Saudi Journal of Biological Sciences 26 (2019) 449–454

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Isolation and characterization of *Aspergillus* sp. for the production of extracellular polysaccharides by response surface methodology

Balamuralikrishnan Balasubramanian [a,*], Soundharrajan Ilavenil [b], N.A. Al-Dhabi [c], Paul Agastian [d], Ki Choon Choi [b,*]

[a] Department of Food Science and Technology, Sejong University, Republic of Korea
[b] Grassland and Forage Division, National Institute of Animal Science, RDA, Seonghwan-Eup, Cheonan-Si, Chungnam 330-801, Republic of Korea
[c] Department of Botany and Microbiology, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia
[d] Research Department of Plant Biology and Biotechnology, Loyola College, Nungambakkam, Chennai 34, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 9 September 2018
Revised 18 October 2018
Accepted 29 October 2018
Available online 31 October 2018

Keywords:
Apergillus sp.
Extracellular polysaccharide
Response surface methodology

ABSTRACT

In this study, *Aspergillus* sp. was isolated for the production of extracellular polysaccharide. The process parameters were initially optimized by traditional methods. The cheap substrate, wheat bran was used for the production of extracellular polysaccharide in solid state fermentation. Supplementation of (1%, w/w) maltose, gelatin enhanced EPS production (5.36 mg/g). The salts such as, Cu^{2+} (4.9 mg/g), Ca^{2+} (3.5 mg/g), Zn^{2+} (2.9 mg/g), Mn^{2+} (3.4 mg/g) and Mg^{2+} (1.8 mg/g) stimulated EPS production. In two level full factorial experimental designs, the EPS yield varied from 3.18 to 11.65 mg/g wheat bran substrate with various combinations of the components supplemented with wheat bran substrate. Among these selected factors in central composite design, maltose significantly influenced on extracellular polysaccharide production.

1. Introduction

Extracellular polysaccharides (EPSs) are extracellular biopolymers produced by fungi, bacteria and blue-green algae (Amjres et al., 2014). These EPSs could be either covalently associated with the cell surface forming a capsule, or be loosely attached, or secreted into the surrounding medium during the growth of cells. EPSs are formed macromolecules during growth of organisms such as, bacteria and fungi (Hongyan et al., 2018). These environment friendly natural polymers are available in the form of heteropolysaccharide and homopolysaccharide and show many biological activities such as antitumor activity and immunostimulating activity (Chen et al., 2004; Manzo et al., 2017; Mayer et al., 2017). Production of EPSs by various organisms, including *Pleurotus pulmonarius* have been reported. Optimization of EPSs by statistical method is useful to explore the medium components to enhance its production (Yan et al., 2016). Traditional one-variable-at – a-time approach followed by statistical method are useful for EPS production. The contour plot and the orthogonal array are useful to locate the requirements of nutrients and environmental factors in liquid culture of various fungal culture (Feng et al., 2010).

The cost of the nutrient sources, such as, carbon, nitrogen has direct impact on EPS production, which limits the market potential of polysaccharides (Sutherland, 2001; Olson and Kellogg, 2010; Ilavenil et al., 2015). To achieve high EPS production, it is necessary to optimize growth conditions, which require an understanding of the various production parameters involved (Velasco et al., 2006). The production of EPS has been found to vary with medium composition, including, nitrogen, carbon and environmental factors (Wu et al., 2017). Statistical optimization method including, response surface methodology, contour plot method and the orthogonal array were used to identify the nutritional requirements and environmental conditions in liquid culture of Phellinus gilvus. The optimization of nutritional and physical parameters is required to enhance production of EPS from any microbial stains. Response surface methodology (RSM) has been used for the production of EPS (Banik et al., 2007). The complex culture media such as, peptone, salt and yeast extract were employed for EPS production, however these are highly expensive. The application

* Corresponding authors.
E-mail addresses: bala.m.k@sejong.ac.kr (B. Balasubramanian), choiwh@korea.kr (K.C. Choi).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2018.10.015
1319-562X/

of cheap substrates could reduce the production cost of EPS (Banik et al., 2007). The present study was aimed to use the cheap agro-industrial waste for the production of EPS in solid state fermentation (SSF) by *Aspergillus* sp.

2. Materials and methods

2.1. Isolation and screening of fungi for EPS production

The fungi were isolated from the soil samples from agricultural field by standard method. They were sub-cultured in potato dextrose agar medium (g/l): potato: 5; dextrose 30 and agar 15, pH 6.0. The plates were incubated at 30 ± 2 °C for 5 days in an incubator. The isolated fungi was further inoculated individually in Erlenmeyer flask containing screening medium (g/L): sucrose, 60; $NaNO_3$, 3; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.05; KH_2PO_4, 1; $FeSO_4{\cdot}5H_2O$, 0.05 an initial pH 6.0 and incubated at 30 ± 2 °C in an orbital shaker at 150 rpm. After five days of fermentation, EPS was extracted and two volumes of ethanol were added to the supernatant. The precipitate was dissolved in appropriate volume of double distilled water and the phenol – sulphuric acid method was used for quantitative analysis of the sugar contents (Dubois et al., 1956).

2.2. Identification of the microbial strains

The isolated fungi were identified by standard method as suggested by Sutherland (1996).

2.3. Inoculum preparations

The selected *Aspergillus* sp. was inoculated into the culture medium containing (g/L) sucrose – 30; sodium nitrate 3.0; and di - potassium hydrogen phosphate (pH 6.0). The Erlenmeyer flask was inoculated with *Aspergillus* sp. and incubated for about five days in an orbital shaker at 150 rpm. After incubation, the stock was stored at 2–8 °C for further use.

2.4. Solid state fermentation

The culture medium was prepared by mixing two grams of wheat bran and sterilized. Then the culture was inoculated and incubated for 7 days. Extracellular polysaccharide was extracted by standard method (Douanla-Meli and Langer, 2009).

2.5. Optimization of fermentation process by one-variable-at-a-time approach

The nutrient factors were optimized by traditional method. Effect of carbon sources (1%) (lactose, dextrose, sucrose, maltose and glucose), nitrogen sources (1%) (gelatine, ammonium sulphate, oat meal, skimmed milk and casein), ions (0.1%) (calcium, magnesium, copper, manganese and zinc) were optimized. Five hundred micro liter of inoculum was introduced on the solid substrate in Erlenmeyer flasks. After 7 days of incubation, the EPS was extracted and assayed.

Table 1
The factors and levels (low and high) selected for 2^5 full factorial design.

Symbol	Variables	Units	Coded levels	
			−1	1
A	pH		5	7
B	Moisture	%	60	90
C	Maltose	%	0.1	1
D	Gelatin	%	0.1	1
E	Copper sulphate	%	0.1	1

2.6. Analysis of variables for EPS production by statistical approach

In our study the selected variables were, pH, moisture, maltose, gelatine, and copper sulphate to explore the significant factors for EPS production by statistical approach. The FFD experimental design consists of 32 experimental runs. The designed experiment is based on the following first order polynomial model.

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ij} \alpha_{ij} x_i x_j + \sum_{ijk} \alpha_{ijk} x_i x_j x_k + \sum_{ijkl} \alpha_{ijkl} x_i x_j x_k x_l + \sum_{ijklm} \alpha_{ijklm} x_i x_j x_k x_l x_m$$

2.7. Central composite design and response surface methodology

Central composite experimental design consists of 20 experiments for the selected three variables. The significance of selected variables were analyzed by five different levels in 20 experiments (−1.682, −1, 0, +1, and +1.682). A second – order polynomial model was established (Lee et al., 2003). The relationship could be expressed by the following equation.

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2$$

Analysis of variance was used to explore the impact of variables on EPS production. 2D contour ad 3D response surface plot were made using Design expert software.

Table 2
Response of 2^5 factorial design for EPS production by *Aspergillus* sp. in SSF.

Run	Factor 1 A: pH	Factor 2 B: Moisture	Factor 3 C:Maltose	Factor 4 D:Gelatin	Factor 5 E:Copper sulphate	EPS mg/g
1	−1	−1	1	1	−1	4.91
2	1	1	−1	−1	1	3.59
3	1	1	1	−1	−1	8.65
4	−1	−1	1	1	1	9.47
5	1	1	−1	1	−1	7.41
6	1	1	1	1	1	10.17
7	1	1	−1	1	1	5.91
8	−1	−1	−1	−1	1	7.92
9	1	−1	1	1	1	5.46
10	−1	−1	−1	−1	−1	6.68
11	1	−1	1	1	−1	7.68
12	−1	1	1	1	1	5.05
13	−1	1	−1	−1	1	7.05
14	−1	−1	1	1	1	4.4
15	−1	1	−1	−1	1	3.36
16	1	−1	−1	1	−1	6.14
17	1	1	1	1	−1	7.24
18	−1	−1	−1	−1	−1	5.86
19	1	1	1	−1	1	5.75
20	−1	1	1	1	−1	11.65
21	−1	1	−1	1	1	3.19
22	−1	1	1	1	1	8.36
23	1	−1	−1	−1	1	5.86
24	1	−1	1	−1	−1	3.62
25	−1	−1	1	−1	−1	6.52
26	−1	1	−1	−1	−1	6.41
27	−1	1	1	−1	−1	8.15
28	−1	1	−1	1	−1	3.18
29	1	−1	−1	1	1	4.96
30	−1	−1	−11	1	−1	8.38
31	1	1	1	−1	1	6.09
32	1	−1	−1	−1	−1	6.15

Table 3
ANOVA for 2^5 factorial experimental designs for the production of EPS.

Source	Sum of squares	df	Mean square	*F*-value	*p*-value	
Model	1.31E+02	29	4.52E+00	69.84	0.0142	Significant
A-pH	5.40E−01	1	5.40E−01	8.27	0.1026	
B-Moisture	5.80E+00	1	5.80E+00	89.52	0.011	
C-Maltose	1.39E+01	1	1.39E+01	215.26	0.0046	
D-Gelatin	9.14E+00	1	9.14E+00	141.11	0.007	
E. Copper sulphate	4.53E+00	1	4.53E+00	69.96	0.014	
AB	7.10E−01	1	7.10E−01	11.03	0.08	
AC	1.40E−01	1	4.00E−01	6.12	0.1319	
AE	1.40E−01	1	1.40E−01	2.17	0.2787	
BC	1.27E+01	1	1.27E+01	195.35	0.0051	
BD	5.53E+00	1	5.53E+00	85.36	0.0115	
BE	2.00E+01	1	2.00E+01	308.41	0.0032	
CD	8.32E+00	1	8.32E+00	128.53	0.0077	
CE	6.90E−01	1	6.90E−01	10.66	0.0824	
DE	2.00E+00	1	2.00E+00	30.89	0.0309	
ABC	2.20E−01	1	2.20E−01	3.47	0.2037	
ABD	1.03E+00	1	1.03E+00	15.9	0.0575	
ABE	1.14E+01	1	1.14E+01	175.68	0.0056	
ACD	2.74E+00	1	2.74E+00	42.28	0.0228	
ACE	1.26E+00	1	1.26E+00	19.4	0.0479	
ADE	1.11E+00	1	1.11E+00	17.14	0.0537	
BCD	1.73E+00	1	1.73E+00	26.71	0.0355	
BCE	1.61E+00	1	1.61E+00	24.88	0.0379	
CDE	1.65E+00	1	1.65E+00	25.44	0.0371	
ABCD	1.00E−01	1	1.00E−01	1.56	0.3376	
ABCE	1.10E−01	1	1.10E−01	1.67	0.3255	
ABDE	1.86E+01	1	1.86E+01	287.31	0.0035	
ACDE	7.00E−01	1	7.00E−01	10.84	0.0812	
BCDE	1.19E+00	1	1.19E+00	18.43	0.0502	
ABCDE	3.37E+00	1	3.37E+00	52	0.0187	
Residual	1.30E−01	2	6.50E−02			
Core Total	1.31E+02	31				

Table 4
The factors and levels (low and high) selected for central composite design and response surface methodology.

Variables	Symbol	Coded values				
		−α	−1	0	1	+α
Moisture	*A*	49.7731	60	75	90	100.227
Maltose	*B*	−0.206807	0.1	0.55	1	1.30681
Gelatin	*C*	−0.206807	0.1	0.55	1	1.30681

Table 5
Experimental design and results of CCD for the production of EPS.

Run	Factor 1 A:Moisture %	Factor 2 B:Maltose %	Factor 3 C:Gelatin %	EPS (mg/g)
1	0	0	0	11.2
2	0	1.30681	0	17.1
3	0	0	0	21.1
4	−1	1	−1	13
5	0	0	0	22.2
6	−1	−1	1	4.1
7	0	−0.206807	0	6.02
8	0	0	0	11.3
9	−1	1	1	1.7
10	1	−1	1	7
11	1	1	1	12.1
12	0	0	0	14.2
13	0	0	0	12.2
14	−1	−1	−1	1.1
15	100.227	0	0	5.2
16	0	0	1.30681	2.3
17	49.7731	0	0	1.8
18	0	0	−0.206807	8
19	1	1	−1	7.91
20	1	−1	−1	2.01

Table 6
ANOVA for the experimental results of the CCD.

Source	Sum of Squares	df	Mean Square	F -Value	p-Value	
Model	6.14E+02	9	6.82E+01	4.25	0.0169	Significant
A-Moisture	1.61E+01	1	1.61E+01	1.01E+00	0.3396	
B-Maltose	1.12E+02	1	1.12E+02	7	0.0245	
C-Gelatin	5.55E+00	1	5.55E+00	0.35	0.5693	
AB	2.80E−01	1	2.80E−01	0.018	0.8972	
AC	3.82E+01	1	3.82E+01	2.38	0.1537	
BC	2.85E+01	1	2.85E+01	1.78	0.212	
A^2	2.56E+02	1	2.56E+02	15.98	0.0025	
B^2	2.69E+01	1	2.69E+01	1.68	0.2242	
C^2	1.90E+02	1	1.90E+02	11.87	0.0036	
Residual	1.60E+02	10	16.03			
Lack of Fit	35.43	5	7.09	0.28	0.9034	Not significant
Pure Error	1.25E+02	5	24.97			
Cor Total	7.74E+02	19				

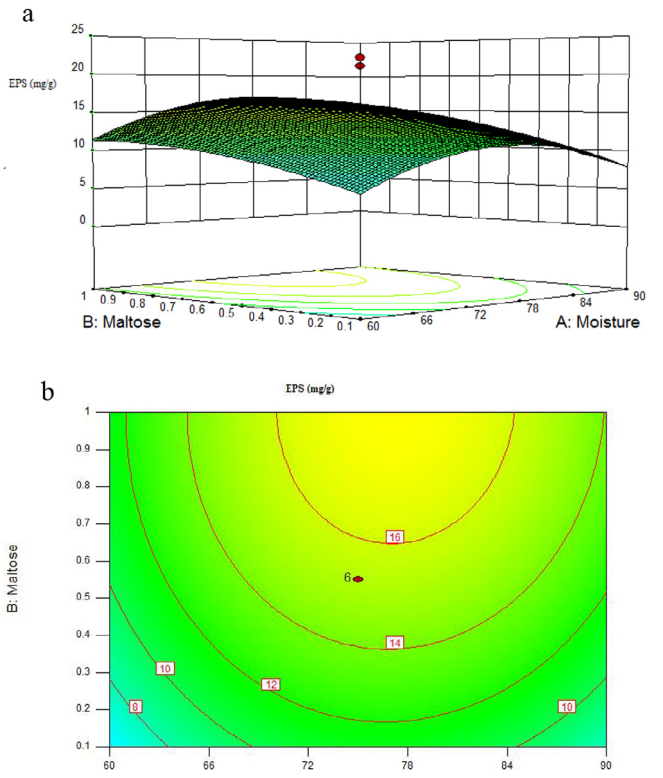

Fig. 1. The 3D-response surface (a) and 2D-contour plots (b) of EPS yield (mg/g) between moisture and maltose.

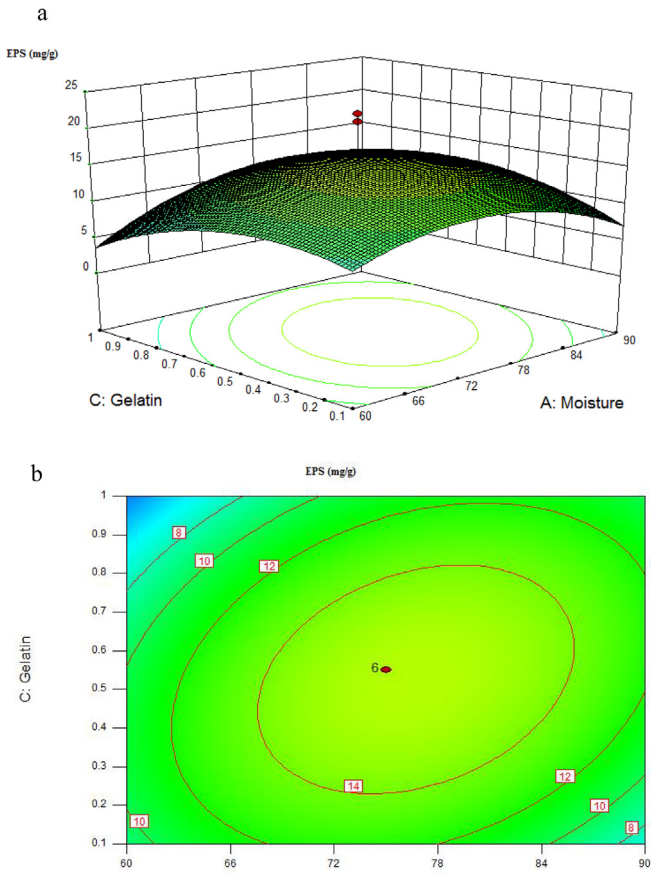

Fig. 2. The 3D-response surface (a) and 2D-contour plots (b) of EPS yield (mg/g) between moisture and gelatine.

3. Results and discussion

3.1. Screening and identification of Aspergillus sp. for the production of EPS

In our study, *Aspergillus* sp. produced maximum amount of polysaccharides. Hence this strain was used for optimization of EPS production. In microorganisms, EPSs are synthesized by intracellular mechanism and secreted out to the environment. Studies on optimization of EPS production by fungi is limited (Sutherland, 1996).

3.2. Optimization of EPS production by traditional method

Many carbon sources namely, glucose, sucrose, maltose, lactose, and dextrose were used to determine the production of EPS. Supplementation of 1% maltose supported more EPS production. The other carbon sources such as sucrose, glucose, lactose and dextrose supported 2.4 mg/g, 3.9 mg/g, 3.2 mg/g, and 2.9 mg/g, respectively. Among the nitrogen sources, gelatine induced more EPS production (5.36 mg/g). Addition of other nitrogen sources such as ammonium sulphate (3.1 mg/g), oat meal (1.2 mg/g), skim milk (3.4 mg/g), and casein (2.87 mg/g) also enhanced EPS production than control. Various inorganic salts (1%) were supplemented with the wheat bran substrate. Among the supplemented salts, Cu^{2+} (4.9 mg/g), Ca^{2+} (3.5 mg/g), Zn^{2+} (2.9 mg/g), Mn^{2+} (3.4 mg/g) and Mg^{2+} (1.8 mg/g) supported EPS production (Table 1).

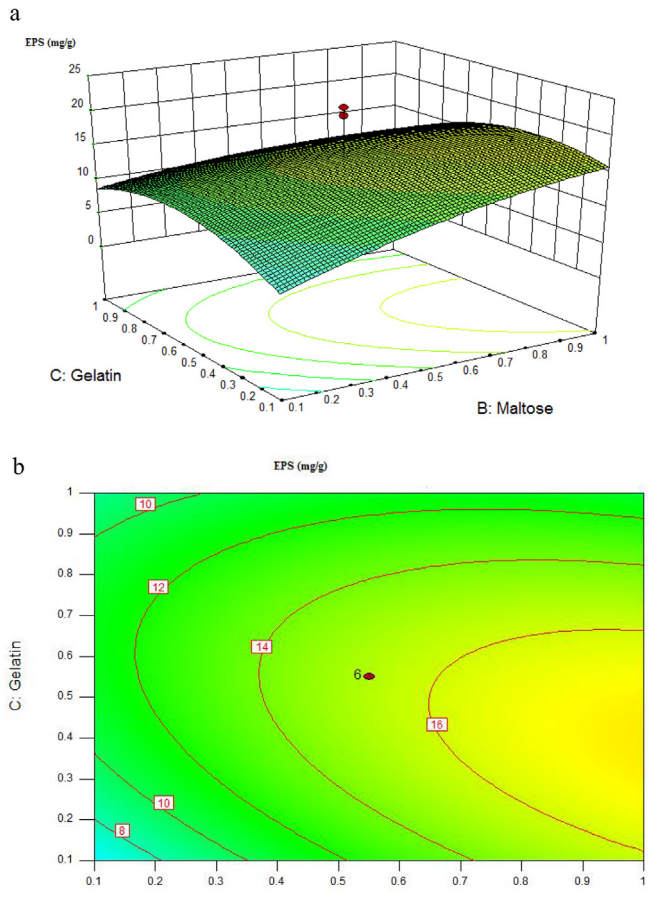

Fig. 3. The 3D-response surface (a) and 2D-contour plots (b) of EPS yield (mg/g) between gelatin and maltose.

3.3. Two level factorial designs

EPS production varied from 3.18 to 11.65 mg/g with various combinations of the components supplemented with wheat bran substrate (Table 2). The nutrients such as, maltose, gelatine and copper sulphate were significantly increased the production of EPS. In this model the analyzed all factors were significantly influenced on EPS production. In most of the organisms, maltose, sucrose and glucose were selected as the potential carbon sources for fungal EPSs production (Mahapatra and Banerjee, 2013). Addition of nitrogen sources is also important variables that induce EPS production (Yuan et al., 2008). Both organic and inorganic nitrogen sources were evaluated by various researchers to find the suitable sources. Mahapatra and Banerjee (2013) reported the induced effect of corn steep powder and yeast extract for the production of EPS. Among the ionic sources ammonium sulphate, ammonium chloride, sodium nitrate, urea and potassium nitrate are commonly applied by researchers. EPS production was found to be high in the presence of high quantity of gelatine (Sun et al., 2009). The Model F-Value of 69.84 implied the designed model was statistically significant (Table 3). EPS production has been optimized using the statistical methods such as, Plackett-Burman design, orthogonal matrix method using Box-Behnken design and fractional factorial design (Feng et al., 2010; Liu et al., 2003).

3.4. Optimization of EPS production by central composite designs and response surface methodology

CCD consists of five level of variables (Table 4) and the designed matrix is listed in Table 5. The Model F-value of 4.25 implied that the designed model was statistically significant (Table 6). Lack of fit should be non-significant to the designed experimental model "Adequate precision" measures the signal (response) to noise (deviation) ratio. In this study, contour plot and response surface plot was used to explore the optimum response of the factors. Maltose critically enhanced on EPS production than gelatine and moisture level. Moisture and gelatine enhanced EPS yield, however not statistically significant (Figs. 1–3).

4. Conclusion

The present study revealed the optimization of important factors with CCD designs to enhance EPS production by Aspergillu sp. The optimized medium yielded a maximum of 22.2 mg/g EPS production. Various fungi should be explored to find EPS with novel properties to replace the synthetic polymers.

Conflicts of interest

The authors declare no conflict of interest.

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