17	4	0.042		
总和 Sum	3.16	11			

*表示差异5%水平显著性。

* means significant at 0.05 level.

表4 试验因素水平及编码

Tab.4 Coded values and corresponding actual values of the optimization parameters used in central composite design(CCD)

因素 Factors	水平 Levels				
	−1		0		1
A 硫酸镁/($mg·L^{-1}$) Magnesium sulfate	50	100	150	200	250
B 磷酸氢二钾/($g·L^{-1}$) Dipotassium hydrogen phosphate	0.01	0.03	0.05	0.07	0.09
C 葡萄糖/($g·L^{-1}$) Glucose	9	12	15	18	21
D 柠檬酸/($mg·L^{-1}$) Citric acid	0	3	6	9	12

2.2.2 响应面优化 根据P-B因素筛选结果,依据中心组合试验原理通过Design expert 8.0软件进行响应面中心旋转组合试验设计及分析,寻找最优化组合,以总脂量为响应值,进行4因素5水平的响应面优化,因素及编码水平如表4所示,设计编码与响应值结果如表5所示,回归模型及系数的显著性检验见表6。

表5 中心旋转组合试验设计与响应值

Tab.5 Design and response value of central rotation combined test

序号 Number	硫酸镁/($mg·L^{-1}$) Magnesium sulfate X_1	磷酸氢二钾/($g·L^{-1}$) Dipotassium phosphate X_2	葡萄糖/($g·L^{-1}$) Glucose X_3	柠檬酸/($mg·L^{-1}$) Citric acid series X_4	总脂量/($g·L^{-1}$) Total lipids Y
1	150	0.01	15	6	0.85
2	150	0.05	15	6	1.83
3	100	0.03	18	3	2.39
4	200	0.07	12	9	1.79
5	100	0.03	12	9	1.44
6	100	0.03	12	3	0.84
7	100	0.07	18	3	2.74
8	150	0.05	15	6	2.17
9	150	0.09	15	6	2.24
10	150	0.05	15	0	0.63
11	150	0.05	15	6	1.73
12	150	0.05	15	12	2.57
13	150	0.05	21	6	3.17
14	150	0.05	9	6	0.80
15	150	0.05	15	6	2.24
16	200	0.03	18	3	2.56
17	100	0.07	12	3	1.45
18	150	0.05	15	6	2.24
19	150	0.05	15	6	2.24
20	100	0.07	12	9	1.92
21	200	0.07	12	3	1.77
22	200	0.03	18	9	1.98
23	200	0.07	18	9	2.45
24	250	0.05	15	6	2.49
25	50	0.05	15	6	2.43
26	100	0.03	18	9	2.61
27	200	0.07	18	3	2.77
28	200	0.03	12	3	1.02
29	200	0.03	12	9	1.74
30	100	0.07	18	9	1.90

利用Design-expert 8.0软件对结果进行多元回归拟合后，经过分析可以得到硫酸镁（X_1）、磷酸氢二钾（X_2）、葡萄糖（X_3）、柠檬酸（X_4）等4个因素对油脂含量的多元回归模型：

$$Y=2.08+0.037\times X_1+0.21\times X_2+0.51\times X_3+0.17\times X_4+0.046\times X_1X_2-0.034\times X_1X_3-0.038\times X_1X_4-0.098\times X_2X_3-0.10\times X_2X_4-0.21\times X_3X_4+0.11\times X_1^2-0.12\times X_2^2-0.011\times X_3^2-0.11\times X_4^2 \quad (4)$$

从表6中可知，模型的F值为5.11，$P<0.05$，显示具备显著效应（表示为*），决定系数R^2=0.826 6，校正系数R_{Adj}^2=0.664 8，说明实际总脂量与模型的回归值具有良好的一致性，同时该模型能解释66.48%的响应值的变化。另外失拟项的F=3.44，P=0.926>0.05，说明失拟项不显著，所以该方程能较好地反映实际情况。因此，该模型可用于微藻油脂产量的分析和预测。从回归模型来看，X_3对总脂量影响极显著，X_2、X_4以及交互项X_3X_4对总脂量影响显著，其余项不显著。说明试验因素不是简单地线性关系；4个因素对总脂量的影响程度大小分别为：葡萄糖、磷酸氢二钾、柠檬酸和硫酸镁。

最终通过Design expert 8.0软件分析，得出藻株Z_8异养培养的最佳工艺条件为：葡萄糖18 g/L、柠檬酸9 mg/L、磷酸氢二钾0.07 g/L、硫酸镁200 mg/L，在此条件下总脂量最大理论值为3.17 g/L，未优化培养基的总脂量1.39 g/L，相比之下优化后提高了128%。

采取上述优化条件进行3次重复试验，结果测得藻株Z_8的总脂量为3.17 g/L，与预测值基本一致（估读后数值一样），说明该回归方程与实际情况拟合较好，该模型可以较好地模拟和预测油脂产量。

表 6　中心旋转组合试验设计方差分析

Tab.6　Analysis of variance in the design of central rotation combined experiment

方差来源 Source	平方和 Sum of squares	自由度 *df*	均方 Mean square	*F*值 *F* value	*P*值 *P* value
模型	10.23	14	0.73	5.11	0.001 7*
X_1	0.033	1	0.033	0.23	0.639 4
X_2	1.04	1	1.04	7.29	0.016 5*
X_3	6.17	1	6.17	43.15	<0.000 1*
X_4	0.73	1	0.73	5.10	0.039 2*
X_1X_2	0.034	1	0.034	0.24	0.630 9
X_1X_3	0.019	1	0.019	0.13	0.722 7
X_1X_4	0.023	1	0.023	0.16	0.695 5
X_2X_3	0.15	1	0.15	1.07	0.317 0
X_2X_4	0.17	1	0.17	1.15	0.299 6
X_3X_4	0.69	1	0.69	4.86	0.043 6*
X_1^2	0.31	1	0.31	2.20	0.159 1
X_2^2	0.41	1	0.41	2.86	0.111 6
X_3^2	0.003 7	1	0.003 6	0.025	0.876 6
X_4^2	0.32	1	0.32	2.25	0.154 1
残差 Residual	2.14	15	0.14		
失拟性 Lack of Fit	1.87	10	0.19	3.44	0.092 6
净误差 Pure error	0.27	5	0.054	3.44	0.926
总和 Cor total	184.24	29			
R^2	0.826 6				
R_{Adj}^2	0.664 8				

*表明$P<0.05$差异显著。

* indicates that the difference of $P<0.05$ is significant.

3　讨论与结论

单因素试验只考虑单一因素的影响，采用这种试验方法必须首先假定各因素间没有交互作用，如果

各因素间存在交互作用,利用这种方法一般会得出错误的结论[21]。正交试验虽然弥补了单因素的不足,但是无法对试验水平之外的最优值进行预测,所得的最优结果仍不够精确[22]。由于响应面法能在整个区域上找到因素和响应值之间的函数表达式,是一种能研究几种因素间交互作用并对区域外的响应值进行预测的回归分析方法[23],可以克服传统经验方法的局限性[24]。发酵培养基各组分往往具有一定的相互作用,不同碳氮比[25]、氮磷比[26]、金属离子[27]及有机酸比例[28-29]都会对发酵过程及结果产生不同程度的影响。为提高藻株Z_8的油脂产量,本论文首先通过单因素试验获得藻株Z_8油脂生产的最佳工艺条件:葡萄糖 15 g/L、硫酸镁 75 mg/L、硝酸钠 1.5 g/L、氯化钙 0.04 g/L、磷酸氢二钾 0.12 g/L、柠檬酸 4 mg/L。然后,通过软件 Design-expert 8.0 依据单因素试验结果,设计 P-B 试验和中心组合试验。通过 P-B 试验的结果,可以看出硫酸镁、磷酸氢二钾、葡萄糖、柠檬酸对藻株Z_8总脂量具有显著影响且呈现正向作用。因此,分别选择这 4 个因素的高水平作为中心组合试验设计的中心点,再进行进一步的优化。最终得出藻株Z_8油脂生产的最佳工艺条件:硫酸镁 200 mg/L、磷酸氢二钾 0.07 g/L、葡萄糖 18 g/L、柠檬酸 9 mg/L,其它条件与单因素优化结果一致。藻株Z_8在此条件下,总脂量达到 3.17 g/L,与未优化前培养条件相比提高了 128%。

本论文不仅可丰富产油微藻的藻种资源,而且从本论文结果可知响应面优化策略是一种简单易行的提高藻株Z_8生物量及油脂含量的方式,本研究结果可为它通过递进式优化策略提高微生物发酵性能提供可供参考的实践经验。

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