Simultaneous Improvement of Yields of Intracellular Polysaccharide and Extracellular Polysaccharide in Submerged Cultivation from a Strain of *Lachnum* sp.

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***Abstract*— The correlations between the yield of intracellular polysaccharide (LIP), the yield of extracellular polysaccharide (LEP) and the biomass of a *Lachnum* YM281 in submerged fermentation were studied. Results showed that there was a significant linear relationship between the yield of LIP (Y) and the biomass (Z): Z=12.561Y-0.0148 (R^2=0.985). The yield of LIP (Y) and yield of LEP (X) were positively correlated, but the linear relationship was insignificant. Accordingly, in order to improve the two synchronously, the two response variables (X,Y) were integrated into a single response variable using the desirability functions and the fermentation conditions of LIP and LEP of *Lachnum* YM281 were optimized synchronously by uniform design to obtain the optimum condition: 46.6g/L glucose, 14.6g/L yeast extract, 6.50mg/L glycine, initial pH value =7.5, 12 days' fermentation at 25℃, the other factors being the same as those of the basic fermentation condition. Under this condition, the yields of LIP and LEP increased by 2.35g/L and 6.28g/L, respectively, improving by 1.42 times and 1.23 times, respectively, compared to those before optimization.**

***Index Term*— *Lachnum*; polysaccharide; desirability functions; uniform design; simultaneous improvement**

I. INTRODUCTION

Since the end of the 1950s when it was found that polysaccharide had the anti-tumor and antioxidant activities, fungal polysaccharides have been the research focus both in and outside China [1], [2]. *Lachnum* is a category of saprophytic fungi that is widely distributed throughout the world [3], [4]. Research on its metabolites in and outside China has concentrated in the antibiotic active substances [5]. In recent years, it has been found that some *Lachnum* strains can produce such active substances with strong antioxidant and antibacterial activities as polysaccharide and pigment under submerged culture [6], [7], [8]. Some *Lachnum* strains of them can produce intracellular polysaccharide and extracellular polysaccharide simultaneously [6], [8], but there are no researches available on the improvement of the yields of the two polysaccharides.

Desirability functions is an effective tool for the simultaneous optimization of the multi-response system. It can integrate two or more response variables into a single target response variable, optimizing two or more responses simultaneously, and has been applied in process optimization and system management, etc [9], [10], [11]. In this study, the desirability functions has been used, for the first time, to integrate the yields of the intracellular and extracellular active polysaccharides of *Lachnum* into a single target variable, and the method of uniform design was adopted to simultaneously optimize the fermentation conditions of the intracellular and extracellular polysaccharides of YM281 (LIP, LEP)to improve their yields simultaneously, laying a foundation for the research & development and industrial production of polysaccharides of *Lachnum*.

II. MATERIALS AND METHODS

A. Strain and medium

Strain: *Lachnum* YM281was separated and preserved in the Laboratory of Microbial Resources and Application of Hefei University of Technology.

Basic fermentation medium [7]: 20g/L glucose, 5g/L yeast extract, 1g/L $MgSO_4 \cdot 7H_20$, 1g/L KH_2PO_4, and natural pH.

B. Methods

Basic Fermentation Condition

Basic fermentation medium; 100mL conical flask with 30mL liquid in it; fermentation temperature: 25℃; rotation speed: 160r/min; and fermentation time: 10 days.

Measurement of the LEP and LIP Yields and the Biomass

The fermentation broth was leached, and the mycelia was washed 2-3 times with distilled water and then dried in a drying oven at 60℃ to constant weight. The weight was converted to dry weight concentration (DWC) of the mycelia (DWC of the mycelia = (Dry weight of the mycelia) / (Volume of the fermentation broth)). Hot water extraction used by Ye et al [6] and Xi et al [9] was adopted to extract LIP for determination of the LIP yield, and then the Phenol - sulfuric acid method [12] was used to measure the concentration. The LIP yield can also be obtained directly by the regression equation of the biomass (Z) and the yield of LIP (Y) derived from the experiment. Fermentation broth of 10mL was added into 95% ethanol (3 times the volume of the fermentation broth), precipitated at 4℃ for 12 hours, and then centrifuged at 4000r/min for 10min. After the supernatant was discarded, the precipitate was dissolved in distilled water till the total value was 10mL, and the LEP mass concentration was determined by the Phenol - sulfuric acid method [12].

Correlations between the LIP, LEP Yield and the Biomass

Under the basic fermentation condition, 9 experiments based on 3 factors and 3 levels were designed according to the method of Hao et al [13] and Pan and Chen [14] with minor modification: glucose (10g/L, 20g/L, 30g/L), yeast extract (2g/L, 5g/L, 8g/L) and initial pH value (4.0, 5.0, 6.0). The experiments were repeated 3 times, and the averages were obtained. F-test and t-test were used to analyze the correlations between the LIP yield (Y), LEP yield (X) and biomass (Z).

Calculation of the Associated Desirability Value (D) of the LIP and LEP Yields

Let's define y as the LIP or LEP yield, y_{min} and y_{max} as the minimum and maximum of the LIP yield or the LEP yield, respectively, and d_m and d_p as the desirability of the LIP and LEP yields, respectively. The desirability value can by calculated as follows [9], [10]: d=0 ($y < y_{min}$); d=1 ($y > y_{max}$); $d=\frac{y-y_{min}}{y_{max}-y_{min}}$ ($y_{min} \le y \le y_{max}$). It can be found that the values of the desirability d_m and d_p increased linearly in the range of y_{min} and y_{max} with the increase of y, varying within 0-1 and having no unit. The associated desirability (D) is expressed as the geometric mean of d_m and d_p, namely, $D=(d_m \times d_p)^{1/2}$. According to the preliminary experiment, y_{max}, i.e., the expected maximum of the LIP or LEP yield, were set as 5g/L and 15g/L, respectively, and y_{min} were both set as 0g/L. If the LIP and LEP yields are both higher than the expected maximum, namely, both d_m and d_p are 1, then D is 1. If one of the two is smaller than the expected minimum, namely, either dm or dp is 0, then D is 0. If both of the two are between the expected maximum and minimum, namely, both dm and dp are less than 1, then D is in the range of 0-1. For convenience of analysis and illustration, the D values in this research are multiplied by 20 times.

Simultaneous Optimization of the LEP and LIP Yields and the Associated Desirability by the Single Factor and Uniform Experiments

The two response variables (LEP yield and LIP yield) were integrated into one single response variable (the associated *desirability*) using the desirability functions for the single factor experiment of *Lachnum* YM-281 to examine the effect of the carbon source (glucose, sucrose, soluble starch, and 1:1 mixtures of the three with glycerol), nitrogen source (urea, yeast extract, peptone, beef extract, soybean seed meal, and 1:1 mixtures of yeast extract and peptone, yeast extract and beef extract, and beef extract and peptone), growth factor (naphthylacetic acid, 2.4-D, VB_1, creatine, glycine and glutamic acid), initial pH value (3.5, 4.5, 5.5, 6.5, 7.5 and 8.5) and fermentation time (6, 8, 10, 12 and 14d) on the LEP and LIP yields and the associated desirability(D). Uniform experiments were carried out to verify the results of the single factor experiments. The experiments were repeated 3 times, and the averages were obtained. Taking D as the single response value, DPS (Data Processing System) was used for quadratic polynomial stepwise regression analysis, and test of significance was performed.

III. RESULTS AND DISCUSSIONS

A. Correlations between the LIP, LEP Yield and the Biomass

TABLE I

CORRELATIONS BETWEEN LIP, LEP YIELD AND BIOMASS

Indexs	Experimental No. and results								
($g.L^{-1}$)	1	2	3	4	5	6	7	8	9
LEP（X）	2.25	4.77	2.91	1.75	2.36	2.81	1.9	2.25	3.70
LIP（Y）	0.39	0.94	0.61	0.48	0.91	1.04	0.76	1.01	1.19
Biomass（Z）	4.75	11.78	7.45	6.51	11.3	12.5	9.56	12.13	15.70

By correlation experiments between the LIP yield and the biomass, we obtained: F= 466.17>$F_{0.01}(1,7)$=12.25; t = 21.59 > $t_{0.025}(7)$=2.3646, indicating that there is a significant linear relationship between the LIP yield (Y) and the biomass (Z). The regression equation is: Z=12.561Y-0.0148 (R^2=0.985), by which the intracellular polysaccharide yield of YM281 can be calculated based on its biomass.

By correlation experiments between the LEP yield (X) and the biomass (Z), we obtained: F = 2.5473; t= 1.6917, then F > $F_{0.25}(1,7)$ = 1.57, F < $F_{0.1}(1,7)$= 3.59, t < $t_{0.025}(7)$= 2.3646, which indicates that X and Z are linearly correlated, but the correlation is not significant [14]. Similarly, by correlation experiments between the LIP yield (Y) and LEP yield (X), we obtained: F = 2.3460 > $F_{0.25}(1,7)$ = 1.57, F < $F_{0.1}(1,7)$= 3.59, t =1.5320 < $t_{0.025}(7)$= 2.3646, indicating that X and Y are linearly correlated, but the correlation is insignificant. This implies that the extracellular polysaccharide yield and intracellular polysaccharide yield of YM281 are correlated, but are not absolutely dependent on each other, that is, the linear, which is a common phenomenon in fermentation kinetics，for example, the yields of mycelium and exopolysaccharide of *Schizophyllum commune* were correlated, and the correlation was also insignificant [13]. Therefore, we can use the desirability functions to integrate the two into one single response variable, and then optimize the yields of the two simultaneously by applying the single factor and uniform experiments.

B. Single Factor Experiment

Effect of the Carbon and Nitrogen Source on the LIP and LEP Yields and the Associated Desirability(D)

Glucose (Glc), sucrose (Suc) and soluble starch (SS) of 20 g/L, and mixtures of the three with glycerol (1:1) (GG, SG and SSG) were used as carbon sources, urea (UR), yeast extract (YE), peptone (PEP), beef extract (BE), and soybean seed meal (SSM) of 5g/L, and yeast extract + peptone (1:1, YP), yeast extract + beef extract (1:1, YB), and beef extract + peptone (1:1, BP) were used as nitrogen sources, to study the influence of different carbon and nitrogen sources on the LIP and LEP yields and the desirability of the two. As shown in Fig. 1 (A), the LEP yield was highest (2.91g/L) when Glc was used as the carbon source, and the LIP yield was highest (0.93g/L) when soluble starch + glycerol (SSG) was used as the carbon source. But when Glc was taken as the carbon source, D had the maximum value (3.72). Although the yields of LIP and LEP failed to reach maximum simultaneously in this case, the associated desirability of both achieved the highest value, enabling the two response variables to reach equilibrium, which was most conducive to the simultaneous improvement of the two, so glucose was chosen as the optimum carbon source. For some fungal fermentations, mixed carbon source is more conducive to the improvement of the yields of mycelia and polysaccharides than single carbon source [17]. But in his study, it has been found that the single carbon source (glucose) was most conducive to the improvement of the mycelium (intracellular polysaccharide) and extracellular polysaccharide. As shown in Fig. 1 (B), different nitrogen sources have great effect on the yields of LIP and LEP. When YE was used as the nitrogen source, D had the maximum value (4.2), and both the yield of LIP (0.76g/L) and yield of LEP (3.61g/L) were also highest, so YE was the optimum nitrogen source.

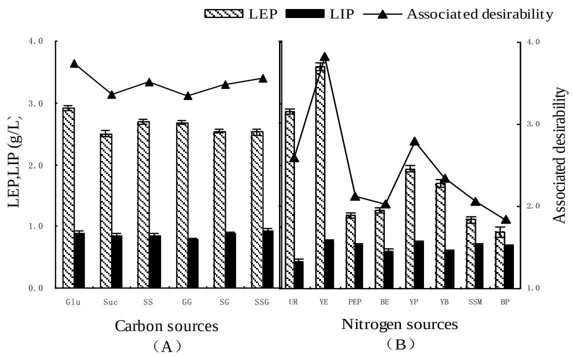

Fig. 1. Effect of the carbon source (A) and nitrogen source (B) on the LIP and LEP yields and the associated desirability(D)

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Effect of the Growth Factor on the LIP and LEP Yields and the Associated Desirability(D)

Naphthylacetic acid (NAA) and 2.4-D of 0.20mg/L, and VB_1, creatine (CRE), glycine (GLY) and glutamic acid (GLUA) of 1mg/L were used as growth factors [7], [16], to study the effect of different growth factors on the LIP and LEP yields and the associated desirability (D) of the two. As shown in Fig. 2 (A), compared with the control (C), except that CRE had an inhibition effect on the production of the intracellular polysaccharide, the other growth factors all promoted the yields of LIP and LEP. When NAA was used as the growth factor, the yield of LIP was highest (0.79g/L), increasing by 22.1%. NAA is an organic naphthalene plant growth-stimulating hormone with extremely low toxicity. Its role is similar to that of indole acetic acid. It has been reported that NAA can promote the growth of mycelium and generation of polysaccharide of *Schizophyllum commune* [16]. In this study, it has also been found that NAA has a great promotion effect on the growth of mycelium (production of intracellular polysaccharide) of *Lachnum*. When GLY was used as the growth factor, the yield of LEP was highest (3.68g/L), increasing by 23.6% compared with the control, and the associated desirability (D) achieved the maximum value so that the yields of LIP and LEP can be improved to the maximum extent simultaneously, so GLY was chosen as the optimum growth factor. This result is in agreement with the report on research of the pigment production condition of *L. brasiliense 84-2* [7].

Effect of the Initial pH Value on the LIP and LEP Yields and the Associated Desirability(D)

The initial pH is an important factor to influence the yields of mycelia and metabolites in fugal fermentation. pH will affect the membrane function, morphology and nutritional requirements of cells [9]. As shown in Fig. 2 (B), the optimal initial pH values for the generation of LIP and LEP were 6.5 and 7.5, respectively, with the corresponding yields of 0.73 and 3.22g/L, respectively, which increased by 20.3% and 25.5%, respectively, compared to those when the initial pH was not controlled (natural pH). When the initial pH value was in the range of 3.5-7.5, D increased with the increase of the initial pH value. When the initial pH value was 7.5, D reached maximum, which was most favorable to the simultaneous improvement of the two. When the initial pH value was higher than 7.5, the associated desirability(D) began to decrease gradually. It may be because too high or too low pH values will inhibit the growth of mycelia and the yields of LIP and LEP. Accordingly, 7.5 was chosen as the optimum initial pH value, which is similar to the optimum initial pH value of *L. brasiliense 84-2* producting pigment [7].

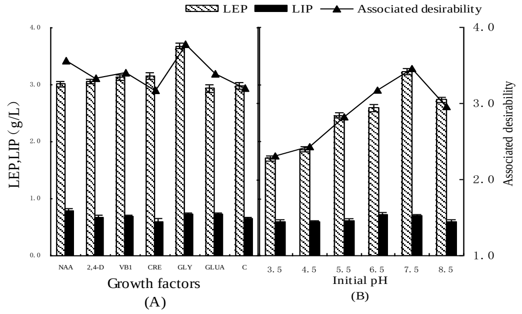

Fig. 2. Effect of the growth factor (A) and the initial pH (B) on the LIP and LEP yields and the associated desirability(D)

Effect of the Fermentation Time on the LIP and LEP Yields and the Associated Desirability(D)

As shown in Fig. 3, from the 6th day to the 12th day during the fermentation process, both the yields of LIP and LEP increased continuously, and the associated desirability of both were also increasing. D reached maximum on the 12th day of fermentation. After the 12th day, the yield of LIP began to decrease, whereas the yield of LEP was still increasing, but the associated desirability of both began to decline. The reason may be that the fermentation of *Lachnum* YM281 reached stable on the 12th day, and autolysis of some mycelia might begin to occur after over 12 days' fermentation, leading to the decline of the biomass and the LIP yield. And as the autolysis of mycelia may affect the determination of the yield of LEP, the yield of LEP appeared to be still increasing. Accordingly, the optimum fermentation time is 12 days.

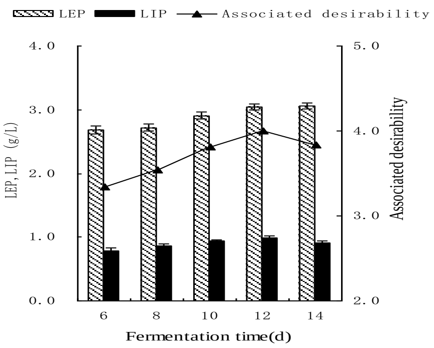

Fig. 3. Effect of the fermentation time on the LIP and LEP yields and the associated desirability(D)

C. Uniform Experiment

On basis of the single factor experiments, U^*_{13} (13^4) uniform design table [14], [15] was adopted to design experiments to examine the effect of the carbon source (glucose), the nitrogen source (yeast extract) and the growth factor (glycine) on the LIP and LEP yields and the associated desirability (D) of them. The initial pH value of the medium was 7.5, with the fermentation time at 25℃ of 10 day, the rotation speed of 160 r/min, and the 100mL conical flask filled with 30mL liquid. The uniform design and the experimental results are given in Table II.

TABLE II
UNIFORM EXPERIMENT DESIGN AND RESULTS

No.	Glu X_1 (g/L)	YE X_2 (g/L)	GLY X_3 (mg/L)	LEP (g/L)	LIP (g/L)	associated desirability (D) Y
1	1（10)	9（13.5)	11（5.5)	2.12	0.63	2.67
2	2（15)	4（6.0)	8（4.0)	1.99	0.95	3.17
3	3（20)	13（19.5)	5（2.5)	1.84	1.12	3.32
4	4（25)	8（12.0)	2（1.0)	2.16	1.61	4.30
5	5（30)	3（4.5)	13（6.5)	4.37	1.24	5.39
6	6（35)	12（18.0)	10（5.0)	5.45	2.21	8.02
7	7（40)	7（10.5)	7（3.5)	5.89	2.42	8.72
8	8（45)	2（3.0)	4（2.0)	3.17	1.01	4.13
9	9（50)	11（16.5)	1（0.5)	4.66	1.71	6.51
10	10（55)	6（9.0)	12（6.0)	5.47	1.98	7.60
11	11（60)	1（1.5)	9（4.5)	2.14	0.98	3.34
12	12（65)	10（15.0)	6（3.0)	3.37	1.82	5.71
13	13（70)	5（7.5)	3（1.5)	2.49	1.95	5.08

Direct Analysis

Direct analysis of the above results showed that the assessment indicators of the 7th experiment were best, with the highest yields of LIP and LEP and the maximum associated desirability (D). Therefore, the corresponding condition for the 7th experiment can be taken as the favorable fermentation condition by direct analysis.

Regression Analysis and Establishment of the Regression Equation

Taking D as the single response value, DPS (Data Processing System) was used for quadratic polynomial stepwise regression analysis, and test of significance was performed, to obtain the regression equation as follows:

$$Y=-6.4500+4.0493X_1+6.9086X_2-0.4348X_1^2-3.0673X_2^2+0.3067X_2X_3$$

The regression results of this equation are given in Table III.

TABLE III
TEST OF SIGNIFICANCE OF THE COEFFICIENTS OF THE REGRESSION EQUATION

Factor	partial correlation	T-test value	p-value
X_1	0.9294	6.6630	0.0002
X_2	0.8228	3.8300	0.0050
X_1^2	-0.9119	5.8778	0.0004
X_2^2	-0.8162	3.7374	0.0057
X_2X_3	0.7266	2.7980	0.0233

The correlation coefficient of this model R=0.9514, F value =13.3622, P=0.0018, the residual standard deviation=0.8030, indicating that this equation is effective. The P values of various variables in Table III indicated that the order of the effects of different factors on D was $X_1 > X_1^2 > X_2 > X_2^2 > X_2X_3$. It can be found by analysis of the results that the factor X_3 is insignificant, but the interaction item of X_2 and X_3 is significant, implying that there is a certain interaction between the nitrogen source (YE) and the growth factor (GLY). The optimum indicator and combinations of various factors can be obtained by assigning the maximum value to the regression equation, as shown in Table IV.

TABLE IV

OPTIMUM INDICATOR AND COMBINATIONS OF VARIOUS FACTORS

Y_{max} Desirability(D)	X_1 glucose (g/L)	X_2 yeast extract (g/L)	X_3 glycine (mg/L)
9.4369	46.581	14.599	6.5000

Verification of the Uniform Experimental Results

Verification experiments were conducted under the two favorable conditions obtained by direct analysis and regression analysis. The results were as follows: the associated desirability(D)of the later experiment (8.87) was greater than that of the former one (8.23), and greater than the D values of all uniform experiments, which suggested that this optimized results can effectively improve the associated desirability of production of LIP and LEP by fermentation of *Lachnum* YM281, thus increasing the yields of both simultaneously.. There is a certain deviation (relative error being 6.43%) between the D value obtained by verification experiment (8.87) and the predicted value by the regression model (9.44). Accordingly, the fermentation condition obtained by regression analysis is the optimum condition. Under this condition, the yields of the intracellular and extracellular polysaccharides of *Lachnum* YM281 can be simultaneously improved to the greatest extent, with the yields of the two being 2.35g/L and 6.28g/L, respectively, increasing by 1.42 and 1.23 times, respectively, compared to those before optimization.

IV. CONCLUSION

There is a significant linear relationship between the intracellular polysaccharide (Y) and the biomass (Z) of *Lachnum* YM281: Z=12.561Y-0.0148. The extracellular polysaccharide yield (X) and the intracellular polysaccharide yield (Y) are positively correlated, but the linear correlation is not significant.

With the use of the desirability functions to integrate the two response variables (the yield of the intracellular polysaccharide (X) and the yield of the extracellular polysaccharide (Y)) into a single target variable, and combining the single factor experiment and uniform experiment, the optimum fermentation condition of the production of the intracellular and extracellular polysaccharides of YM281was obtained: 46.6g/L glucose, 14.6g/L yeast extract, 6.5 mg/L glycine, 1g/L $MgSO_4 \cdot 7H_2O$, 1g/L KH_2PO_4, initial pH value= 7.5, 100mL conical flask with 30mL liquid, the rotation speed=160r/min, and fermentation at 25℃ for 12 days. Under the optimum fermentation condition, the associated desirability (D) of the yields of the intracellular and extracellular polysaccharides and of *Lachnum* YM281 reached maximum, and the yields of intracellular and extracellular polysaccharides were simultaneously improved to the greatest extent, which were 2.35g/L and 6.28g/L, respectively, increasing by 1.42 and 1.23 times, respectively, compared to those before optimization.

In this study, the yields of the intracellular and extracellular polysaccharides of *Lachnum* YM281 were increased simultaneously by the effective use of the desirability functions combining the uniform design. This method can also simultaneously improve two or more related metabolites of other microorganisms. In the further work, we will breed high polysaccharide-producing strain by protoplast mutagenesis, and study the fermentation conditions of the mutant strain to enhance the yield of *Lachnum* polysaccharide.

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