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Optimization of a bioactive exopolysaccharide production from endophytic *Fusarium solani* SD5

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ABSTRACT

Endophytic fungi were less investigated for exopolysaccharide production. In this study endophytic *Fusarium solani* SD5 was used for optimization of exopolysaccharide production. One variable at a time method and response surface methodology were employed to explore the optimum medium compositions and fermentation conditions. The organism produced maximum exopolysaccharide after 13.68 days of incubation at 28 °C in potato dextrose broth supplemented with (g%/l) glucose, 9.8; yeast extract, 0.69; KCl, 0.05; KH_2PO_4, 0.05 with medium pH 6.46. Use of 50 ml medium in 250 ml Erlenmeyer flask gives highest exopolysaccharide production. The organism produced more than two times higher exopolysaccharide (2.276 ± 0.032 g/l EPS) at optimized condition compared to pre-optimized condition (0.96 ± 0.021). *In vivo* toxicity test established nontoxic nature of the EPS (≤400 mg EPS/Kg of body weight). The EPS slightly altered intestinal indigenous bacteria and influenced the growth of beneficial *Lactobacillus* spp.

1. Introduction

Fungal polysaccharides had emerged as an important class of bioactive substances since last two decades (Cho, Oh, Chang, & Yun, 2006). These polysaccharides were either attached to the cell surface or found in the extracellular medium (Banik, Kanari, & Upadhyay, 2000). Various types of endo and exopolysaccharides (EPSs) were synthesized by *Ganoderma lucidum*, *Agaricus blazi*, *Cordyceps* sp., *Lentinus edodes*, *Grifola frondosa*, etc. through submerged cultures and had been reported for different interesting biological activities (Yang & He, 2008). Exopolysaccharide (EPS) had several advantages over intracellular polysaccharide like easy isolation, purification, etc. At present, scientists intend to find out novel fungi with potentiality to synthesize new EPS with unique structural and biological features. Thus, microorganisms from unexplored habitat are preferred. Endophytic fungi are such type of fungi which reside in the plant tissues without apparent harm to their host and have got special interest as they are reported as rich source of different bioactive substances (Strobel & Daisy, 2003). Though endophytic fungi and fungal EPS seem promising field of research, literature regarding EPS production from endophytic fungi is still inadequate (Banerjee, Jana, & Mahapatra, 2009; Chen et al., 2011).

Process optimization is considered very essential in any microbial metabolite production as product yield massively varies between pre and post optimization (Banerjee et al., 2009; Fariña, Siñeriz, Molina, & Perotti, 1998; Lee, Park, Ahn, Ka, & Park, 2007). Many factors such as medium composition, initial medium pH, incubation time and temperature could influence the production efficiency. A fungal endophyte of *Alstonia scholaris*, *Fusarium solani* SD5 was already reported for a novel extracellular rhamnogalactan production which had anti allergic, anti inflammatory and antioxidant activity (Mahapatra & Banerjee, 2012, 2013). Though this EPS has several applications, optimization of this valuable EPS production from *F. solani* SD5 has not been evaluated. The *in vivo* toxicity of this EPS and its effects on intestinal microbial population were also not accounted.

Hence, in the present study, for the first time an attempt was taken to optimize the EPS production from endophytic *F. solani* SD5 by liquid submerged fermentation following combined one variable at a time (OVAT) technique and response surface methodology (RSM). The *in vivo* toxicity and effect of the EPS on intestinal bacteria were also evaluated.

2. Materials and methods

2.1. Microorganism

Endophytic *F. solani* SD5 (GenBank accession number JQ657824) isolated from *A. scholaris* was used in the present investigation. The

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organism was maintained and stored on potato dextrose agar (PDA) slants at 25 °C and 4 °C, respectively.

2.2. Production of EPS

Experiments were carried out in 250 ml Erlenmeyer flasks. Initially the organism was cultured in 50 ml potato dextrose broth (PDB) medium (Himedia) having pH 6.0 at 28 °C on a rotary shaker incubator at 120 rpm. shaking speed for 14 days. Unless otherwise specified, during optimization culture conditions and medium compositions were used as optimized in its previous experimental step.

2.3. Optimization of EPS production by OVAT method

To find out the optimum incubation time, temperature and medium pH for mycelial growth and EPS production, organism was grown at different time interval (2–18 days) with different medium pH (4.0–8.0) and incubation temperature (20–30 °C).

To investigate the requirement of additional nutrients for mycelial growth and EPS production, various carbon sources (1 g%, w/v, including glucose, rhamnose, galactose, starch, fructose, maltose, raffinose) and various nitrogen sources (0.3 g%, w/v, including yeast extract, ammonium chloride, ammonium nitrate, glycine and urea) were used separately in the PDB medium. After selection of additional carbon and nitrogen supplements, different concentrations of these two selected nutrients were applied to the culture medium to decide on the effectual concentration necessary for maximum exopolysaccharide and biomass production. Various metal ion (0.05 g%, w/v, including $MnCl_2$, $CaCl_2$, $MgCl_2$, $FeCl_3$, KCl, NaCl, $HgCl_2$) and phosphate sources (0.05 g% and 0.1 g%, w/v, K_2HPO_4, KH_2PO_4 and NaH_2PO_4) were supplemented in the culture medium to determine their effect on biomass and EPS production.

To evaluate the oxygen requirement, organism was grown in 250 ml Erlenmeyer flasks with different medium volume (25, 50, 75, 100 ml). Oxygen requirement for growth and EPS production were indirectly predicted by computing surface area, head space volume, medium volume, total volume, medium depth in flask culture as described by Wonglumsom, Vishnubhatla, and Fung (2000).

2.4. Optimization of EPS production using Box–Behnken design

After OVAT optimization, further optimization was performed with the RSM. The investigational design was a Box–Behnken experimental design with four key factors selected from one variable at a time experiments. The experimental design concerned four independent factors each at three different levels where the levels were −1.0, 0 and +1.0. Production of EPS was fitted to a second order polynomial equation by means of a multiple regression technique. An empirical model related to the most significant factors was also obtained. The performance of the system is explicated by the subsequent second order polynomial equation:

$$Y = \beta_0 + \sum \beta_1 x_1 + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2$$

where Y is the predicted response or dependant variable, x_i and x_j are independent factors, β_0 is the intercept of the regression equation, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

2.5. EPS estimation

The culture was filtered and centrifuged at 10,000 × *g* to separate fungal biomass. The supernatant was concentrated in rotary evaporator under low pressure at 40 °C. Absolute ethanol was added to the concentrated supernatant (5:1, v/v), mixed thoroughly and kept 24 h at 4 °C. The precipitate was recovered by centrifugation at 10,000 × *g* for 10 min, and dialyzed in a cellulose membrane (molecular weight cut off 10,000) against distilled water for 24 h. The concentration of the EPS was estimated spectrophotometrically by phenol sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose (10–500 μg/ml) as the standard.

2.6. Biomass estimation

Broth culture of the organism was centrifuged at 10,000 × *g* for 10 min, then mycelial pellets were collected and dried at 60 °C for 24 h. Then dry weight of mycelium was measured.

2.7. In vivo activity assessment

Depending on the previous reports of this EPS (Mahapatra & Banerjee, 2012, 2013) and literature about nontoxic nature of common fungal exopolysaccharides, a single experimental design was constructed for evaluation of *in vivo* toxicity and effect of this EPS on intestinal bacterial population.

2.7.1. Animals

Experiments were performed using male Wistar rats weighing 100–110 g. The animals were fed standard pellet diet with vitamins, antibiotic and were given *ad libitum* access to water and housed in polypropylene cages (Tarson) with 12 h light:dark cycle. The animals were allowed to acclimatize for 1 week. The animals used did not show any sign of malignancy or other pathological processes. Animals were maintained in accordance with the guidelines of the ethical committee of Vidyasagar University, Midnapore, West Bengal, India.

2.7.2. Experimental design

Total 25 rats were divided into 5 groups containing 5 rats in each. The following groups were considered for the experiment:

Group I: Control, received saline water; Group II–V: received different concentrations of EPS (100, 200, 300 and 400 mg EPS/Kg of body weight). The experimental procedures were as follows.

2.7.3. Toxicity assessment

The experimental groups (Section 2.7.2) were orally administered different concentrations of EPS and saline water (as described in Section 2.7.2) daily for 30 successive days. To evaluate oral toxicity in rats, mortality and clinical signs were continually observed at one time per day for 30 days of treatment. Clinical annotations included changes in skin, eyes, occurrence of secretions and excretions and behavioral changes like, self-mutilation, walking backwards (Almeida-Lima et al., 2011). After 30 days of treatment the animals from each group were weighed, anesthetized and blood samples were collected from each animal through retro-orbital puncture. Hematological studies were performed by counting TLC (total leukocyte cell) and DLC (differential leukocyte cell). After blood collection all animals were sacrificed by cervical dislocation. The kidneys, spleen, liver, testicle, heart and lungs were carefully isolated weighed and examined macroscopically for any alterations (Almeida-Lima et al., 2011).

2.7.4. Intestinal bacterial status analysis

Feces from each rat were collected just after dropping onto clean paper underlying the cage. Fecal samples were homogenized and suspended in sterilized phosphate-buffered saline (PBS; pH 7.0 and 9 g/l NaCl). The suspension was then centrifuged (1000 × *g* for 5 min) and clear supernatant was used for microbial analysis.

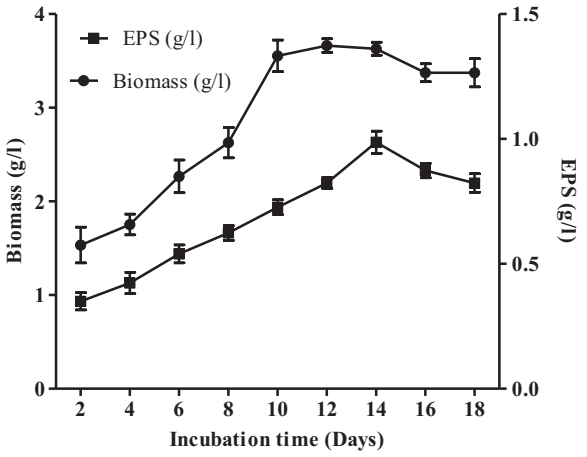

Fig. 1. Effect of incubation time on mycelia growth and EPS production by *Fusarium solani* SD5 in submerged culture.

All the fecal indicator bacteria were enumerated by standard plate count technique. Total aerobic bacteria were isolated in Nutrient agar medium (Hi-medi, India). Total anaerobic bacteria, *Lactobacillus* spp., *Escherichia coli*, *Clostridium perfringens* were isolated in reduced Wilkins Chalgren agar (WCA, Micromaster, India), MRS (Hi-media, India) Mac-Conkey agar (Hi-media, India), EMB agar (Hi-media, India) perfringen agar (Hi-media, India), respectively. For anaerobic culture (Total anaerobic bacteria, *Lactobacillus* spp., *C. perfringens*) an anaerobic jar was used from which oxygen was removed catalytically and replaced with 10% of both CO_2 and H_2 gas using a packet kit made by Micromaster, Mumbai.

All experimental data were taken at day 0 and after 30 days of treatment.

2.8. Statistical analysis

The RSM experimental design and subsequent regressional analysis of the experimental data were performed using Design-expert 8.0 software. The same software was also used for drawing response surface curves and corresponding contour plots from the results of RSM experiment. Each experiment was repeated thrice. All the results were expressed as mean ± SEM of the number of the experiments. The data analysis was performed using Graph Pad Prism software. The data analysis and graphical drawing of one variable at a time experiments were also carried out by Graph Pad Prism software. Statistical significance of data (Section 2.7.4) was performed by one way ANOVA followed by Newman–Keuls multiple comparison test.

3. Results and discussion

3.1. OVAT optimization

3.1.1. Effect of time course on fermentation for biomass and EPS production

F. solani SD5 was cultured in 250 ml Erlenmeyer flasks for 2–18 days. The growth and EPS production profile were presented in Fig. 1. It was noticed that there was a smooth increase in EPS production from 2 to 14 days and a slight decrease from 14 to 18 days. Cell biomass and EPS production reached the highest level after 14 days of fermentation (0.987 ± 0.045 g/l EPS). After 14 days the fungal strain was entered its death phase and as a consequence, biomass decreased. This type of fermentation period optima for EPS production was also noticed in findings of Nehad and El-Shamy (2010) and Lee et al. (2007) where fermentation period of 9 and 12 days were reported as most suitable for EPS production in submerged fermentation of *Alternaria alternate* and *Ganoderma applanatum* KFRI 646, respectively.

3.1.2. Effect of fermentation temperature on biomass and EPS production

EPS production related to growth of *F. solani* SD5 was studied at various incubation temperatures (20–30 °C). It was found that the organism was able to grow at a range of temperature 20–30 °C, whereas, maximum EPS and biomass production occurred at 28 °C (data not shown here). It was comparable with many other fungi, like *Paecilomyces japonica*, *A. alternate*, *Phellinus gilvus* that had similar lower temperature optima between 20 °C and 30 °C (Bae, Sinha, Park, Song, & Yun, 2000; Hwang, Kim, Xu, Choi, & Yun, 2003; Nehad & El-Shamy, 2010) for EPS production in submerged culture. It might be due to the fact that temperature played a key role in enzymatic induction or inhibition of the production of fungal EPS.

3.1.3. Effect of initial fermentation medium pH on biomass and EPS production

Production of any type of microbial metabolite is highly sensitive to its medium pH (Mahapatra & Banerjee, 2009). Generally, medium pH influenced the activity of enzymes and some metabolic channels might become available at some pH ranges, but not at others. Initial medium pH ranging from 4.0 to 8.0 was considered to investigate the EPS and biomass production by *F. solani* SD5. Below the pH 5.0 and above pH 7.0, EPS production was strongly inhibited, although biomass productions in both cases were noticeable. The maximum EPS and biomass production was found at initial medium pH value of 6.5 (data not shown here). The acidic pH optima for EPS production from fungal cultures were quite common. Formerly, Banerjee et al. (2009), Nehad and El-Shamy (2010), Nour El-Dein, El-Fallal, Toson, and Hereher (2004), and Roukas and Biliaderis (1995) reported optimum medium pH 6.5, 6.0, 5.5, 5.0 for EPS production by *Aureobasidium pullulans*, *Stemphylium* sp., *Pleurotus pulmonarius*, *A. alternate*, respectively.

3.1.4. Effect of additional carbon sources on biomass and EPS production

Most of the fungi preferred carbohydrates as their basic carbon and energy source, which were directly linked with growth of the organism and metabolite synthesis (Kim, Xu, Hwang, Choi, Kim, & Yun, 2003; Yang & He, 2008). Among all the tested additional carbon sources, glucose had the most advantageous effect on EPS production, whereas growth of *F. solani* SD5 was very efficient on rhamnose containing medium. However, fructose, maltose and starch might also serve as alternative carbon sources which supported considerable biomass and EPS production (Fig. 2a). Although galactose and raffinose showed quite similar effects on biomass and EPS production but they were not considered considering cost effectiveness. These observations might be due to varied effects of catabolic repression of different sugars for EPS synthesis or different sugar uptake fascinations of the fungus. Earlier, maximum EPS production by *Stemphylium* sp. and *A. alternate* were reported when sucrose and glucose was used as soul carbon source, respectively (Banerjee et al., 2009; Nehad & El-Shamy, 2010).

After selection, different concentrations of glucose were tested to evaluate its effect on EPS and biomass production. It was clearly seen that biomass and EPS production simultaneously increased with increasing glucose concentrations within a certain range (2–10 g%) above which EPS and biomass production decreased (Table 1). At 10 g% glucose concentration maximum EPS (1.715 ± 0.020 g/l EPS) and biomass production was noticed. Previously Choi, Maeng, Ding, and Cha (2007) reported that *Fomitopsis pinicola* produced maximum EPS in a medium containing 4% glucose.

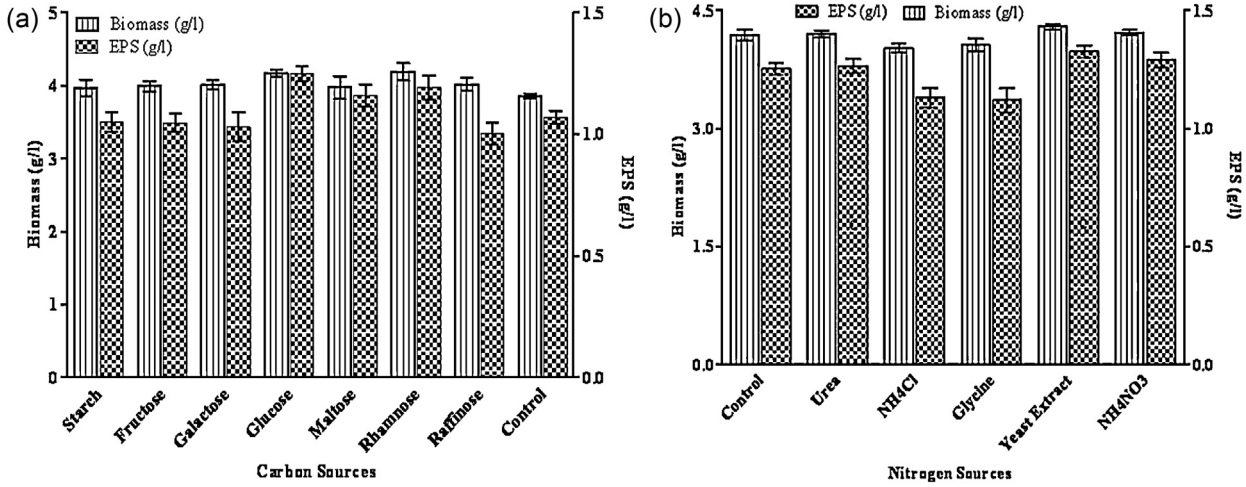

Fig. 2. Effect of different additional carbon sources (a) and nitrogen sources (b) on mycelia growth and EPS production by *Fusarium solani* SD5 in submerged culture.

3.1.5. *Effect of additional nitrogen sources on biomass and EPS production*

Nitrogen is also a major nutrient for both biomass and EPS production (Banerjee et al., 2009). Studies on supplementation of both inorganic and organic nitrogen sources to the fermentation medium showed a mixed trend on EPS production. Among the tested nitrogen sources maximum biomass and EPS production was found when yeast extract employed and was followed by NH_4NO_3 and urea (Fig. 2b). These findings were in accordance with required nitrogen supplements of *Shiraia bambusicola*, *A. alternate*, *Stemphylium* sp. for maximal EPS and biomass production in submerged fermentation (Banerjee et al., 2009; Nehad & El-Shamy, 2010; Yang & He, 2008). It was also noticed that medium containing NH_4Cl and glycine produced biomass near to control but EPS production was decreased. It might be due to metabolic shifting induced by these compounds; influenced other metabolite production that needs to be explored.

Different concentrations of additional yeast extract were also examined. It was observed that with the increase of yeast extract concentration EPS yield also increased up to a certain limit, above which the growth and production of EPS both decreased (Table 1). *F. solani* SD5 produced highest EPS and biomass when 0.7 g% yeast extract was added to the culture medium. Earlier Choi et al. (2007) reported that maximum EPS production by *F. pinicola* was noticed in a medium containing 0.5% yeast extract and 0.1% malt extract as nitrogen supplements.

Table 1
Effect of different glucose concentrations, yeast extract concentrations, salts and phosphates on biomass and EPS production by *Fusarium solani* SD5 in submerged fermentation.

Parameters tested	Effectors	Percentage added (g%)	Biomass (g/l)	EPS (g/l)
Glucose concentration	Glucose	2	4.456 ± 0.031	1.436 ± 0.026
		4	4.974 ± 0.076	1.496 ± 0.035
		6	5.122 ± 0.113	1.519 ± 0.020
		8	5.637 ± 0.079	1.650 ± 0.027
		10	6.297 ± 0.147	1.715 ± 0.020
		12	5.977 ± 0.075	1.621 ± 0.035
		14	5.043 ± 0.063	1.453 ± 0.024
Yeast extract concentration	Yeast extract	0.1	6.295 ± 0.043	1.680 ± 0.035
		0.3	6.533 ± 0.056	1.724 ± 0.017
		0.5	6.855 ± 0.050	1.764 ± 0.034
		0.7	7.143 ± 0.036	1.874 ± 0.031
		0.9	7.066 ± 0.044	1.813 ± 0.035
Different metal ions	$HgCl_2$	0.05	0.633 ± 0.120	0.117 ± 0.009
	$MnCl_2$	0.05	5.220 ± 0.064	1.450 ± 0.029
	$FeCl_3$	0.05	0.353 ± 0.048	0.050 ± 0.026
	KCl	0.05	7.307 ± 0.035	1.904 ± 0.011
	$BaCl_2$	0.05	6.383 ± 0.091	1.722 ± 0.016
	NaCl	0.05	7.233 ± 0.039	1.886 ± 0.003
	$CaCl_2$	0.05	7.200 ± 0.045	1.894 ± 0.004
	$MgCl_2$	0.05	7.176 ± 0.037	1.893 ± 0.007
	Control	–	7.172 ± 0.040	1.873 ± 0.005
Different phosphates	K_2HPO_4	0.05	7.333 ± 0.073	1.960 ± 0.061
		0.1	7.323 ± 0.071	1.906 ± 0.056
	NaH_2PO_4	0.05	7.370 ± 0.064	1.961 ± 0.064
		0.1	7.340 ± 0.038	1.956 ± 0.062
	KH_2PO_4	0.05	7.577 ± 0.084	2.093 ± 0.049
		0.1	7.400 ± 0.067	1.948 ± 0.049
	Control	–	7.313 ± 0.032	1.901 ± 0.052

3.1.6. Effect of additional salts and phosphate sources on biomass and EPS production

In the present study KCl showed the best affirmative effect on EPS (1.904 ± 0.011 g/l EPS) and biomass production (Table 1). Similar type of inducing effect of $MgSO_4 \cdot 7H_2O$ on EPS production from fungi was also reported by Banerjee et al. (2009), Hwang et al. (2003), and Yang and He (2008). The mechanism of stimulation of EPS production by metal ions was not clearly understood till now. In this study $CaCl_2$, $MgCl_2$ and NaCl also stimulated the biomass and EPS production like KCl. These findings influenced to assume that these metal ions increased the cell membrane permeability which consequently persuades EPS excretions (Banerjee et al., 2009) or functioned as cofactor of key enzymes for EPS synthesis.

It was noticed that the organism utilized all the tested phosphorous sources very well, which reflected by the average biomass production (Table 1). But enhancement of EPS production was noticed only with KH_2PO_4 at 0.05 g%/l (2.093 ± 0.049 g/l EPS). It might be due to phosphate played an important role in function of the proton-ATPase that control trans-membrane movement of different materials or involved in phosphorylation and dephosphorylation of important enzymes required for EPS synthesis.

3.1.7. Oxygen requirement for biomass and EPS production

In flask culture system an oxygen gradient was occurred between the head space gas and the medium layer (Wonglumsom et al., 2000). As system always preferred equilibrium so, oxygen is transported to liquid phase. Although cotton plug did not stop air penetration in culture flask but, plugging could restrict free air passing. Thus, the air volume of head space could influence the oxygen level in liquid medium. Another factor, surface area directly controls the oxygen diffusion in liquid. With increase in surface area oxygen diffusion in liquid medium had been increased (Wonglumsom et al., 2000). Furthermore, medium depth also regulates the level of oxygen circulation in liquid. Oxygen diffusion in liquid decreased with increase in medium depth. In the present experiment, fermentations were carried out in different 250 ml Erlenmeyer flasks with different volumes of culture medium (25 ml, 50 ml, 75 ml and 100 ml). Considering the surface area, head space volume and medium depth (Table 2), it could be assumed that, lower the medium volume, higher the dissolve oxygen and higher the medium volume, lower the dissolve oxygen. The result showed that maximum biomass and EPS production (2.105 ± 0.059 g/l EPS) by the organism was achieved when grown in 50 ml of medium (Table 2). Growth and EPS production both were sharply decreased with decrease or increase in medium volume than 50 ml. Therefore, it was clearly seen that medium level of dissolved oxygen was most suitable for the EPS production in Erlenmeyer flask by *F. solani* SD5.

OVAT optimization process evidently helped us in selection of different physical conditions and nutrients useful in increasing the yields of EPS production by *F. solani* SD5. At optimized condition 2.105 ± 0.059 g/l EPS was produced.

3.2. Optimization by Box–Behnken design

For optimization, conventional OVAT approach had some limitations, especially for interpretation about interactions among different significant factors. To resolve this type of problem, a coupled OVAT and RSM was strongly suggested by different researchers (Feng, Li, Wu, Cheng, & Ma, 2010; Xiao, Xiao, Xiong, Liang, & Zhong, 2010). In fermentation technology, RSM was applied adequately as it considered most effective, economic and reasonable design for integrate test analysis and mathematical modeling with suggested values of most probable optimum conditions of significant variables and their corresponding product yield level (Zhang, Yan, Chen, & He, 2012). Here, after OVAT, response surface methodology was adopted for optimization of EPS production by *F. solani* SD5. In comparison to other common RSM, i.e., orthogonal design and variance analysis, Box–Behnken design permits computation of factors and corresponding response at transitional levels which were not experimentally studied (Dong, Xie, Wang, Zhan, & Yao, 2009). Hence, a three level Box–Behnken design of four factors (glucose concentration, yeast extract concentration, pH of the fermentation medium and fermentation time) with five replicates at the center point of each factor was implicated as model for analysis of EPS production. Table 3 showed the experimental design including predicted and measured values of EPS production. The measured results showed considerable variations in EPS production in different fermentation conditions. The replicated center point conditions always showed maximum EPS production than any others. The predicted response Y for EPS production obtained from further multiple regression analysis on the experimental data was described as follows:

$$\begin{aligned} Y_{EPS} = {} & 2.2544 - 0.09808x_1 - 0.153x_2 - 0.01458x_3 - 0.1355x_4 \\ & - 0.0525x_1x_2 + 0.003x_1x_3 + 0.04075x_1x_4 + 0.00475x_2x_3 \\ & + 0.11775x_2x_4 - 0.012x_3x_4 - 0.31978x_1^2 - 0.28516x_2^2 \\ & - 0.26628x_3^2 - 0.38466x_4^2 \end{aligned}$$

where Y_{EPS} is the predicted EPS yield; x_1, x_2, x_3, x_4 are coded factors of yeast extract concentration (g%), glucose concentration (g%), pH of the fermentation medium and fermentation time (day), respectively. A regression analysis was performed to analysis of goodness of fit of the RSM with the experimental outputs (Table 4). The *F*-test data was checked and the model *F*-value of 547.34 indicated that the model was significant. There was only a 0.01% chance that a model *F*-value this large could occur due to noise. The adjusted determinant coefficient (R^2_{ADj}) of the polynomial model was measured for testing the goodness-of-fit of the regression equation. The value of R^2_{ADj} was determined as 0.9964 which indicated that there was a high degree of correlation between the experimental and predicted values and more than 99% variations in the response could be explained by that second order polynomial prediction equation. Adeq precision measured the signal to noise ratio with a desired value greater than 4. In the present experimental model the ratio, 71.533 indicated an adequate signal and the model could be used to navigate the design space. The lack of fit *F*-value of 0.45 implies the lack of fit was not significant relative to the pure error and the fitness of the model was good. The resulted model *P*-value ($P < 0.0001$) indicated that the model equation was suitable to describe the response of experiment pertaining to EPS production. The *P*-value of lack of fit for the equation 0.8612 was higher than 0.05 which indicated a high degree of accuracy and consistency of the experimental values. The *P*-value of prob > *F* less than 0.05 indicated model terms were significant. In this model the linear and quadratic effects of glucose concentration (g%), yeast extract concentration (g%), pH of the fermentation medium and fermentation time (day) were significant ($P < 0.05$). Furthermore, the interactions among the variables for the production of EPS were also analyzed. The most sound interaction was found between glucose concentration (g%), yeast extract concentration (g%); yeast extract concentration (g%), fermentation time (day) and glucose concentration (g%), fermentation time (day) ($P < 0.05$).

Based on the constructed model, three dimensional response surface plots were constructed by Design Expert 8.0 software to get better understanding of the effects of the variables and their interactions on the yield of EPS. The 3D plots visualized interactions between the variables and helped to rapidly determine the optimum values of each variable for maximum EPS production (Fig. 3). It was seen that all the 3D plots had significant quadratic surface rather than linear plane. The model predicted a maximum response

Table 2
Effect of different medium volume on dissolved oxygen level in submerged culture and corresponding biomass and EPS production by *Fusarium solani* SD5.

Medium volume (ml)	Total volume of flask (ml)	Head space volume (ml)	Medium depth (cm)	Surface area (cm)	Biomass (g/l)	EPS (g/l)
25	320	295	1	2.65	3.673 ± 0.033	0.897 ± 0.042
50	320	270	1.5	2.58	7.587 ± 0.215	2.105 ± 0.059
75	320	245	2.1	2.53	5.287 ± 0.154	1.404 ± 0.039
100	320	220	2.6	2.41	3.777 ± 0.081	0.908 ± 0.024

Table 3
Experimental design and results of the Box–Behnken design for optimization of the exopolysaccharide production by *Fusarium solani* SD5.

Run	Independent variables				Response [EPS yield (g/l)]	
	X_1: YEC[a] (g%)	X_2: GC[b] (g%)	X_3: M pH[c]	X_4: FT[d] (day)	Measured	Predicted
1	+1 (=0.75)	0 (=10)	0 (=6.5)	+1 (=15)	1.357	1.357
2	0 (=0.7)	+1 (=10.5)	−1 (=6)	0 (=14)	1.547	1.560
3	0	−1 (=9.5)	−1	0	1.877	1.875
4	0	−1	0	−1 (=13)	1.978	1.991
5	0	0	0	0	2.241	2.254
6	0	−1	0	+1	1.472	1.484
7	+1	0	−1	0	1.57	1.582
8	+1	0	+1 (=7)	0	1.551	1.559
9	0	0	0	0	2.292	2.254
10	−1 (=0.65)	0	−1	0	1.785	1.784
11	−1	−1	0	0	1.86	1.848
12	+1	0	0	−1	1.544	1.547
13	0	0	−1	−1	1.754	1.741
14	0	−1	+1	0	1.835	1.837
15	0	+1	0	+1	1.42	1.414
16	0	0	0	0	2.233	2.254
17	0	0	+1	−1	1.748	1.736
18	−1	0	0	−1	1.81	1.824
19	−1	0	+1	0	1.754	1.749
20	−1	0	0	+1	1.46	1.472
21	0	0	+1	+1	1.45	1.441
22	0	+1	0	−1	1.455	1.449
23	0	0	0	0	2.254	2.254
24	0	0	−1	+1	1.504	1.494
25	0	+1	+1	0	1.524	1.540
26	+1	+1	0	0	1.355	1.346
27	0	0	0	0	2.252	2.254
28	+1	−1	0	0	1.77	1.757
29	−1	+1	0	0	1.655	1.647

The data of response are mean value of triplicate experimental result.
[a] Yeast extract concentration.
[b] Glucose concentration.
[c] Culture medium pH.
[d] Fermentation time.

Table 4
ANOVA for response surface quadratic regression model of EPS production.

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value Prob > *F*
Model	2.389766	14	0.170698	547.3435	<0.0001
x_1	0.115444	1	0.115444	370.1727	<0.0001
x_2	0.280908	1	0.280908	900.7345	<0.0001
x_3	0.002552	1	0.002552	8.183283	0.0126
x_4	0.220323	1	0.220323	706.4681	<0.0001
x_1x_2	0.011025	1	0.011025	35.35178	<0.0001
x_1x_3	0.000036	1	0.000036	0.115434	0.7391
x_1x_4	0.006642	1	0.006642	21.29845	0.0004
x_2x_3	0.00009	1	0.00009	0.289388	0.5991
x_2x_4	0.05546	1	0.05546	177.8339	<0.0001
x_3x_4	0.000576	1	0.000576	1.84695	0.1956
x_1^2	0.663317	1	0.663317	2126.933	<0.0001
x_2^2	0.52745	1	0.52745	1691.275	<0.0001
x_3^2	0.459936	1	0.459936	1474.79	<0.0001
x_4^2	0.959754	1	0.959754	3077.461	<0.0001
Residual	0.004366	14	0.000312		
Lack of fit	0.002309	10	0.000231	0.448944	0.8612
Pure error	0.002057	4	0.000514		
Cor total	2.394132	28			
R-Squared	0.998176				
Adj R-Squared	0.996353				
Pred R-Squared	0.993102				
Adeq Precision	71.53303				

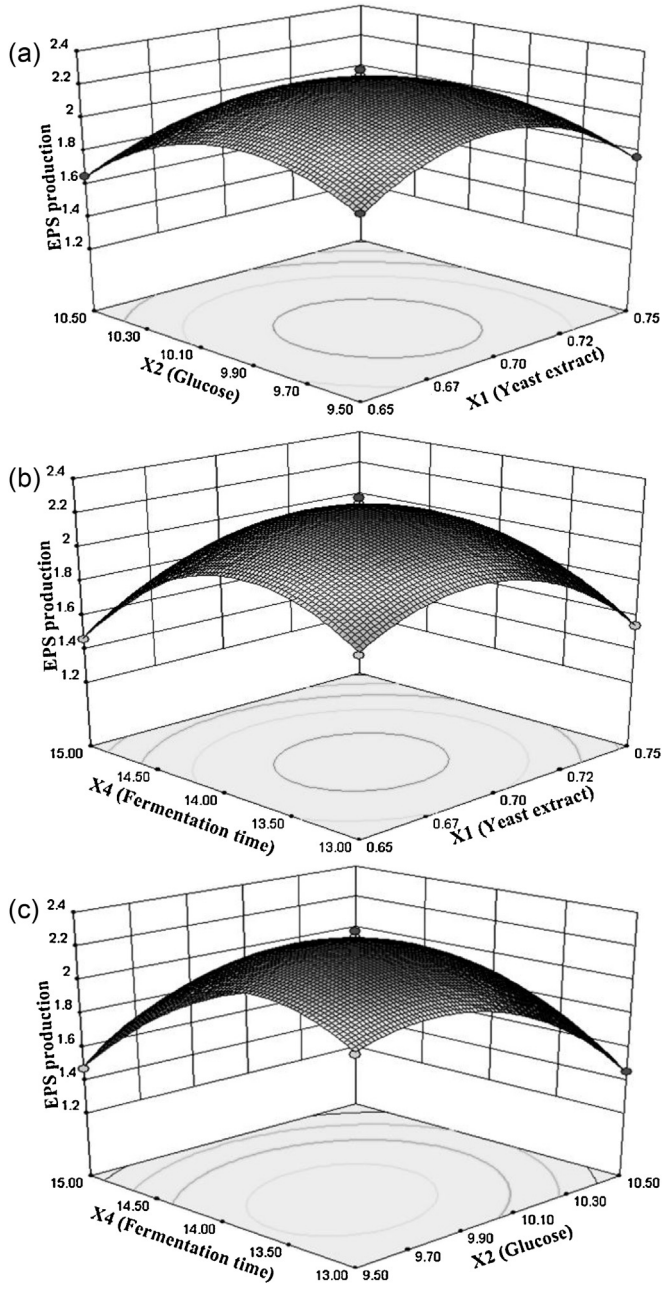

Fig. 3. The 3D-plot and 2D-projection showing the most important interactions, (a) between glucose and yeast extract at pH 6.5 after 14 days fermentation; (b) between yeast extract and fermentation time at pH 6.5 with 10 g% glucose concentration; (c) between glucose and fermentation time at pH 6.5 with 0.7 g% yeast extract concentration.

point of 2.294 g/l when the uncoded levels of the four most significant variables were 9.8 g% glucose concentration, 0.69 g% yeast extract concentration, medium pH 6.46 and fermentation time 13.68 days within the experimental range.

Validation of these predictions related to EPS production was appraised by triplicate laboratory trials in flask culture. With the predicted stipulations 2.276 ± 0.032 g/l EPS was produced by *F. solani* SD5. This finding established a very momentous connection between expected and investigational EPS production level. Experimental verification established the fitness and validation of the model and the best possible conditions were finally selected through a least numerical experiments compared to any other design. In comparison with OVAT, use of RSM reduced the fermentation time by 7 h 28 min and also reduced the required of glucose and yeast extract by 0.2 g% and 0.01 g%, respectively with modest induction in EPS production.

3.3. Toxicity assessment

It was noticed that no rats were died during and after 30 days of EPS treatment up to its maximum dose (400 mg/kg of body weight). Neither any symptom of diseases nor any changes of behavior were found in rats. The body weight, organ weight, hematological counts did not show any significant alteration compared to control groups. So, the EPS was assumed to be safe for consumption up to a dose 400 mg/kg of body weight.

3.4. Intestinal bacterial status

Intestinal microflora had several beneficial effects over its host. Colonic microflora fermented non-digestible dietary residue and generated short-chain fatty acids, those stimulated absorption of calcium, magnesium, iron ions and regulated epithelial cell proliferation, differentiation in the large and small bowel (Guarner & Malagelada, 2003). Colonic microorganisms played a role in vitamin synthesis and were functioned as a crucial line of resistance to colonization by exogenous harmful microbes (Guarner & Malagelada, 2003). Therefore, they were highly relevant in prevention of invasion of tissues by pathogens.

The measurements of total anaerobes, total aerobes, *Lactobacillus*, *C. perfringens*, *E. coli* and other enterobacteria in rat feces were presented as $\log_{10}$ CFU/g wet fecal sample. At the day 0, microbial counts from different rat feces showed a similar type of bacterial load. Although differences were found from individual to individual, but those differences were not noticeable (data not shown here). The counts of day 30 were presented here in Table 5. In control group densities of different considered microbiota were more or less same as estimated before and end of the experiments. So, the data of control group could also be considered as average counts of day 0. As shown in Table 5, the treatment with EPS at different dose resulted in higher densities of total anaerobes, *Lactobacillus* and reduced the count of *C. perfringens* as evident by comparing values for day 0 with those for days 30. Counts of *C. perfringens* were decreased significantly in the entire tested group compared to control group. Among the tested groups, up to dose 200 mg/kg of body weight showed significant decrease ($P < 0.05$) in *C. perfringens* counts above which the differences were not significant ($P > 0.05$) compared to the mentioned dose (200 mg EPS/kg of body weight). The increasing trends in densities of *Lactobacillus* and total anaerobes were highest when 200 mg/kg of body weight EPS were feed. At the dose 300 and 400 mg EPS/kg of body weight *Lactobacillus* and total anaerobes were also increased but these were not significant compared to 200 mg EPS/kg of body weight dose. Enrichment of *Lactobacillus* and reduction of *C. perfringens* indicated healthy intestinal physiological conditions evident by previous literatures (Deng et al., 2007; Lidbeck et al., 1991; Romond, Bezirtzoglou, & Romond, 1998). The counts of *E. coli* showed that up to 200 mg/kg of body weight EPS feed, the count was increased faintly as measured after 30 days. It was also noticed that with the same treatment count of other enterobacteria was quite same as initial count. Enterobacteria, other than *E. coli* were generally pathogens or opportunistic pathogens. The differences of counts of *E. coli* and other enterobacteria were not significant in all the tested doses. These findings were also indicated a good intestinal health (Deng et al., 2007; Lidbeck et al., 1991). Treatments with the EPS above 200 mg/kg of body weight did not show significant alteration of microbial count compare to that dose. This might be due to growth of intestinal flora were controlled by themselves or because intestinal microenvironment favors the growth of the microbes to certain limit favorable for host. In comparison to control all the EPS treated

Table 5
Quantification of intestinal (fecal) bacteria of rat after 30 days of different EPS dose feed.

Treatments	Bacterial count ($\log_{10}$ CFU/g wet fecal sample)					
	Total aerobes	Total anaerobes	*Lactobacillus* spp.	*Escherichia coli*	Other enterobacteria	*Clostridium perfringens*
Control	6.23 ± 0.003	11.40 ± 0.010	4.48 ± 0.010	5.93 ± 0.010	3.18 ± 0.029	4.43 ± 0.012
EPS (100 mg/kg body weight)	6.25 ± 0.017	11.49 ± 0.015^{c}	4.59 ± 0.031^{c}	5.93 ± 0.020	3.18 ± 0.015	4.08 ± 0.046^{c}
EPS (200 mg/kg body weight)	6.25 ± 0.006	12.50 ± 0.006^{c,a}	5.68 ± 0.046^{c,a}	5.94 ± 0.015	3.15 ± 0.021	3.38 ± 0.020^{c,a}
EPS (300 mg/kg body weight)	6.28 ± 0.015	12.49 ± 0.029^{c}	5.69 ± 0.006^{c}	5.93 ± 0.015	3.20 ± 0.020	3.40 ± 0.022^{c}
EPS (400 mg/kg body weight)	6.25 ± 0.025	12.50 ± 0.020^{c}	5.71 ± 0.010^{c}	5.94 ± 0.023	3.20 ± 0.006	3.41 ± 0.006^{c}

Statistical level of significant difference: Control vs. EPS treated group ($c = P < 0.05$); 100 vs. 200, 200 vs. 300, 300 vs. 400 mg EPS/kg of body weight treated groups ($a = P < 0.05$).

groups showed noticeable positive alteration of gastrointestinal microflora. Results indicated that the EPS exhibited beneficial role on intestinal health by selectively stimulating, restricting and reducing the growth of limited number of bacteria in the colon.

4. Conclusion

In this study, for the first time, endophytic *F. solani* SD5 was used for optimization of EPS production in submerged fermentation by OVAT method and followed by response surface method using a Box–Behnken design. During OVAT optimization process in flask culture, with common factors, effect of medium volume in term of oxygen level was also investigated in this study. This approach is relatively new in fungal exopolysaccharide production. At optimized conditions selected after RSM, the organism produced 2.276 ± 0.032 g/l EPS, i.e., more than two times higher than pre optimized conditions (0.96 ± 0.021 g/l EPS). The tested EPS had no toxicity on rats and it altered intestinal microbial count to a beneficial way. Not only that, as the EPS of *F. solani* SD5, rhamnogalactan; was already reported as biological response modifier, hence, the fundamental information obtained from the present investigation will be beneficial for further development of this useful bioactive EPS production process in large scale bioreactor intended for its industrialization in drug and food industries. This work might be helpful to optimize culture conditions for EPS production by other filamentous fungi.

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