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ORIGINAL ARTICLE

Schizophyllan production by newly isolated fungus *Schizophyllum commune* IBRC-M 30213: optimization of culture medium using response surface methodology

Aref Mohammadi[1] · Seyed Abbas Shojaosadati[1] · Hajar Jamshidian Tehrani[1] · Seyed Mohammad Mousavi[1] · Tayebeh Saleh[2] · Alireza Chackoshian Khorasani[1,3]

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Abstract

Schizophyllan (SPG) is a commercially attractive biopolymer produced by *Schizophyllum commune*. An investigation on the potential for SPG production by Iranian native *S. commune* was conducted based on culture medium, fermentation conditions and bioreactor type, . Nine native fungal strains were isolated from the northern forest of Iran at different times. Based on growth rate and SPG production, one strain was selected for further study. Optimal medium composition and inoculum size for maximizing SPG production and minimizing biomass were determined using central composite design by setting sucrose, yeast extract, inoculum size, carboxymethyl cellulose and oleic acid in the ranges of 50–200 g/L, 1–4 g/L, 2–10%, 2–12 g/L and 0.032–0.222%, respectively. The results showed that optimal results were obtained at 93.47 g/L sucrose, 1.87 g/L yeast extract, 7.68% inoculum size, 9.07 g/L carboxymethyl cellulose and 0.13% oleic acid, with maximum SPG production of 9.97 g/L and minimum biomass of 35.18 g/L. Under these optimal conditions, the production of SPG was studied in stirred tank and bubble column bioreactors. The results revealed greater production in the stirred tank because of better mixing of the culture medium. The SPG produced was characterized using rheometery, Fourier transform infrared spectroscopy, nuclear magnetic resonance), scanning electron microscopy and gel permeation chromatography. The results of these characterizations demonstrated the similarity of the SPG produced by *S. commune* IBRC-M 30213 to commercial SPG. Thus, the SPG produced shows good potential as a polysaccharide for use in various industries.

Keywords Schizophyllan · *Schizophyllum commune* · Iranian native fungi · Biopolymer · Polysaccharide · Optimization

Introduction

Polysaccharides are abundant natural biopolymers, representing by far the largest group of polymers manufactured worldwide (Poli et al. 2011). Schizophyllan (SPG), as a biopolymer, is a non-ionic and water-soluble extracellular polysaccharide built of a backbone chain of 1,3-β-D-glucopyranose units linked with single 1,6-bonded β-D-glucopyranoses at about every third glucose molecule in the basic chain (Yanaki et al. 1985; Rau 1999; Kumari et al. 2008; Abdel-Mohsen et al. 2014). This biopolymer is produced by submerged and solid-state culture strains of the wood-rotting and filamentous fungus *Schizophyllum commune* (Rau et al. 1992; Sutivisedsak et al. 2013c). *S. commune* is an edible mushroom belonging to the phylum Basidiomycetes, order Agricales and family Schizophllaceae (Bolla et al. 2008). SPG has unique physical properties, such as high viscosity, film formation and thermal stability, which make this biopolymer useful for various industrial applications such as strengthening the immune system in vaccines, and in anticancer therapies. SPG is also used as an oxygen impermeable film for food preservation, a thickener for cosmetic lotions, an emulsifier enhancing oil recovery and a food supplement (Sutivisedsak et al. 2013a, b; Zhang et al. 2013; Zhong et al. 2013). Due to the

✉ Seyed Abbas Shojaosadati
shoja_sa@modares.ac.ir; http://www.modares.ac.ir/en/Schools/chemE/academic-staff/~SHOJA_SA

[1] Biotechnology Group, Faculty of Chemical Engineering, Tarbiat Modares University, PO Box 14155–4838, Tehran, Iran

[2] Department of Nanobiotechnology, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

[3] National Elites Foundation of Iran, Tehran, Iran

important applications of this biopolymer, it has attracted much attention in recent years.

Most studies have focused on the production of SPG by the identified strains such as ATCC 38548, and a few have investigated its production using newly isolated strains. Therefore, little information exists on SPG production by native strains. *S. commune* can be isolated from all continents except Antarctica. It usually grows abundantly in areas with high humidity, frequently forming on carious wood (Imtiaj et al. 2008; Ohm et al. 2010).

The aim of this study was to newly isolate native strains of *S. commune* for optimizing the production of SPG. New native strains of *S. commune* were isolated from the trunks of carious trees in various regions in the north of Iran. The best strain was identified by sequence analysis of the D1/D2 regions of ribosomal DNA. SPG production was optomized using central composite design (CCD). Before optimization, the effects of sucrose, yeast extract, inoculum size, carboxymethyl cellulose (CMC) and oleic acid were extensively investigated using the "one factor at a time" method. Subsequently, SPG production under optomized conditions was studied in stirred tank and bubble column bioreactors. The SPG produced was characterized using rheometery, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), scanning electron microscopy (SEM) and gel permeation chromatography (GPC).

Materials and methods

Isolation of SPG-producing fungi

For isolation of SPG-producing fungi, the body of rotting trees was cut off from the main trunk of the tree. In order to prepare samples, they were first washed with detergent after removing the fruiting bodies from the wood, then rinsed with distilled water and soaked in a bleaching agent for 15 min. The bleached samples were washed with ethanol (75%) for 20 s and finally washed with sterile distilled water under sterile conditions. The prepared samples were transferred to potato dextrose agar (PDA) medium and incubated at 28 °C for 7–8 days. Grown fungi were stored in stock medium at 4 °C for further studies.

Monthly subculture of cultures grown on PDA was necessary for the maintenance of strains. As the first step in the preparation of seed culture, an 8 × 8 mm square of mycelia was used to inoculate 50 mL sterile culture medium (30.0 g/L glucose, 3 g/L yeast extract, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L KH_2PO_4) in a 250-mL Erlenmeyer flask that was incubated at 28 ± 2 °C and 180 rpm for 2 days in a shaker incubator. At the later step, to increase the contact surface of the mycelium with medium, the broth obtained from the first step was homogenized. Then, 5 mL homogenized broth was inoculated into 50 mL sterile culture medium in a 250-ml Erlenmeyer flask and incubated at 28 ± 2 °C and 180 rpm for 2 days in a shaker incubator. The optimal pH for culture of *S. commune* was pH 5.5 (Smirnou et al. 2011).

Various factors influenced SPG production. Initial screening was carried out using the "one factor at a time method. According to previous studies, sucrose and glucose as carbon sources and yeast extract along with malt extract as nitrogen sources were chosen to investigate the production of SPG (Rau 1999; Kumari et al. 2008). After preparation of the inoculant of all isolated strains, 2.5 mL of each sample was inoculated into each Erlenmeyer flask and the production of SPG was evaluated. Thereafter, the isolated strain of *S. commune* with the highest production of SPG was selected for further studies. To compare the results obtained from the native strain, *S. commune* ATCC 38548 was purchased from the IHEM fungal collection in Belgium with a code of 5263 as control strain.

Molecular identification of isolated fungi

Total DNA was extracted according to a previously described protocol, with some modifications (Cubero et al. 1999). Fresh mycelia collected from 7th-day cultures grown on PDA medium were washed with Tris-EDTA (TE) buffer and kept in a 1.5 mL microtube at −20 °C for 15 min; 130 μL cool lysis buffer (Tris-HCl 450 mM, EDTA 150 mM, NaCl 120 mM, pH ~7.8) was added to each microtube and briefly ground using a glass rod. Then, 75 μL 10% sodium dodecyl sulfate (SDS) was added to the microtube, and kept at room temperature for 15 min. After gentle shaking, 370 μL lysis buffer was added to the microtube, which was then incubated at 65 °C for 20 min. After incubation, 75 μL 10% SDS was added to the microtube, which was then kept at room temperature for 15 min. After gently shaking, again, 370 μL lysis buffer was added and the microtube was incubated at 65 °C for 20 min. Thereafter, the vial was transferred to −20 °C for 20 min. After another round of 65 and −20 °C incubations, 150 μL cool potassium acetate buffer (pH 4.8) was added. Afterwards, the microtube was vortexed for 10 s and centrifuged at 11,000 *g*. Subsequently, the upper phase was transferred to a 1.5 mL microtube, 800 μL 99.9% isopropanol was added, and the mixture was then centrifuged at 12,000 *g* at 4 °C for 5 min. Then, the supernatant was removed, 300 μL 70% alcohol was added to the precipitated DNA, vortexed and centrifuged at 12,000 *g* at 4 °C for 5 min. Again, the supernatant was removed and the residual alcohol evaporated. The DNA pellet was air dried at 37 °C and resuspended in 80 μL sterile sterilized distilled water. The DNA concentration was measured using a spectrophotometer.

The D1-D2 fragment (nearly 600 bp) of the rDNA in selected strains was amplified by polymerase chain reaction (PCR) using universal primers NL-1 (5′-GCAT

ATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTC CGTGTTTCAAGACGG-3′) (Romanelli et al. 2010). Amplification reactions were prepared in a total volume of 50 μL containing 5 μL PCR buffer (Fermentas, Waltham, MA), 10 mmol dNTPs, 2.5 mM $MgSO_4$, 10 pmole of each primer, 0.5 μL 99% dimethyl sulfoxide (DMSO), 5 U *Taq* DNA polymerase, 2 μL genomic DNA, and an appropriate volume of sterilized distilled water. The PCR was performed using a MyCycler thermal cycler (Bio-Rad, Hercules, CA) with an initial denaturation stage of 4 min at 94 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing for 45 s at 56 °C, extension for 85 s at 72 °C, and one additional cycle with a final 7 min chain elongation at 72 °C. Amplification products were analyzed by gel electrophoreses on a 1.5% agarose gel sequenced by Takapouzist Biotechnology Corporation (Tehran, Iran).

The sequence of the D1-D2 fragment of large subunit (LSU) loci was used for similarity investigations after being traced. According to the results obtained from the similarity assessments in the nucleotide database of GenBank, NCBI, and identification utilities in CBS databases, 69 hits were downloaded for further sequence analyses. Sequences were aligned using the ClustalW option in the MEGA5 package (default settings) (Nasr et al. 2014). Flanking regions and sequences that caused undesirable gaps were excluded from the analysis. The alignment was checked by eye, and, finally, the resulting multiple sequence alignment was used for phylogenetic assessments. Phylogenetic trees were rooted with *Fistulina antarctica* (CBS 701.85). Maximum likelihood (ML) and maximum parsimony distance analyses were conducted with the MEGA5 package (Nasr et al. 2014). Jukes-Cantor and Kimura 2-parameter substitution models were used for ML analysis. The robustness of the trees was evaluated by 500 bootstrap replications.

Optimization of medium for SPG production

A design of the experimental method using CCD packaged in Design Expert version 7.0.1.0 (Stat-Ease, Minneapolis, MN) was applied to optimize the production of SPG. The independent variables selected for optimization were as follows: sucrose, yeast extract, inoculum size, CMC and oleic acid. The factors assessed at five various levels are given in Table 1. The total experiments were 50 runs comprising 32 (2^5) factorial points, 10 axial points and 8 replicates at the center points. The dependent responses were SPG production (y_1) and biomass production (y_2).

The results were analyzed to develop a significant model for SPG and biomass production using analysis of variance (ANOVA) through the *F*-values and *P*-values. The quality of the fit of the polynomial model was expressed by the determination coefficient R^2. The objective function of the optimization was to maximize the production of SPG and minimize the biomass by optimizing the concentrations of sucrose, yeast extract, inoculum size, CMC and oleic acid. The mathematical relationships between the responses and independent variables were represented by a quadratic polynomial equation as given in Eq. 1:

$$y = \beta_i + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{i \neq j=1}^{k} \beta_{ij} x_i x_j \qquad (1)$$

where y refers to the predicted responses, x_i and x_j are independent variables, B_0 is the offset term, B_i, B_{ii} and B_{ij} are the linear, quadratic and interaction coefficients, respectively, and K is number of factors case study.

SPG and biomass measurement

In order to determine the amount of dry cell weight, fermentation broth was centrifuged at 10,000 *g* for 20 min. The obtained pellet was washed with distilled water, dried at 65 °C, and then maintained in this temperature to constant weight. All measurements were carried out with two repetitions.

To estimate the amount of SPG, the supernatant obtained from the centrifuge was filtered using a membrane filter with 200 μm pore size. Then, two volumes of 96% (*v*/v) ethanol were gradually added to the filtrate until the SPG precipitated. The filtrate was kept at 4 °C for 12 h to complete the precipitation process. Thereafter, the obtained SPG was centrifuged at 10,000 *g* for 20 min. The SPG deposited was dried at 65 °C to constant weight (Kumari et al. 2008; Limin et al. 2013).

SPG production in bioreactor

The formation of extracellular SPG was dependent on fungal growth. After determining optimum fermentation conditions, the production of SPG was investigated using batch cultures in a bioreactor with the optimal medium. A stirred tank and a bubble column bioreactor were used. Oxygen consumption and shear stress were two important factors in the growth of fungi, and, ultimately, in the production of SPG using *S. commune*. Using a 3-L stirred tank bioreactor with a 2-L working volume, the secretion of SPG by *S. commune* under the conditions of 0.5 vvm as an initial aeration rate, agitation at 150 rpm, and cultivation temperature of 28 °C was studied. Further, a bubble column bioreactor with a 1-L working volume was used under the terms of 0.5 vvm initial aeration rate and the cultivation temperature of 28 °C. The initial pH of the medium was set at 5.3 (Rau 2004). Dissolved oxygen concentration, and its pH levels in the medium, were monitored by a polarographic oxygen probe and pH sensor during the fermentation process.

Table 1 Experimental independent variables and their levels in the central composite design (CCD)

Variable factor	Symbol	Units	Low axial -α	Low factorial −1	Center 0	High factorial +1	High axial +α
Sucrose	x_1	g/L	50	93.4	125	156.6	200
Yeast extract	x_2	g/L	1	1.8	2.6	3.2	4
Inoculum size	x_3	(v/v)%	2	4.32	6	7.68	10
CMC	x_4	g/L	2	4.8	7	9.2	12
Oleic acid	x_5	(v/v)%	0.032	0.087	0.127	0.166	0.222

Rheological measurement

SPG rheological properties at concentration of 3 g/L were measured using a MCR 301 rheometer (Anton Paar, Blankenfelde-Mahlow, Germany) with a low shear viscometer. Steady shear was performed at 21 °C in the range of 0.1–1000 1/s. Oscillatory tests of complex viscosity, storage modulus (G'), and loss modulus (G") were investigated at frequencies from 10 Hz to 0.1 Hz and 21 °C (Zhong et al. 2013).

FTIR spectroscopy

The FTIR spectrum of SPG was recorded using a FTIR spectrometer (Perkin Elmer, Waltham, MA) and the spectrum was scanned at 25 °C over the wave number range of 4000–400 cm^{-1}. The sample was prepared using a mixture of dry potassium bromide and polysaccharide.

Nuclear magnetic resonance

The ^{1}H–NMR spectrum was recorded on a 500 MHz NMR Spectrometer (Bruker, Billerica, MA). In this study, 1% (w/v) SPG solution was prepared at 80 °C using DMSO as solvent.

Molecular weight measurement

GPC was used to determine the average molecular weight of the SPG. The GPC was equipped with a PL Aquagel-OH Mixed-H gel column (Agilent Technologies, Santa Clara, CA), and eluted with 0.05 M sodium nitrate at room temperature with a flow rate of 0.5 mL/min. The column was calibrated using dextran with molecular weight standards ranging from 10^3 Da to 10^6 Da. The separations were detected using a refractive index detector (RID) (Shimadzu, Kyoto, Japan). In this study, a 1% (w/v) SPG solution was prepared using dissolution of 10 mg dried powder SPG in 1 mL distilled water with increasing the temperature to 135 °C for 20 min.

Compositional analysis

The sugar composition of SPG was determined by hydrolysis of 30 mg SPG with 2 *N* TFA at 110 °C for 2 h. Aldononitrile acetate derivatives were prepared according to the Price method (Price 2004). Hydroxylamine DMAP reagent (0.2 mL) was added, and the sample was stirred in a heating block at 60 °C for 30 min prior to the addition of 0.5 mL Ac_2O to complete the peracetylation. The reactions were quenched using 2 mL water and extracted with 1 mL ethylacetate. For GC/MS analysis, the upper layer (1 μL injection) was applied.

GC/MS analysis was carried out using an Agilent 5973 gas chromatograph equipped with a HP-5MS capillary column (30 m × 0.25 mm) and a Mass Selective Detector (MSD) (Agilent Technologies). The GC was interfaced with a HP 5973 Series mass spectrometer configured in electron impact (EI) mode. Helium was used as the carrier. The column temperature was ramped over a linear gradient from 40 °C to 300 °C at 15 °C /min, holding for 10 min, and finally reset to 35 °C. The temperatures of the injector and detector were set at 300 °C. The sugars such as glucose, xylose, etc. were used as standards (Leathers et al. 2006).

Scanning electron microscopy

The surface morphology of the samples was analyzed using the Sigma VP (Zeiss, Jena, Germany) field emission scanning electron microscope. The micrograph of the surface of SPG was obtained by operating the FE-SEM with an accelerating voltage of 15 kV in magnification of 1.5 Kx.

Results and discussion

Isolation of fungi and screening for SPG production

Nine fungal strains were isolated from the trunks of trees in the forests of northern Iran. The produced SPG and biomass in different culture media are shown in Table 2. The results were determined on the 7th day. One of the isolated strains of *S. commune*, from a walnut tree, was found to have the highest production of SPG (1.08 g/L), and was competitive with *S. commune* ATCC 38548 producing 1.47 g/L. Therefore, this isolated strain was used for further studies. According to the results, the medium containing yeast extract was better than medium with malt extract in SPG production. Thus, yeast extract was selected as a nitrogen source. The evaluation of carbon source was carried out more accurately using growth

Table 2 Production of schizophyllan (SPG) and biomass based on various native strains isolated from tree samples on different media on the 7th day

Source of fungus	Glucose and yeast extract				Glucose and malt extract				Sucrose and yeast extract				Sucrose amd malt extract			
	SPG (g/l)	Biomass (g/l)	$Y_{P/S}$ [c]	$Y_{P/X}$ [d]	SPG (g/l)	Biomass (g/l)	$Y_{P/S}$	$Y_{P/X}$	SPG (g/l)	Biomass (g/l)	$Y_{P/S}$	$Y_{P/X}$	SPG (g/l)	Biomass (g/l)	$Y_{P/S}$	$Y_{P/X}$
Walnut [a]	1.08 ± 0.04	7.84 ± 0.51	0.048	0.13	0.46 ± 0.01	1.78 ± 0.061	0.020	0.25	1.24 ± 0.07	9.23 ± 0.10	0.055	0.13	0.92 ± 0.03	4.29 ± 0.66	0.040	0.21
Walnut [b]	0.57 ± 0.03	5.42 ± 0.35	0.025	0.10	0.41 ± 0.06	2.27 ± 0.032	0.018	0.18	0.9 ± 0.01	6.98 ± 0.31	0.04	0.12	0.82 ± 0.02	5.28 ± 0.31	0.036	0.15
Ulmus glabra	0.85 ± 0.02	8.72 ± 0.66	0.037	0.09	0.39 ± 0.02	2.57 ± 0.040	0.017	0.15	0.81 ± 0.08	6.82 ± 0.41	0.036	0.11	0.71 ± 0.06	5.62 ± 0.23	0.031	0.12
Salix alba	0.31 ± 0.03	5.2 ± 0.38	0.013	0.06	0.42 ± 0.08	1.56 ± 0.02	0.018	0.26	0.29 ± 0.01	7.07 ± 0.62	0.012	0.04	0.24 ± 0.09	7.19 ± 0.51	0.010	0.03
Greengage	0.49 ± 0.05	4.47 ± 0.45	0.021	0.11	0.4 ± 0.07	0.74 ± 0.06	0.017	0.54	0.4 ± 0.07	3.37 ± 0.21	0.017	0.11	0.45 ± 0.07	4.23 ± 0.65	0.02	0.10
Mespilus germanica	0.27 ± 0.02	4 ± 0.41	0.012	0.06	0.14 ± 0.04	1.08 ± 0.02	0.006	0.13	0.33 ± 0.05	5.03 ± 0.53	0.014	0.06	0.41 ± 0.05	5.63 ± 0.42	0.018	0.07
Albizia julibrissin	0.03 ± 0.01	3.05 ± 0.52	0.001	0.01	0.4 ± 0.04	3.08 ± 0.09	0.017	0.13	0.2 ± 0.01	2.11 ± 0.07	0.008	0.09	0.29 ± 0.01	2.84 ± 0.26	0.012	0.10
Alnus serrulata	0.18 ± 0.04	4.73 ± 0.32	0.008	0.03	0.38 ± 0.02	2 ± 0.04	0.016	0.19	0.38 ± 0.03	6.07 ± 0.41	0.016	0.06	0.48 ± 0.03	6.31 ± 0.58	0.021	0.07
Pyrus boissieriana	0.38 ± 0.05	3.94 ± 0.28	0.016	0.09	0.39 ± 0.01	1.82 ± 0.03	0.017	0.21	0.72 ± 0.06	6.89 ± 0.52	0.032	0.10	0.52 ± 0.06	6.29 ± 0.50	0.023	0.08
ATCC 38548	1.47 ± 0.01	7.01 ± 0.62	0.065	0.20	1.2 ± 0.02	3.07 ± 0.03	0.053	0.39	1.6 ± 0.09	6.85 ± 0.12	0.071	0.23	1.26 ± 0.03	6.97 ± 0.83	0.056	0.18

[a] Walnut tree location 36°26′12.5″N, 53°13′13.7″E

[b] Walnut tree location 36°31′51.3″N, 53°04′10.3″E

[c] $Y_{P/S}$: g SPG/ g substrate

[d] $Y_{P/X}$: g SPG/ g biomass

curves within 10 days (Fig. 1) since the difference in the SPG produced in medium with glucose-yeast extract and sucrose-yeast extract was not clear. In addition, the 8th day was chosen as the stop point for the analysis of polysaccharide and carbon source. As shown in Fig. 1a, the best strain had more SPG production in the medium containing sucrose as the carbon source. According to these results, it was concluded that sucrose was a more suitable carbon source than glucose for the production of SPG in the same conditions. When glucose was used as a carbon source, it was easily consumed by fungi and used for biomass production. Further, β-glucanase secretion by *S. commune* could cause a reduction in the amount of SPG produced after the 7th day (Fig. 1a) (Rau et al. 1992). At low concentrations of carbon source (Fig. 1c), β-glucanase activity was greater at the end of the growth period to hydrolyze

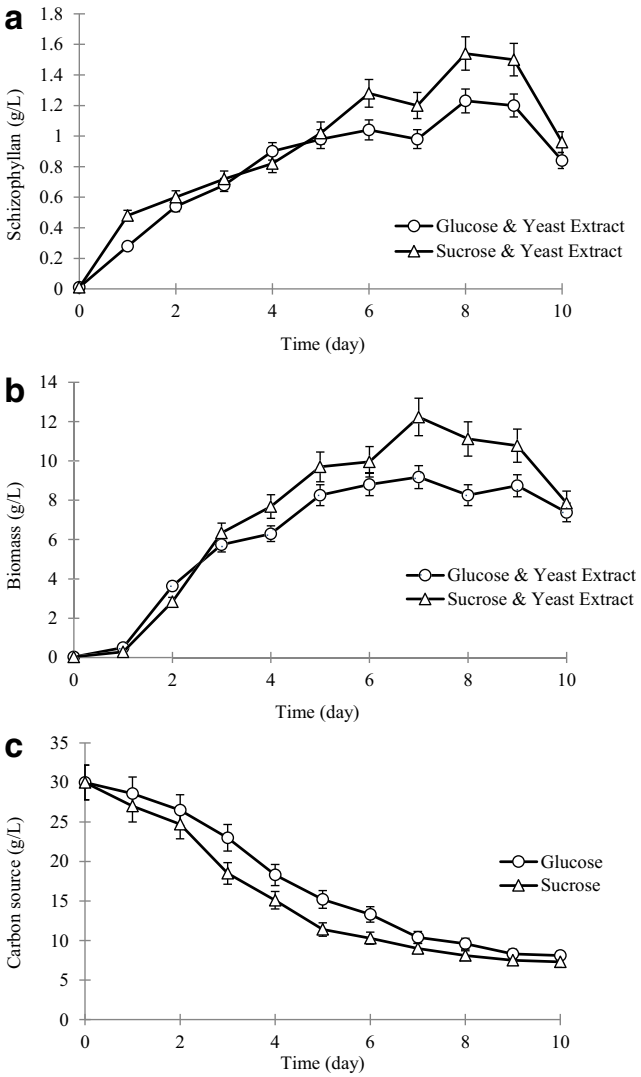

Fig. 1 **a** Schizophyllan (SPG) production and **b** biomass production by *Schizophyllum commune* IBRC-M 30213, and **c** carbon source concentration in both glucose-yeast extract and sucrose-yeast extract medium

glycoside links in beta-D-glucan. This resulted in increasing the glucose concentration in the culture medium since glucose constitutes the highest proportion of SPG constituent units. Furthermore, SPG could be consumed as a carbon source by the fungus to reduce the viscosity of the culture medium (White et al. 2002). When sucrose was used as a carbon source, the β-fructosidase broke down sucrose into α-D-glucose 1-phosphate and fructose (Goedl et al. 2008). This metabolic pathway was considered as a limitation for the fungal strain producing SPG and consuming fructose as a lateral carbon source. Maximum production of biomass was obtained using sucrose when compared with glucose. Similar results were reported by other researchers studying *Schizophyllum commune* for SPG production (Jamshidian et al. 2016). Moreover, high concentrations of sucrose were not recommended since they may increase the osmotic pressure of the culture medium, thus leading to the difficult secretion of exopolysaccharide from the cell wall (Farina et al. 1998).

Molecular identification and phylogenetic analysis

Phylogenetic analysis using the GenBank database indicated that the LSU sequences of this database were mostly of *S. commune*, and there was trace records of other species of the genus *Schizophyllum*. This native strain was recorded and coded as IBRC-M 30213 in the Iranian Biological Resource Center (IBRC) useful for the production of SPG. The sequence similarity investigations of NCBI's GenBank nucleotide database showed *Schizophyllum* sp. PDD 103380 [GenBank KF727350; identities = 573/575 (99%), gaps = 1/575 (0%))] *S. commune* isolate FK7 [GenBank JQ695912; identities = 573/575 (99%), gaps = 1/575 (0%)], and an uncultured fungus (clone C72) [GenBank JQ231247; identities = 573/575 (99%), gaps = 1/575 (0%)], as the three most similar hits. However, dozens of hits were also shown with >99% similarities to strain IBRC-M 30213, and most of them were identified as members of *S. commune*. Phylogenetic studies confirmed the affiliation of strain IBRC-M 30213 to *S. commune* as depicted in Fig. 2. Where, A refered to KP012878, KP012945, KF727350, KC414834, KC414832, KC414831, KC414829, KC414827, KC414826, KC414821, KC414815, KC414822, JQ807984, KC414817, JQ695912, KC414830, KC414836, JQ231247, HM595606, HM595605, GQ241260, FJ372712, FJ372710, DQ071725, AY858374, AY571023, AM269873, FJ471576, KF679518, KC414825, AJ406555, KC414839, AB733341, GQ254661, AM269872, KC414824, AB733340, KC414820, FJ372704, KC414819, AB733322, DQ674815, GQ351562, KC414837, AM269871, AB363768, AF261587, KC414833, KC414838, FJ372705, FJ372711, KC414823, KC414840, AB080723, AF334751 and IBRC-M 30213.

Optimization of medium for SPG produced by *S. commune* IBRC-M 30213

As considered in previous studies, the effective factors, such as carbon and nitrogen sources, and the appropriate range of concentrations, were investigated by the one-factor-at-a-time method in this study (Kumari et al. 2008; Hao et al. 2010). Based on the results obtained from the one-factor-at-a-time experiments, CCD was utilized as a statistical design to investigate the effects on biomass and SPG production of five different medium components (independent variables) (Table 3). The CCD presented in Table 3 was employed to optimize the medium composition for maximizing SPG production (y_1) and minimizing biomass production (y_2) in the submerged cultivation of *S. commune* IBRC-M 30213. The concentration of KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ were fixed at 1 g/L and 0.5 g/L, respectively. Throughout the runs, run number 31, with the conditions of 156.60 g/L sucrose, 1.80 g/L yeast extract, 7.68% inoculum size, 9.20 g/L CMC and 0.09% oleic acid, yielded the highest SPG production (11.06 g/L) with low biomass production (42.08 g/L). The lowest production of SPG (4.42 g/L) was obtained under run number 25, with a combination of 93.40 g/L sucrose, 1.80 g/L yeast extract, 4.32% inoculum size, 4.80 g/L CMC and 0.17% oleic acid.

In the other study, *Ganoderma* polysaccharide was produced by fed-batch fermentation of *Ganoderma lucidum* in which sucrose was a suitable carbon source for the extracellular polysaccharide production, although the cells could not grow well. The maximum cell density and production of exopolysaccharide reached 20.95 g/L and 0.75 g/L, respectively (Tang and Zhong 2002). In comparison, in our study, biomass and exopolysaccharide production, and also production yield, was greater since the productivity of a metabolite was related to the fungal species and culture conditions. In contrast, *Volvariella volvacea* strains were studied to produce intracellular polysaccharides in which the production of biomass and intracellular polysaccharides reached 16.70 g/L and 8.37 g/L, respectively (Diamantopoulou et al. 2016). The higher production of intracellular polysaccharides in comparison to exopolysaccharides was due to the presence of cell wall, which limited and decreased secretion of exopolysaccharides into the surroundings.

Depending on the fungal strain and culture medium, the production yield of various exopolysaccharides can be different. *Botryosphaeria rhodina* produced β-glucan exopolysaccharide up to 17.2 g/L when grown on undiluted olive-mill wastewaters (Crognale et al. 2003). *Botryosphaeria rhodina* DABAC-P82 produced 17.7 g/L exopolysaccharide (homopolysaccharide of glucose) after only 24 h fermentation when grown on optimal nitrogen source (Selbmann et al. 2003). Moreover, the highest exopolysaccharide quantity achieved ranged between 1.6 g/L and 1.8 g/L by cultivation of *Ganoderma lucidum* and *Lentinula edodes*, respectively

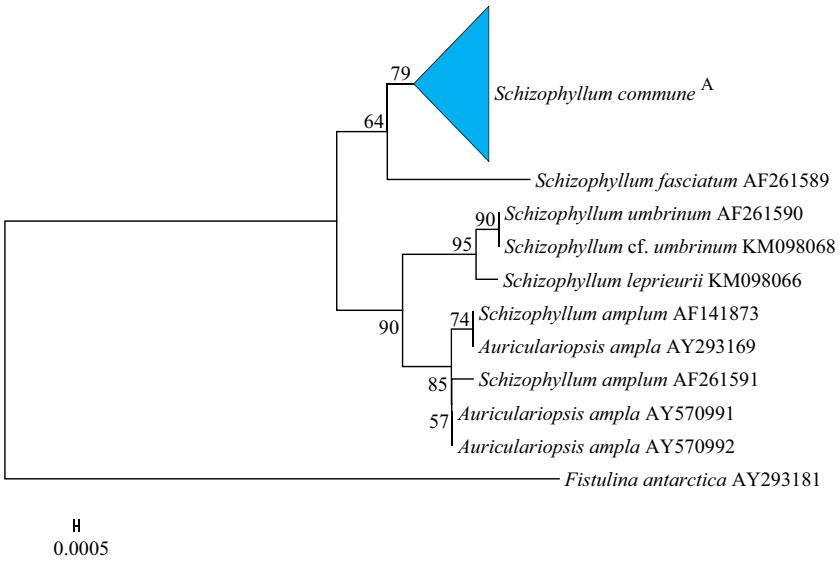

Fig. 2 Neighbor-joining tree illustrating the phylogenetic relationship between members of *S. commune* and other members of this genus based on the available large subunit (LSU) sequences in the GenBank nucleotide database

(Diamantopoulou et al. 2012). In this study, the highest SPG production was approximately 11 g/L. Comparison between this study and others proved that the type of strain, culture medium and fermentation conditions affected exopolysaccharide production the most.

The results were analyzed statistically using ANOVA. The experimental values obtained from the CCD were regressed by a quadratic polynomial equation. Table 4 represents the mathematical model relating the responses (production of SPG and biomass) with factors (sucrose, yeast extract, inoculum size, CMC and oleic acid). The second-order polynomial coefficients for each term of the equation were determined through multiple regression analysis using the software Design Expert. The *P*-values were used to determine the significance of each of the coefficients. The models significantly ($P < 0.05$) represent the relationship between the responses (SPG and biomass production) and the significant input variables. The response surface analysis of the experimental data indicated that factors had quadratic effects on SPG and biomass production with good regression coefficients. R^2 values were 0.8799 and 0.7951 for SPG production and biomass production, respectively. These values were relatively acceptable due to the large number of experiments and measurements.

As shown in Fig. 3, the predicted versus actual graphs for the responses of y_1 (Fig. 3a) and y_2 (Fig. 3b) yielded fairly straight lines at 45°, implying an appropriate relationship between the experimental data and predicted values.

The objective of this study was to obtain the maximum production of SPG and minimum biomass by setting the concentrations of sucrose, yeast extract, inoculum size, CMC and oleic acid in the range of the study conditions. The optimized condition of each factor was 93.50 g/L sucrose, 1.87 g/L yeast extract, 7.68% inoculum size, 9.10 g/L CMC and 0.10% oleic acid. To validate the optimal conditions, a confirmation test was carried out. In the optimum medium, the maximum production of SPG and minimum production of biomass were 9.97 g/L and 35.18 g/L, respectively, approximately equal to predicted values of 10.00 g/L and 35.00 g/L. SPG production of 9.97 g/L in optimumconditions was obtained in experimental tests with *S. commune* IBRC-M 30213, which wasmore than in previously reports of SPG produced by other strains (Kumari et al. 2008; Sutivisedsak et al. 2013b). A high carbon to nitrogen ratio was a key factor in improving the production of more polysaccharides (Survase et al. 2007). In our study, the maximum SPG production in optimum conditions occurred at a C/N ratio of 49.9, while (Kumari et al. 2008) reported maximum SPG production 8.03 g/L in optimum conditions and at a C/N ratio of 83.6.

The response surface contour plots (Figs. 4, 5) illustrate how production of SPG (y_1) and biomass (y_2) relate to the factors of sucrose (x_1), yeast extract (x_2), inoculum size (x_3), CMC (x_4) and oleic acid (x_5) through the quadratic models in Table 4. CMC and oleic acid had positive effects on the production of SPG. The results indicated that the CMC and oleic acid increased SPG production to 50% and 33.8%, respectively. As a fatty acid, oleic acid could stimulate the production of other fungal metabolites, such as exopolysaccharides, and facilitate the immediate uptake of nutrients from the medium (Li et al. 2011). CMC might also increase the oxygen transfer rate (Hao et al. 2010), and promote the uptake of nutrients from the culture medium (Li et al. 2011). In addition, our results showed that, at a low level of nitrogen source, the amount of produced SPG increased with the increase of the inoculum size due to the increasing density of fungi, while, at a high level of nitrogen source, the amount of produced SPG decreased with the increase in the inoculum size and carbon source, because additional nitrogen source was consumed for significant biomass growth (Rau et al. 1992; Survase et al. 2007). In addition, by increasing the amount of inoculant

Table 3 CCD-based on medium components for the production of SPG and biomass as responses

Run	Medium components					Response		Yield		Residual sugar (g/L)
	x_1 (g/L)	x_2 (g/L)	x_3 (v/v)%	x_4 (g/L)	x_5 (v/v)%	y_1[a] (g/L)	y_2 (g/L)	$Y_{P/S}$	$Y_{P/X}$	
1	156.60	3.20	4.32	4.80	0.17	9.76	42.24	0.083	0.231	37.35
2	125.00	1.00	6.00	7.00	0.13	6.34	44.68	0.067	0.141	32.28
3	93.40	1.80	4.32	9.20	0.17	4.78	34.24	0.068	0.139	19.42
4	93.40	3.20	4.32	9.20	0.17	5.88	36.00	0.083	0.163	22.62
5	156.60	3.20	7.68	9.20	0.17	10.26	47.84	0.087	0.214	38.85
6	125.00	2.60	6.00	7.00	0.13	9.69	44.80	0.103	0.216	36.25
7	156.60	3.20	4.32	9.20	0.09	10.08	51.06	0.085	0.197	31.17
8	93.40	3.20	4.32	9.20	0.09	7.40	38.32	0.105	0.193	25.36
9	156.60	3.20	4.32	4.80	0.09	10.00	41.30	0.085	0.242	40.15
10	93.40	1.80	7.68	9.20	0.09	9.64	34.34	0.137	0.280	23.84
11	93.40	3.20	7.68	4.80	0.09	6.12	26.78	0.087	0.228	24.68
12	125.00	2.60	6.00	7.00	0.13	9.48	42.81	0.101	0.221	33.29
13	125.00	2.60	6.00	7.00	0.13	9.41	42.72	0.100	0.220	30.73
14	50.00	2.60	6.00	7.00	0.13	4.52	22.76	0.120	0.198	11.48
15	125.00	2.60	6.00	7.00	0.13	9.40	39.96	0.100	0.235	32.24
16	156.60	1.80	7.68	4.80	0.17	7.76	39.78	0.066	0.195	37.75
17	125.00	2.60	6.00	7.00	0.13	9.04	39.30	0.096	0.230	31.85
18	93.40	1.80	7.68	4.80	0.09	8.18	31.08	0.116	0.263	21.37
19	93.40	1.80	7.68	9.20	0.17	9.04	35.20	0.129	0.256	22.31
20	156.60	1.80	4.32	4.80	0.09	5.16	37.86	0.043	0.136	38.36
21	156.60	3.20	7.68	4.80	0.17	7.72	45.92	0.065	0.168	39.90
22	93.40	3.20	7.68	9.20	0.09	7.06	36.54	0.100	0.193	23.84
23	156.60	3.20	7.68	4.80	0.09	7.04	38.78	0.059	0.181	37.63
24	125.00	4.00	6.00	7.00	0.13	7.30	37.46	0.077	0.194	32.21
25	93.40	1.80	4.32	4.80	0.17	4.42	29.50	0.063	0.149	21.44
26	93.40	3.20	4.32	4.80	0.09	5.72	29.50	0.081	0.193	23.54
27	156.60	1.80	4.32	4.80	0.17	5.44	37.66	0.046	0.144	36.77
28	156.60	1.80	7.68	9.20	0.17	8.06	40.14	0.068	0.200	37.43
29	156.60	3.20	7.68	9.20	0.09	7.98	40.72	0.067	0.195	40.62
30	125.00	2.60	6.00	7.00	0.13	9.52	42.42	0.101	0.224	33.38
31	156.60	1.80	7.68	9.20	0.09	11.06	42.08	0.094	0.262	37.96
32	125.00	2.60	6.00	7.00	0.22	6.96	43.68	0.074	0.159	31.25
33	93.40	3.20	7.68	9.20	0.17	6.58	35.70	0.093	0.184	22.87
34	93.40	1.80	4.32	9.20	0.09	5.60	33.08	0.079	0.169	21.75
35	125.00	2.60	6.00	2.00	0.13	6.18	41.94	0.065	0.147	30.22
36	125.00	2.60	2.00	7.00	0.13	5.68	43.68	0.060	0.130	31.64
37	200.00	2.60	6.00	7.00	0.13	7.06	40.14	0.047	0.175	48.25
38	156.60	3.20	4.32	9.20	0.17	9.21	48.66	0.078	0.189	36.63
39	125.00	2.60	10.00	7.00	0.13	7.14	43.54	0.076	0.163	30.51
40	93.40	1.80	7.68	4.80	0.17	7.76	33.06	0.110	0.234	20.96
41	125.00	2.60	6.00	12.00	0.13	10.90	34.94	0.116	0.311	29.27
42	125.00	2.60	6.00	7.00	0.13	8.99	41.46	0.095	0.216	30.85
43	156.60	1.80	4.32	9.20	0.09	9.96	39.64	0.084	0.251	38.19
44	93.40	3.20	7.68	4.80	0.17	6.18	38.84	0.088	0.159	22.86
45	93.40	3.20	4.32	4.80	0.17	5.18	33.08	0.073	0.156	21.55
46	156.60	1.80	7.68	4.80	0.09	7.48	43.94	0.063	0.170	37.12
47	125.00	2.60	6.00	7.00	0.13	9.24	40.58	0.098	0.227	30.67

Table 3 (continued)

Run	Medium components					Response		Yield		Residual sugar (g/L)
	x_1 (g/L)	x_2 (g/L)	x_3 (v/v)%	x_4 (g/L)	x_5 (v/v)%	y_1[a] (g/L)	y_2 (g/L)	$Y_{P/S}$	$Y_{P/X}$	
48	156.60	1.80	4.32	9.20	0.17	6.43	39.82	0.054	0.161	38.20
49	125.00	2.60	6.00	7.00	0.03	5.94	41.08	0.063	0.144	31.26
50	93.40	1.80	4.32	4.80	0.09	5.76	27.38	0.082	0.210	21.42

[a] Results are average of two repetitions

Table 4 Predicted quadratic models for SPG and biomass production with statistical analysis

Response	Model	Statistical value	
		R^2	P-value
SPG production	$y_1 = 9.39 + 0.79 \times {}_1 + 0.18 \times {}_2 + 0.48 \times {}_3 + 0.71 \times {}_4 - 0.17 \times {}_5 + 0.49x_1x_2 - 0.45x_1x_3 + 0.19x_1x_4 + 0.048x_1x_5 - 0.80x_2x_3 - 0.18x_2x_4 + 0.27x_2x_5 + 0.11x_3x_4 + 0.23x_3x_5 - 0.23x_4x_5 - 0.59 \times {}_1{}^2 - 0.4 \times {}_2{}^2 - 0.48 \times {}_3{}^2 - 0.1 \times {}_4{}^2 - 0.47 \times {}_5{}^2$	0.8799	< 0.0001
Biomass production	$y_2 = 41.51 + 4.30 \times {}_1 + 0.82 \times {}_2 + 0.26 \times {}_3 + 0.92 \times {}_4 + 0.73 \times {}_5 + 0.58x_1x_2 - 0.30x_1x_3 - 0.37x_1x_4 - 0.37x_1x_5 - 0.92x_2x_3 + 0.63x_2x_4 + 0.79x_2x_5 - 0.87x_3x_4 + 0.60x_3x_5 - 0.68x_4x_5 - 2.04 \times {}_1{}^2 - 0.34 \times {}_2{}^2 + 0.11 \times {}_3{}^2 - 0.80 \times {}_4{}^2 - 0.11 \times {}_5{}^2$	0.7951	< 0.0001

at all levels of carbon sources, polysaccharide production was increased as the concentration of yeast extract and oleic acid set at their optimal conditions (1.87 g/L and 1.30 ml/L, respectively).

SPG production in bioreactor

Various factors that influence the production of SPG can be controlled in the bioreactor. Factors such as temperature, pH, and dissolved oxygen play an important role in the fermentation process. However, it should be emphasized that factors such as the type of impeller, initial size of inoculum and pellet morphology are effective agents for the formation of extracellular polysaccharide.

In this study, SPG production in the bioreactors of stirred tank and bubble column was compared together and the data are listed in Table 5. Based on the results, it was found that the maximum production of SPG in the stirred tank bioreactor was observed on the 6th day. The final production of SPG in the bubble column bioreactor was obtained at the same time.

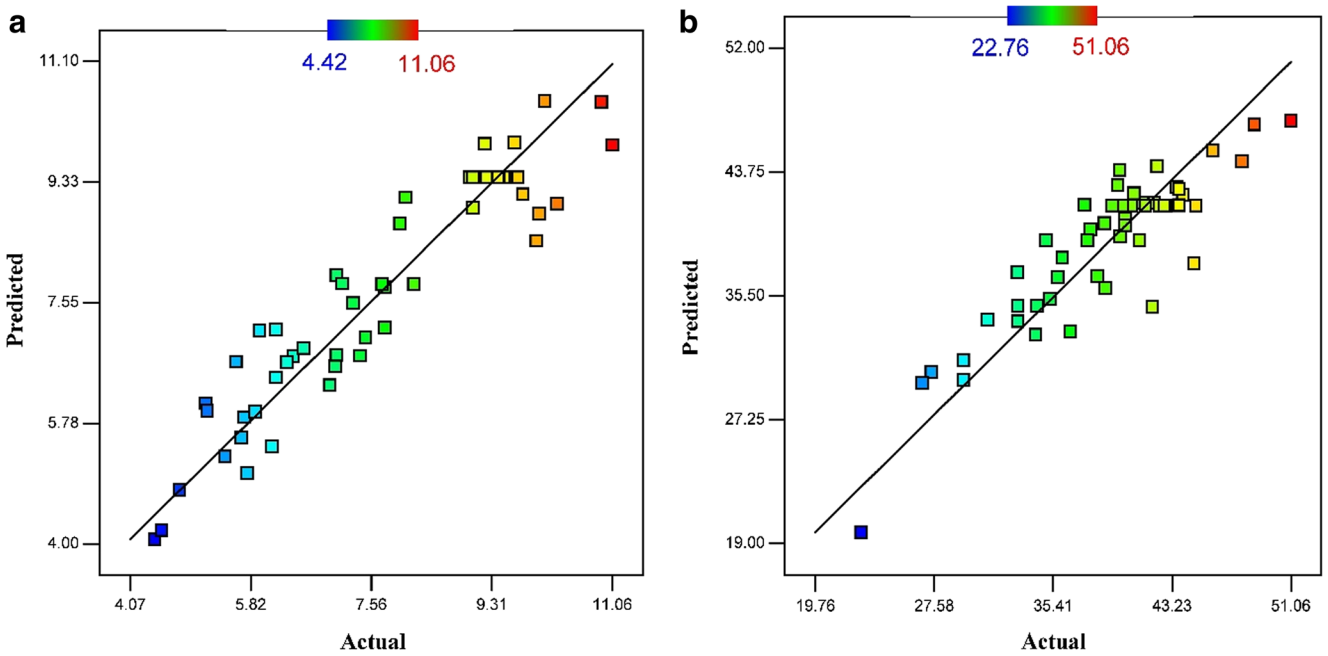

Fig. 3 Predicted vs. actual plot for **a** SPG and **b** biomass production

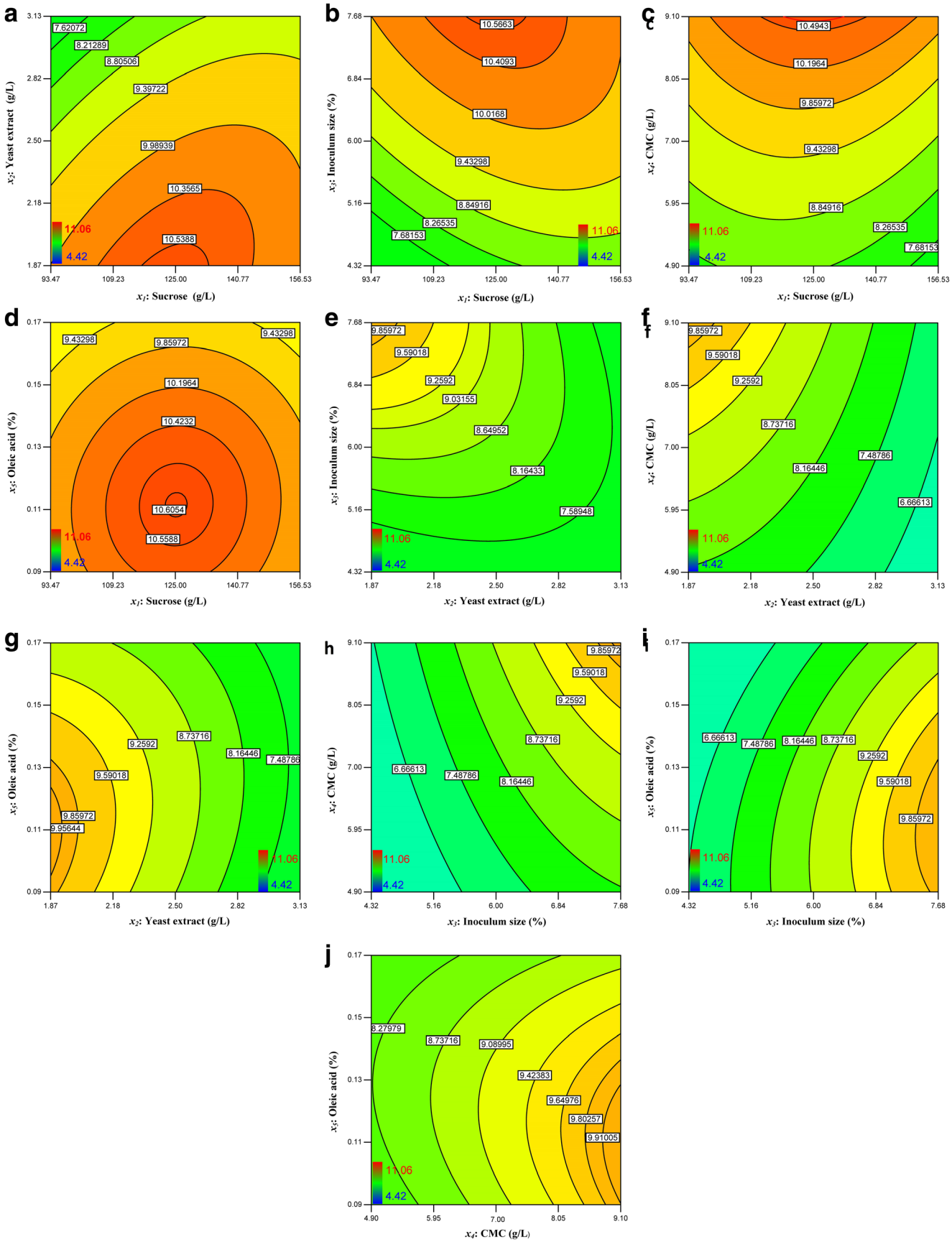

Fig. 4a–j Response surface contour plots for SPG production as a function of the interaction between factors. **a** Sucrose and yeast extract, **b** sucrose and inoculum size, **c** sucrose and carboxymethyl cellulose (CMC), **d** sucrose and oleic acid, **e** yeast extract and inoculum size, **f** yeast extract and CMC, **g** yeast extract and oleic acid, **h** inoculum size and CMC, **i** inoculum size and oleic acid, and **j** CMC and oleic acid

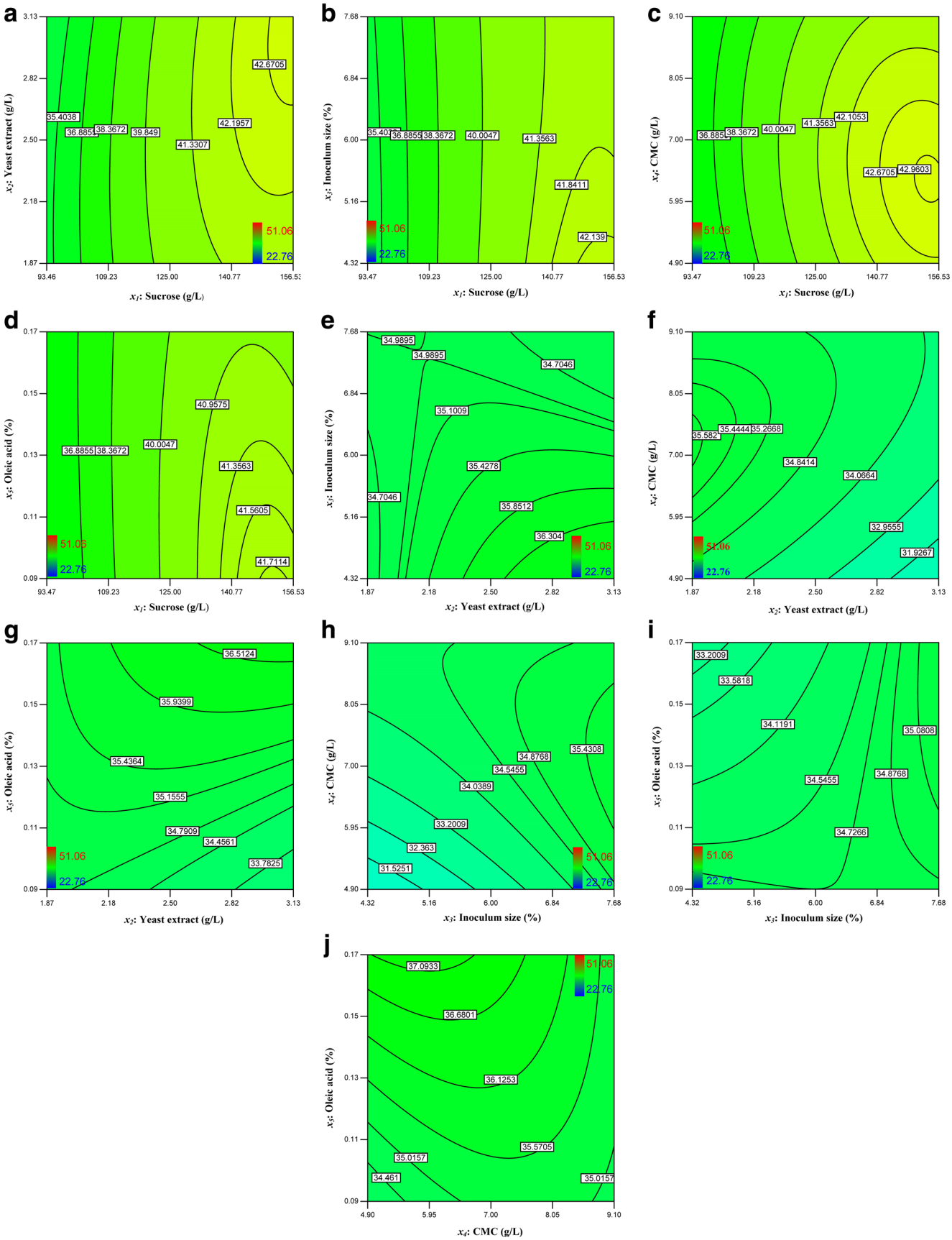

Fig. 5a–j Response surface contour plots for biomass production as a function of the interaction between factors. **a** Sucrose and yeast extract, **b** sucrose and inoculum size, **c** sucrose and CMC, **d** sucrose and oleic acid, **e** yeast extract and inoculum size, **f** yeast extract and CMC, **g** yeast extract and oleic acid, **h** inoculum size and CMC, **i** inoculum size and oleic acid, and **j** CMC and oleic acid

Table 5 Comparison of the performance of bioreactors in the production of SPG

Bioreactor type	Biomass (g/L)	SPG (g/L)	Time (day)	Productivity (g/L day)	$Y_{P/S}$	$Y_{P/X}$
Stirred tank	18.47	5.77	6	0.96	0.061	0.312
Bubble column	11.2	5.25	6	0.87	0.056	0.468

Although the carbon source was not completely consumed by *S. commune*, SPG production ceased in the bioreactors after 6 days (Fig. 6). The amount of SPG obtained in this stage was 5.77 g/L and 5.25 g/L in the stirred tank and bubble column bioreactors, respectively.

Aeration rate plays an important role in SPG production in submerged cultivation of *S. commune*. Results revealed that the growth of biomass was significantly enhanced by increasing the aeration rate to 0.1 vvm; however, an increase in aeration rate decreased the maximum SPG production. In order to enhance polysaccharide yield, the oxygen limitation was applied to a submerged culture of *S. commune* (Shu et al. 2005). The greater amount of SPG produced in the stirred tank bioreactor could be due to the agitator and baffle, which led to the better mixing of the culture medium, and prevented the excessive growth of pellets. In addition, the existence of the agitator and baffles could help release SPG trapped inside pellets. However, a very high speed agitator could possibly damage the mycelium and the SPG product (Rau 1999).

Characterization of SPG produced by *S. commune* IBRC-M 30213

The steady flow behaviors of SPG at a concentration of 3 g/L were investigated over a shear rate range of 0.1–1000 1/s at 21 °C. As shown in Fig. 7a, the viscosity of a 3 g/L SPG solution decreased from 0.19 Pa.s to 0.01 Pa.s when the shear rate increased from 0.1 1/s to 1000 1/s, while values of 0.3 Pa.s to 0.04 Pa.s were reported in previous research (Zhong et al. 2013). Moreover, the shear stress of the SPG solution increased simultaneously with increasing the shear rate, and demonstrated an approximately nonlinear dependence on shear rate; the SPG solution behaved like a pseudoplastic fluid. In this study, for investigation of frequency dependence of the storage modulus (G′) and the loss modulus (G″), a dynamic frequency analysis was carried out in the linear viscoelastic range. As reported, the highest amounts of G′ and G″ were obtained at higher frequencies, and it was revealed that the two modules had a special dependency on the frequency (Zhong et al. 2013). Figure 7b shows "cross-over frequency" (where G′ = G″) occurred at 0.121 1/s and G′ is higher than G″ at all frequencies, and the system displayed solid-like behavior (Bot et al. 2001).

The FTIR spectrum is a powerful tool for the determination of functional groups in the polysaccharide extracts from *S. commune* (Fig. 8). The broad band at 3384.09 cm^{-1} was a characteristic of O−H stretching in hydrogen bonds. The peak at 2887.79 cm^{-1} was assigned by C−H stretching vibrations, and the peak at 1651.90 cm^{-1} related to C−C stretching. The peak at about 1029 cm^{-1} referred to the C−O linkage. It was observed that the peaks at 1431.67, 1372.34, and 1252.28 cm^{-1} represented C−O linkages that these peaks were dependent on the position of the C–O band in the SPG structure. In addition, the band at 929 cm^{-1} and 850 cm^{-1} was assigned to the C—H variable angle vibration of β-pyranoside. Thus, SPG had a β-glycosidic band and pyranose ring (Limin et al. 2013).

The ^{1}H–NMR spectrum of SPG was shown in Fig. 9. As shown in Fig. 9, a large peak was also observed at 2.55 ppm due to DMSO, and two anomeric sugar signals at 4.52 ppm and 4.30 ppm were detected, which represented the β-D-1,3-linked glucose and β-D-1,6-linked glucose, respectively.

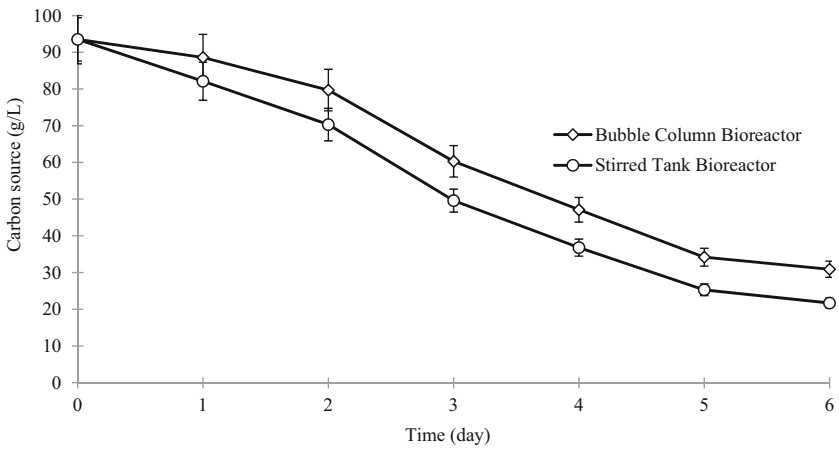

Fig. 6 Consumption of carbon source by *S. commune* IBRC-M 30213 in the bioreactors

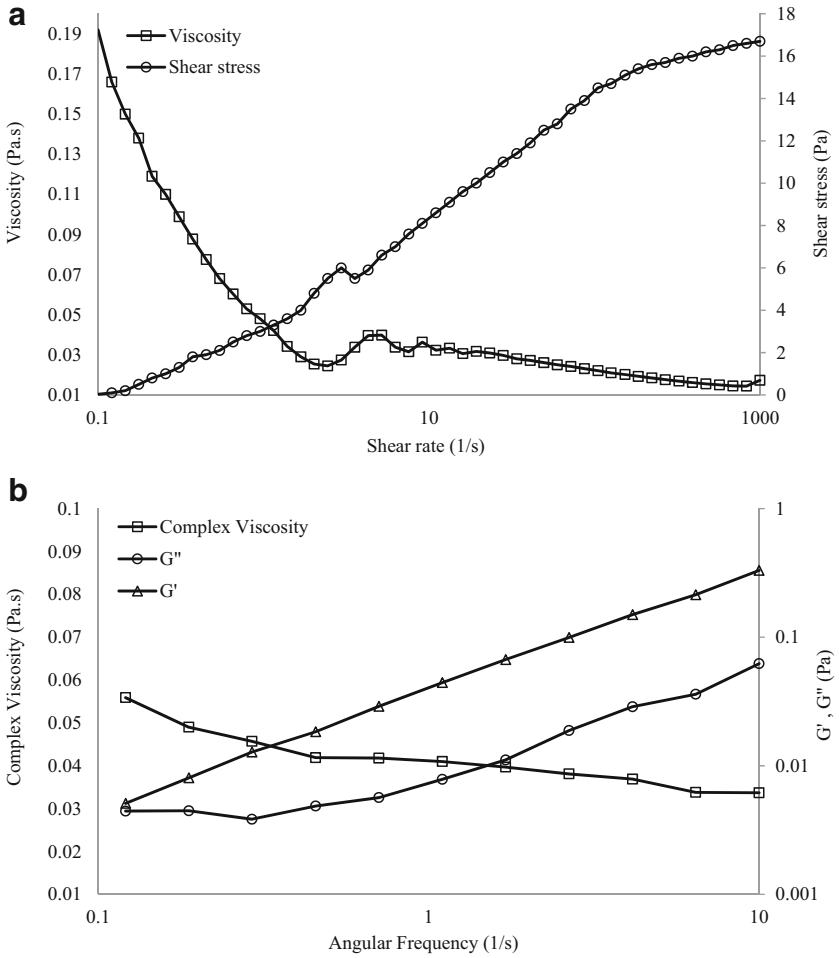

Fig. 7 **a** Shear stress and viscosity graphs of SPG with 3 g/L concentration. **b** Change curves of storage modulus (G′), loss modulus (G″) and complex viscosity with frequency

These two peaks demonstrated that the highest proportion of constituent units in the structure of SPG was glucose. In comparison, other peaks of carbohydrate could be seen in the 2.7–4 ppm region for ^{1}H (Sutivisedsak et al. 2013b; Jamshidian et al. 2016). Therefore, the signals at 3.45 ppm and 3.48 ppm were assigned to CH_2.

GPG is a convenient method to determine the molecular weight of SPG. The weight-average molecular weight of SPG obtained by submerged cultivation of *S. commune* IBRC-M 30213 was calculated to be 37×10^4 g/mol and a polydispersity index of 1.53, while commercial SPG has molecular weight 390 of 6×10^6 g/mol (Rau 2005). Further, SPG with

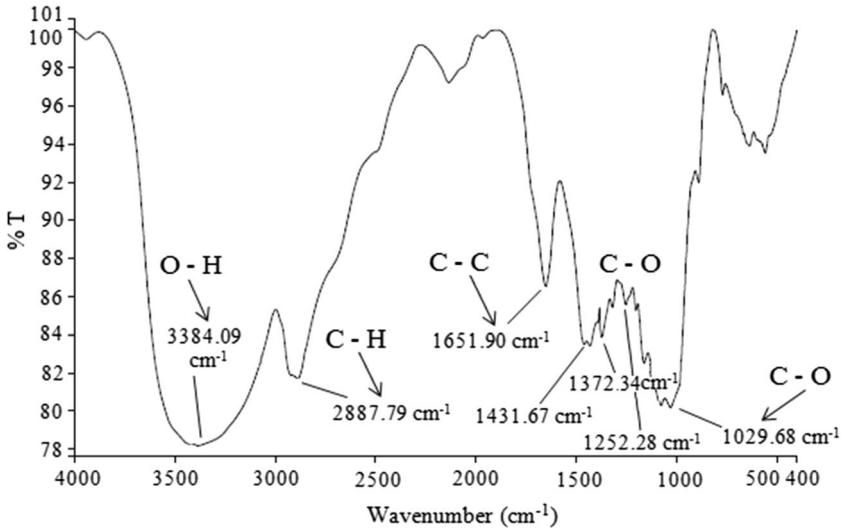

Fig. 8 Fourier transform infrared spectroscopy (FTIR) spectrum of the produced SPG

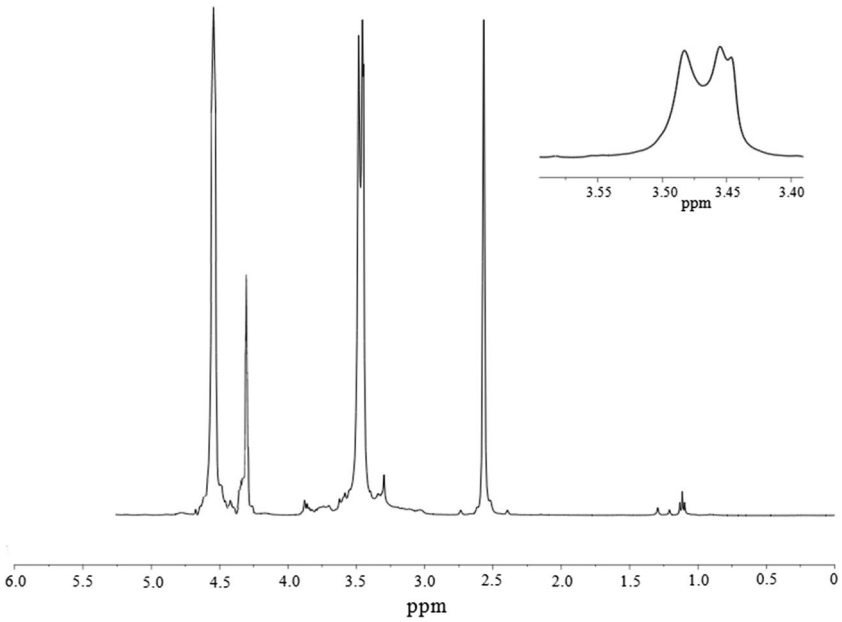

Fig. 9 The ^{1}H–NMR spectrum of the produced SPG

Table 6 Monosaccharide building blocks of SPG

Sample	Sugar component	(%)
SPG produced by *S. commune* IBRC-M 30213	Glucose	78
	Galactose	10
	Mannose	7
	Arabinose	3.5
	Xylose	1.4

molecular weight of 1.4×10^6 g/mol (Jamshidian et al. 2016) and 1.3×10^7 g/mol (Sutivisedsak et al. 2013c) was produced in other studies. Consequently, the type of isolated strains and fermentation conditions affected the molecular weight of the SPG produced.

The precipitated SPG was hydrolyzed for determination of monosaccharide composition using GC/MS. The monosaccharide composition of the extracellular biopolymer produced by *S. commune* IBRC-M 30213 grown on sucrose and yeast extract is presented in Table 6. The SPG obtained in this study was composed of glucose, galactose, mannose, arabinose, and xylose as well as commercial SPG. This polysaccharide was

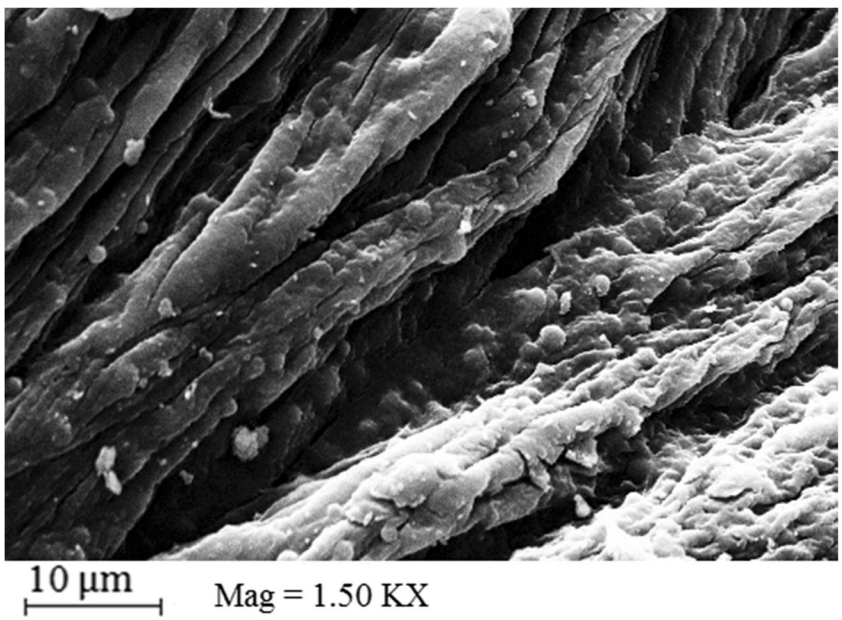

Fig. 10 Scanning electron microscopy (SEM) image of the produced SPG

composed mainly of glucose, containing a low percentage of xylose. Similarly, glucose was characterized as the major constituent unit of SPG studied by other researchers (Limin et al. 2013; Sutivisedsak et al. 2013a; Jamshidian et al. 2016). The composition of other polysaccharides produced by fungal strains was different; however, the major building block might be the same. Fructose was the major building block of polysaccharide produced by *Ganoderma tsugae* (Tseng et al. 2005). *Pleurotus pulmonarius* produced an exopolysaccharide containing mannose as the major building block (Smiderle et al. 2012). Similar to this study, *Volvariella volvacea* produced a polysaccharide that was composed of glucose in the highest proportion (Diamantopoulou et al. 2016).

SEM image shows that, when SPG was extracted by ethanol 95%, it adopted a triple helix structure (Fig. 10). Indeed, intertwined strands in this micrograph and irregular composition were representation of a triple helix structure.

Conclusions

This study was conducted using various parameters to enhance SPG production and reduce biomass in native fungal *S. commune* isolated from the forests of northern Iran. The SPG was found to be produced using different carbon and nitrogen sources. The newly isolated strain *S. commune* IBRC-M 30213 produced a maximum SPG of 9.97 g/L and a minimum biomass of 35.18 g/L under optimal conditions, which were deteremined using the CCD method as 93.47 g/L sucrose, 1.87 g/L yeast extract, 7.68% inoculum size, 9.07 g/L CMC and 0.13% oleic acid. Further, the production of SPG was studied in stirred tank and bubble column bioreactors. The results showed that production was greater in the stirred tank bioreactor since agitator and baffle led to better mixing of the culture medium, and prevented excessive growth of the fungal pellets. Furthermore, rheometery, FTIR, NMR, SEM and GPC characterizations showed that the produced SPG was similar to commercial SPG.

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