

Production optimization, characterization and gene expression of pullulan from a new strain of *Aureobasidium pullulans*

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ABSTRACT

In this study, a new yeast-like fungal was obtained from leaf surfaces collected from Kheyroodkenar forest, Mazandaran, Iran. The properties of this strain, such as morphology, DNA molecular, and product showed that it is related to *Aureobasidium pullulans* family and named *A. pullulans* MG271838. The pullulan production conditions by this strain were optimized using a Box-Behnken design. The results showed that the optimum production yield (37.55 ± 0.45 g/l) was obtained in pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v. The pullulan had a concentration-dependent flow behavior, amorphous structure based on XRD pattern and high thermal stability (decomposition temperature of 300 °C). Also, the chemical structure of pullulan was confirmed by FTIR spectroscopy. In addition, there was a direct relationship between pullulan yield and the gene expression of fks, pgm and ugp as the most important genes in pullulan production.

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1. Introduction

Aureobasidium pullulans is an important saprophytic yeast like fungus that in recent classification, considered as a branch of *Ascomycete* and *Dothideaceae* family [1,2]. This polymorphic fungus, with yellow, pink or light brown colors which ultimately change to black (due to production of melanin at chlamydospore production step), usually found in various habitats, such as phyllosphere of plant especially fruits and leaves [3–5], painted and plastic surfaces [6], bathroom surfaces [2] and human skin [7]. This fungus able to produce different enzymes such as amylases, xylanases, pectinases, β -glucuronidase, β -glucosidase, laccase and also valuable polymers such as polyalamic acid, and pullulan [3,8–10].

Pullulan, as an exopolysaccharide (EPS), is synthesized in the cell wall membrane and then exuded out to the cell surface to form slimy layer. This polymer is a linear glucan with maltotriose (a trisaccharide formed from three glucoses linked by (1 → 4) linkages) repeating units connected by (1 → 6) linkages [11,12]. Therefore, it can be stated that this polysaccharide is composed of Alpha-D-glucopyranosyl units which are linked either (1 → 4) or (1 → 6) linkages. The regular alternation of (1 → 4) and (1 → 6) bonds led to creation of specific properties such as adhesive ability, capability to form fibers and strong oxygen impermeable films [13,14], good water solubility, biocompatibility and biosafety [15]. Because of the good features, pullulan and its derivatives have various applications in food, chemical, pharmaceutical,

photographic, lithographic and electronic industries [16]. Also, genetic studies have shown that α -phosphoglucose mutase (PGM, EC 5.4.2.2), UDP-glucose pyrophosphorylase (UGP, EC 2.7.7.9) and glucosyltransferase (FKS, EC 2.4.1.34) are three essential enzymes involved in pullulan biosynthesis [17,18]. Although the genes encoding these enzymes were identified, the complete mechanism of pullulan biosynthesis has been remained unclear [19].

It is certain that the production yield and the properties (such as flow behavior and emulsifying activity) of pullulan produced by different strains of *A. pullulans* can be different. Therefore, the optimization of production process conditions (pH, concentrations of carbon and nitrogen sources) and also, the properties investigation of the produced pullulan from different strains are necessary. Response Surface Methodology (RSM) is one of the statistical techniques used for modeling and analysis of the effective factors, as independent variables, on the yield of ultimate products as dependent variables [20–23].

So far, pullulan production from *A. pullulans* has been investigated in several studies [24–30]. However, the production of this biopolymer from new strains of *A. pullulans* is very attractive due to its importance and wide application in various industries. Therefore, in this study, we isolated a high yielding and non-melanin producing strain of *A. pullulans* from the Kheyroodkenar forest in Iran and optimized the effect of the process variable on the yield of pullulan production using Box-Behnken response surface design. Also, the relationship between increasing production yield and the expression of the most important genes (pgm: α -phosphoglucose mutase enzyme encoder, ugp: UDP-glucose pyrophosphorylase enzyme encoder and fks: glucosyltransferase enzyme encoder) involved in pullulan production

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was investigated. In final, some properties of produced pullulan by mentioned strain in optimum conditions such as flow behavior, emulsifying, structural and thermal properties were determined.

2. Materials and methods

2.1. Raw material and reagents

Ammonium hydrogen sulfide, sodium chloride, magnesium sulfate, potassium hydrogen phosphate, ferric sulfate, zinc sulfate, sucrose, fructose, glucose, galactose, xylose, mannose, yeast extract, malt extract, urea, sodium nitrite, sodium hydroxide, hydrochloric acid, sodium azide, agar and potato dextrose agar were bought from Merck Chemical Co. (Darmstadt, Germany). pepton was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Also, all other reagents and chemicals were of analytical grade.

2.2. Isolation of *A. pullulans*

A large number of leaf samples were collected randomly from Kheyroodkenar forest of Nowshahr in north of Iran. The leaves were first divided into small pieces and then were soaked in 25 ml distilled water for 3 days ($\sim 25^{\circ}\text{C}$). 0.1 ml of mentioned mixture was transmitted to 10 ml of the MSM (Minimal Salt Medium) that was containing; 1 g $(\text{NH}_4)_2\text{HPO}_4$; 0.5 g NaCl; 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 2 g K_2HPO_4 ; 0.01 g FeSO_4 ; 0.01 g MnSO_4 ; 0.01 g ZnSO_4 ; 10 g sucrose and 10 g chloramphenicol in 1 L of distilled water (pH 4.0; adjusted by HCl). After two days shaking at $\sim 25^{\circ}\text{C}$, about 20 μl of the supernatant containing yeast forms was spread on minimal salt agar medium. After 4 days, the independent colonies (20 colonies) were purified by repeating on the water agar medium. The fungi were maintained on PDA slants at $\sim 4^{\circ}\text{C}$ and were subcultured every two weeks [1].

2.3. Pullulan production by *A. pullulans* isolates

Two loops of yeast colonies were transmitted from PDA medium to 250 ml Erlenmeyer flasks containing 100 ml of inoculum medium (3 g of yeast extract; 5 g of peptone; 3 g of malt extract and 10 g sucrose per liter of distilled water in pH of 5.5). Then, the culture was incubated for 3 days ($\sim 25^{\circ}\text{C}$, 200 rpm) in a shaking incubator (Stuart Orbital Incubator S150, Staffordshire, UK). In the next step, 5 ml (5%v/v) of inoculum medium was transferred to 250 ml Erlenmeyer flasks containing 100 ml synthetic medium (50 g sucrose; 2.0 g yeast extract; 5.0 g K_2HPO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0 g NaCl and 0.01 g FeSO_4 per liter of deionized water in pH of 6.5) and incubated (28°C , 200 rpm). After seven days, the biomass was first separated by centrifuge (Sigma 8KS, Osterode am Harz, Germany) at 15000 $\times g$ for 20 min at 4°C , and then for precipitation of EPSs, the cold ethanol was added to the cell free culture broth in ratio of 2:1 v/v and was placed in a refrigerator (4°C , 12 h). In the next step, the supernatant was separated from mixture by centrifugation (15,000 $\times g$, 4°C , 20 min) and then for purification, the obtained precipitate was dissolved in distilled water and precipitated by ethanol (ratio of 2:1 v/v), low temperature (4°C , 12 h) and centrifugation (15,000 $\times g$, 4°C , 20 min), respectively. This purification process was repeated twice. In final, the purified precipitate was dried at 50°C until reach to constant weight and the production yield of different isolates was reported based on gram pullulan per liter initial solution (g/l) [26,31]. Among these 20 strains, the isolate with the highest production yield was selected for further experiments.

2.4. Morphological analysis

Morphological properties of selected strain were conducted by an optical microscope (Olympus, BH2, Japan). For this purpose, the *A. pullulans* isolate was inoculated on the PDA plate and placed in an

incubator ($\sim 25^{\circ}\text{C}$) for 6 days. Afterward, the obtained plate was used for morphological analysis.

2.5. Molecular characterization

Fresh cells of the selected strain were harvested from the PDA medium by centrifugation (6000 $\times g$, 4°C , 10 min). After adding cetyl trimethylammonium bromid (CTAB) buffer and 5% sodium dodecyl sulfate (SDS), they were placed in incubator (65°C , 2 h). Then, the phenol and chloroform solutions were added in equal amounts and the sample was washed with cold ethanol. In the next step, teris-EDTA (TE) buffer (50 μl) was added and total genomic DNA was extracted. To assessment of phylogenetic relationships among the selected strain and the other identified strains, amplification and sequencing of internal transcribed spacer (ITS) were accomplished using the primers IT1 and IT4. Eventually, the gene sequences and the similarity of gene sequences were analyzed by CLUSTAL X program and Gen-Bank BLASTN, respectively [32,33].

2.6. Effect of different carbon and nitrogen sources on the production of pullulan

After selection of the best strain, one factor at a time design was used to select the best source of carbon and nitrogen for reach to maximum production yield [5,29,34]. For this purpose, the carbon (glucose, sucrose, fructose, mannose, galactose and xylose) and nitrogen (peptone, yeast extract, urea, ammonium sulfate and sodium nitrite) sources were variable, while other factors were constant (for carbon: 50 g carbon source (variable); 2.0 g yeast extract; 5.0 g K_2HPO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0 g NaCl and 0.01 g FeSO_4 per liter of deionized water in pH of 6.5 and for nitrogen: 2.0 g nitrogen source (variable); 50 g sucrose; 5.0 g K_2HPO_4 ; 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.0 g NaCl and 0.01 g FeSO_4 per liter of deionized water in pH of 6.5). Eventually, the carbon and nitrogen sources with maximum production yield were selected for pullulan production optimization.

2.7. Optimization of pullulan production and statistical analysis

Box–Behnken design is a widely used design for optimization of various factors. Its main advantage is the simultaneous investigation of individual and interactive effects of process variables on the response by a low number of experiments [35]. Therefore, after selection of the best carbon (sucrose) and nitrogen (yeast extract) sources, this design with three variables (pH, sucrose concentration and yeast extract concentration) in three levels was used to achieve maximum production yield, when the independent variables were “in range”. (Table 1). It should also be noted that the production process was carried out as stated in Section 2.3. All statistical analysis and graphics were conducted by Design Expert (version 7.0.0, Stat-Ease Inc., Minneapolis, MN, USA) and Excel (version 2010, Microsoft Corporation, Redmond, WA, USA) softwares.

2.8. Gene expression evaluation by Real Time PCR

2.8.1. RNA extraction

In this part, the gene expression was evaluated according to the described method by Ju et al. [36], with minor modifications. For this purpose, the fresh cells of selected strain were first harvested from the PDA medium in the exponential phase (7 days) by centrifugation (6000 $\times g$, 4°C , 10 min) and then twice washed with deionized water. For RNA extraction by Thermo Kit, CTAB buffer (100 μl), 5% SDS buffer (100 μl) and beta-mercaptoethanol (20 μl) were added to the sample under a chemical hood and incubated at 65°C for 10 min. Then, phenol-chloroform-isoamyl was added and the sample was centrifuged (12,000 $\times g$, 4°C , 10 min). The supernatant was transferred to a new tube and 300 μl of chloroform solution was added and centrifuged at mentioned

Table 1

Box-Behnken design matrix with independent variables (pH, sucrose concentration (SC) and yeast extract concentration (YEC)) and their levels, experimental yield (EY) and predicted yield (PY, g/l) of pullulan.

Factors	Unit	Actual levels			
		–1	0	+1	
(X ₁) pH	–	4.0	6.5	9.0	
(X ₂) SC	%w/v	1.0	3.5	6.0	
(X ₃) YEC	%w/v	0.20	0.45	0.70	
Run	pH	SC	YEC	EY	PY
1	4.0	1.0	0.45	6.60	5.77
2	9.0	1.0	0.45	6.00	5.55
3	4.0	6.0	0.45	13.10	13.55
4	9.0	6.0	0.45	16.60	17.42
5	4.0	3.5	0.20	13.70	14.56
6	9.0	3.5	0.20	15.10	15.58
7	4.0	3.5	0.70	5.90	5.41
8	9.0	3.5	0.70	8.90	8.03
9	6.5	1.0	0.20	13.50	13.46
10	6.5	6.0	0.20	39.80	38.48
11	6.5	1.0	0.70	19.00	20.31
12	6.5	6.0	0.70	14.90	14.93
13	6.5	3.5	0.45	16.00	14.26
14	6.5	3.5	0.45	15.20	14.26
15	6.5	3.5	0.45	11.60	14.26

conditions. Afterward, the supernatant was again added to the new tube and the cold isopropanol (1 ml) was poured inside it. The sample was placed at about –20 °C for 1 h and then centrifuged (12,000 × g, 4 °C, 10 min). The cold ethanol (70%) was following added to supernatant and the centrifuge step was repeated in the same conditions (12,000 × g, 4 °C, 10 min). The sample was dried in a plate underneath the hood and 30 µl of Diethyl pyrocarbonate water (DEPC water) was added to prevent degradation by RNases. Nanodrape (2000c, Thermo Scientific) was used to measure the sample concentration.

In the next stage, to purify the extracted RNA, 1.5 µl DNase I enzyme (Fermentase, USA) and 1.5 µl restriction enzyme buffer was added to the 3 µg of RNA and then its volume was reached to 15 µl by DEPC water and placed at ~ 37 °C for 30 min. Then, in order to disable the DNase enzyme, 2 µl of EDTA (50 mM) buffer was added and placed at 70 °C for 15 min. Finally, after nanodrop quantification, the samples were kept at about –80 °C until next experiment.

2.8.2. Synthesis of complementary DNA (cDNA)

For synthesis of cDNA by Termo Fast Kit, 0.5 µl of Oligo (dT) primer, 0.2 µl of RH (Random Hexamer) were added to the 2 µg of RNA and then its volume was reached to 10 µl by DEPC water and placed at ~ 60 °C for 10 min. After that, reverse transcriptase enzyme and RI (RNase inhibitor) were added and placed at ~ 42 °C for 90 min.

2.8.3. Gene expression evaluation by real-time PCR

The real-time PCR (Step One, Applied Biosystem) was used to measure the transcription levels of pgm 1, ugp, and fks genes (PGM, UGP, and FKS enzymes- encoding genes, respectively). The sequences of the primers used for the amplification of the genes were designed by OLIGO7 software and listed in Table 2.

The amplification of cDNA was performed using 0.5 µl of SYBR green master mix (2×) (Takara, Japan), 0.25 µl of forward primers, 0.25 µl of reverse primers, 2 µg of cDNA and DEPC water (up to 10 µl). The amplification reaction was under the following conditions: initial denaturation at 94 °C for 120 s followed by 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and elongation step at 72 °C for 20 s. The experiments were performed in three replications in a 96-well plate (Bio-Rad). The act1 gene was used (as the reference gene) to normalize all samples. The relative expression level of the target genes was calculated using a relative standard curve.

Table 2

Genes, primers, Tm, and the length of amplified PCR products for transcriptional analysis by qRT-PCR.

Gene	Primer	Sequences, 5' → 3'	Tm (°C)	PCR product (bp)
act1	F(ACTIN1)	GTTGTCCCATCTACGAAGTTTC	59.3	163
	R(ACTIN1)	GCTTCTCTTGATGTCACGAACG	59.7	
pgm1	F(PGM1)	CCGCTTCTCATATCATCCGCATC	59.2	175
	R(PGM1)	GTGAGGGTCTTGAGGTGTCG	59.6	
ugp	F(UGP)	TCATCCCCAACGGCAAGTCG	59.8	172
	R(UGP)	AGCATCAAGTCGGAGCAGGTC	59.6	
fks	F(FKS)	AGAAGACCGAGAAGGACACTGC	59.5	126
	R(FKS)	TCTGAGAACGGAGCGATGCC	59.7	

2.9. Flow behavior of pullulan solution

The flow behavior of the aqueous solutions of commercial and produced pullulan in four concentrations of 2, 4, 6 and 8%w/v was investigated by a rotational programmable viscometer (LVDV-II Pro, Brookfield Engineering Inc., USA) with a LV spindle at 25 °C. For this intention, the shear rate was programmed from 1 to 120 s^{–1} and the obtained data was fitted to the Power law model according to (Eq. (1)):

$$\tau = k \cdot \dot{\gamma}^n \quad (1)$$

where τ is the shear stress (Pa), $\dot{\gamma}$ the shear rate (s^{–1}), k the flow consistency index (Pa sⁿ), and n is the flow behavior index.

2.10. Emulsifying properties activity (EA) and emulsion stability (ES)

In this section, emulsifying activity (EA) and emulsion stability (ES) of pullulan solution was measured by adding 5 ml of sunflower oil to 5 ml pullulan solution (1.5%w/v) containing 0.02% bactericide (sodium azide). Thereafter, the samples were mixed with ultra-turax at 10000 × g for 4 min and centrifuged at 4000 × g for 5 min. The EA was computed as follows [37,38]:

$$EA (\%) = \frac{\text{Volume of the emulsion phase}}{\text{Total volume of system}} \times 100 \quad (2)$$

The emulsion stability of pullulan solutions was determined after 1 and 30 days at 4 and 25 °C. For this purpose, the produced emulsions were centrifuged at 4000 × g for 4 min and then, the ES was computed as follows [39]:

$$ES (\%) = \frac{\text{The remaining emulsified layer volume}}{\text{The initial emulsified layer volume}} \times 100 \quad (3)$$

2.11. Fourier transform infrared spectroscopy (FTIR)

FT-IR spectrum of pullulan (in the range of 4000–450 cm^{–1}) was drawn by Perkin Elmer FTIR spectrometer (Perkin Elmer co., MA, USA) with KBr pellet method and the result was compared with the obtained spectrum from commercial pullulan (TCI chemicals, Tokyo).

2.12. X-ray diffraction (XRD) analysis

The XRD pattern of the pullulan sample was obtained by an X-ray diffractometer (EQuniox 3000, Inel, France). For this purpose, the pullulan powder was irradiated with Cu-K α radiation, 2 θ of 5°–100° and scanning speed of 0.01°/s.

2.13. Thermo gravimetric analysis (TGA)

TGA was performed by a thermo gravimetric analyzer (Perkin-Elmer, Pyris) with an alumina cell. In this section, the sample heating,

under nitrogen flow of 100 ml/min, was done in range of 30–600 °C with ramping rate of 10 °C/min.

3. Results and discussion

3.1. Selection of the best pullulan producer

Twenty strains suspected to be *A. pullulans* were isolated from the collected samples, according to the phenotypic properties and ability of exopolysaccharide (EPS) production. Table 3 shows that the EPS production of strains was varied from 4.9 to 29.1 g/l and the highest value of EPS was produced by M11 strain. The strain colony had a pale pink color with slimy surface, which is probably due to hyaline blastospores [40]. Also, a number of specified forms were observed: Branched septate hyphae, endoconidium, conidium, arthroconidium and chlamyospore (Fig. 1A). All these phenotypic properties of the isolate are identical to those of *A. pullulans*.

For confirm of this subject, the molecular analysis was also done. Phylogenetic tree created by internal transcribed spacer (ITS) in Fig. 1B showed that strain M11 (with access number of MG271838), as a new strain, is phylogenetically similar to other *A. pullulans* strains and belongs to this family.

3.2. Selection of the best carbon and nitrogen sources

Carbon and nitrogen sources are the most important factors in the cell metabolism and pullulan synthesis. For selection of the best carbon source, sucrose, glucose, fructose, galactose, mannose and xylose were used in concentration of 50 g/l, while other factors were constant. The obtained results showed that although the pullulan production by *A. pullulans* MG271838 was high for glucose, the maximum production of the exopolysaccharide was due to sucrose (Fig. 2A), which is in line with many studies [41–43].

For selection of the nitrogen source, ammonium sulfate, peptone, yeast extract, urea and sodium nitrite in concentration of 2.0 g/l were used as variable and other factors were considered constant. Fig. 2B shows that the highest production yield of pullulan is relate to yeast extract. This result is in agreement with many reports [44–46].

3.3. Optimization and statistical analysis

After selection of sucrose and yeast extract as carbon and nitrogen sources, Box-Behnken response surface experimental design with

three variables (pH, sucrose concentration and yeast extract concentration) in three levels and three replications at the center point was used for optimization of pullulan production from *A. pullulans* MG271838.

The results related to production yield of runs were shown in Table 1. Optimization with mentioned design indicated that the maximum production was 38.57 g/l in pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v. For confirm, three experiment were done in optimum conditions and production yield was 37.55 ± 0.45 g/l, which was close to predicted value.

Analysis of variance results (ANOVA) were shown in Table 4. As can be seen, *p*-value of model is lower than 0.05 and lack-of-fit is insignificant, which show the model is highly significant and can predict the response [47]. Also, the determination coefficient was 98.03%, which shows only 1.97% of data is unexplained (98.03% of data are in agreement with model [48]. The predicted quadratic model for pullulan production yield is as follows:

$$\begin{aligned} \text{Production yield (\%)} = & 14.27 + 0.91X_1 + 4.91X_2 - 4.71X_3 - 7.30X_1^2 \\ & + 3.60X_2^2 + 3.93X_3^2 + 1.03X_1X_2 \\ & + 0.40X_1X_3 - 7.60X_2X_3 \end{aligned} \quad (4)$$

where X_i is a coded value (see to Table 1).

3.4. The effect of independent variables on the pullulan production yield

The pH factor has effect on the cellular morphology as well as the cellular metabolic activity. Therefore, this factor has an important role in the production of metabolic enzymes and thereby, pullulan production [18]. Fig. 3 showed that with increase in pH, the production yield of pullulan was first increased and then decreased. Optimum pH for production of pullulan is probably due to the type of *A. pullulans* strain and medium condition [13]. So, the various strains in different mediums may have different production yield. For instance, Sugumaran et al. [49] reported the optimum pH of 6.6 for pullulan production from the Asian palm.

The nitrogen source is a key factor in pullulan production and the many studies indicated that the best condition for production of this exopolysaccharide was created in its low concentration [50]. Our results also showed that the yield production of pullulan was decreased with increasing the yeast extract concentration (Fig. 3). Generally, increase in nitrogen source concentration leads to increasing biomass and decreasing product concentration which is in line the results reported by Singh et al. [25] and Sheng et al. [51].

Carbon source is another effective factor on the pullulan production by *A. pullulans*, which its concentration usually had a direct relationship with production yield [3]. Fig. 3 showed that by increasing sucrose concentration, production yield of pullulan was increased. This result was in line with reports of Göksungur et al. [45] and Chi et al. [52]. However, it should also be noted that the excessive increase in carbon source can lead to decreasing the production yield that is due to its osmotic pressure and the negative effect on the fermentation rate and thereby, decrease in pullulan synthesis [53].

3.5. Evaluation of gene expression

As mentioned earlier, *fks*, *pgm* and *ugp* genes are the most important in pullulan production, which their expression has a direct relationship with production of the exopolysaccharide [54]. In this section, the expression of mentioned genes in *A. pullulans* MG271838 separated from basic medium (pH of 6.5, sucrose concentration of 5%w/v and yeast extract concentration of 0.2%w/v), optimal medium (pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v) and the medium with the least production yield (pH of 4, sucrose concentration of 3.5%w/v and yeast extract concentration of 0.7%w/v) was compared. Fig. 4 showed that the expression of all three genes for optimum medium was higher than the medium with the

Table 3

EPS production for each isolate. M_{1–20} is a coded value for isolates obtained from leaves with ability of exopolysaccharide (EPS) production.

Isolate	Pullulan production yield (g/l)
M1	12.0
M2	17.1
M3	12.3
M4	8.9
M5	12.1
M6	15.4
M7	21.0
M8	9.5
M9	12.8
M10	8.5
M11	29.1
M12	19.1
M13	4.9
M14	25.1
M15	11.2
M16	7.8
M17	28.0
M18	12.7
M19	7.2
M20	16.0

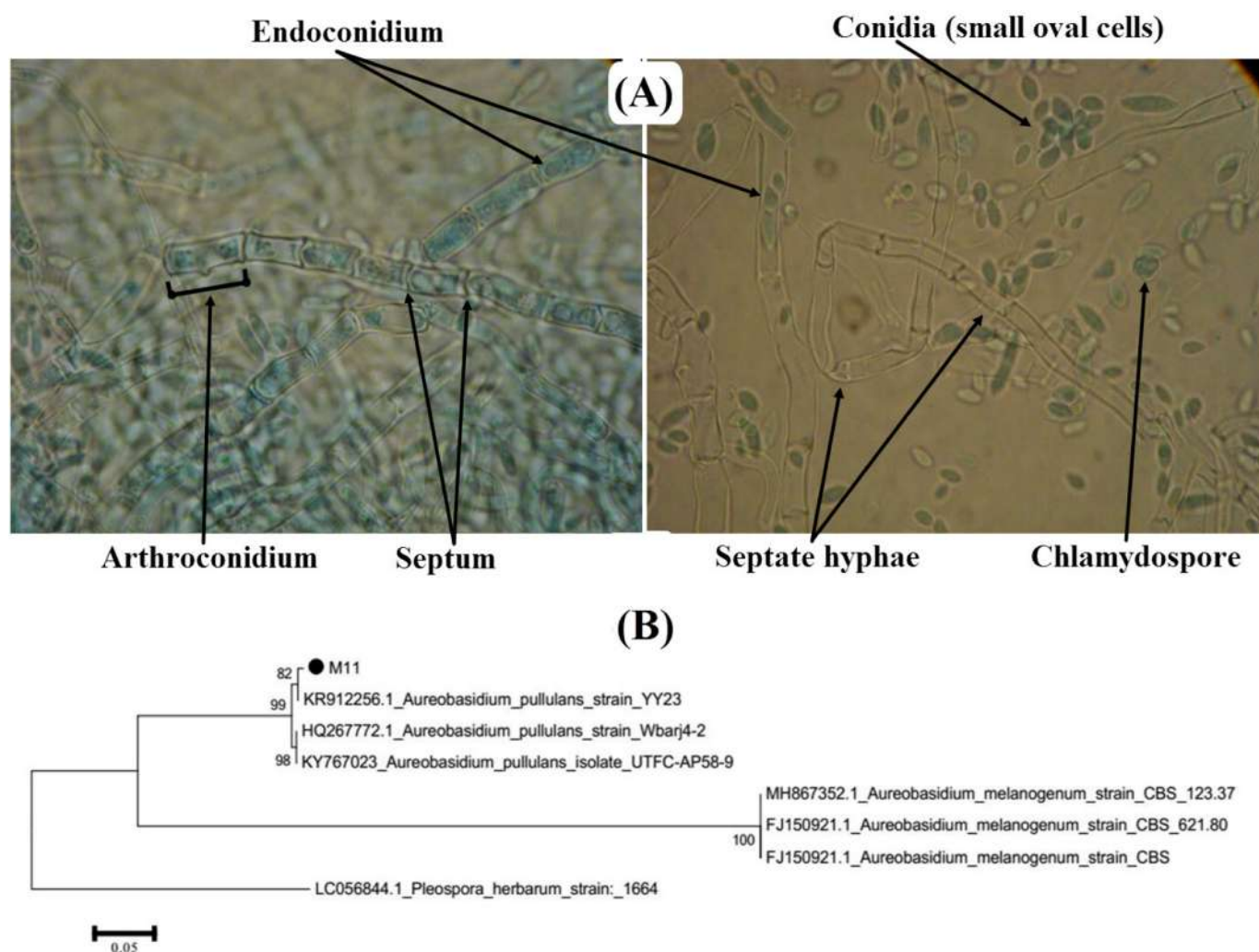


Fig. 1. (A) The obtained morphology of *A. pullulans* strain M11 (MG271838) by an optical microscope (Olympus, BH2, Japan) with 40× magnification; (B) the neighbor-joining tree of *A. pullulans* strain M11 (MG271838).

least yield, which confirmed higher production yield in optimum medium. In another study, Ju et al. [36] obtained a similar result for the transcriptional levels of *pgm* and *fks* genes.

3.6. Viscosity measurement

In this study, the flow behavior of the obtained and commercial pullulan solutions in different concentrations (2, 4, 6 and 8%w/v) was analyzed at room temperature (Fig. 5). As shown in Fig. 5, the apparent viscosity of pullulan solutions was increased by increasing concentration. However, pullulan solutions had the different flow behavior in various concentrations. As can be seen, pullulan solutions in concentrations of lower than 8%w/v had a Newtonian behavior ($n \approx 1$), while with increasing concentration to 8%w/v, the flow behavior was changed to pseudoplastic ($n < 1$). The pseudoplastic flow behavior of pullulan can be due to the separation of exopolysaccharides from each other, alignment of them with the shear field and thereby decrease in viscosity up to an approximately constant value [55]. The similar change in flow behavior with increasing concentration was reported for pullulan and other polysaccharides [53,56,57].

3.7. Emulsifying properties

In this part, emulsifying properties of pullulan produced from *A. pullulans* MG271838 under optimum condition (pH of 6.76, sucrose

concentration of 6%w/v and yeast extract concentration of 0.2%w/v) was evaluated. The emulsifying activity of pullulan was 58% at 25 °C. Also, the stability of emulsions was 88.4 and 81% after 1 day and 88 and 79.5% after 30 days (in 4 and 25 °C, respectively). Therefore, it can be said that the emulsions were more stable in low temperature. These results were in agreement with obtained data from other studies [58,59]. The previous studies indicated that the emulsifying properties of pullulan is probably attributed to the presence of very low amounts of peptide moieties of culture medium in the obtained pullulan and also the viscosity of this polysaccharide solution that can disperse the oil droplets [60].

3.8. Fourier transform infrared spectroscopy

The FTIR spectrum of pullulan produced from *A. pullulans* MG271838 in optimum conditions (pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v) was shown in Fig. 6A. Also, the obtained pullulan spectrum was compared with commercial sample in Table 5. The absorption region between 3300 and 3500 cm^{-1} is related to the hydroxyl groups of sugar units that are widely observed in the polysaccharide structure. The peak between 2800 and 3000 cm^{-1} is due to the symmetric and asymmetric bonds of C—H in the target sample [24]. The absorption peaks of 1617.31, 1369.14, 1156.83, 1016.62 and 925.53 cm^{-1} are attributed to O—C—O, C—OH, C—O—C, C—O and

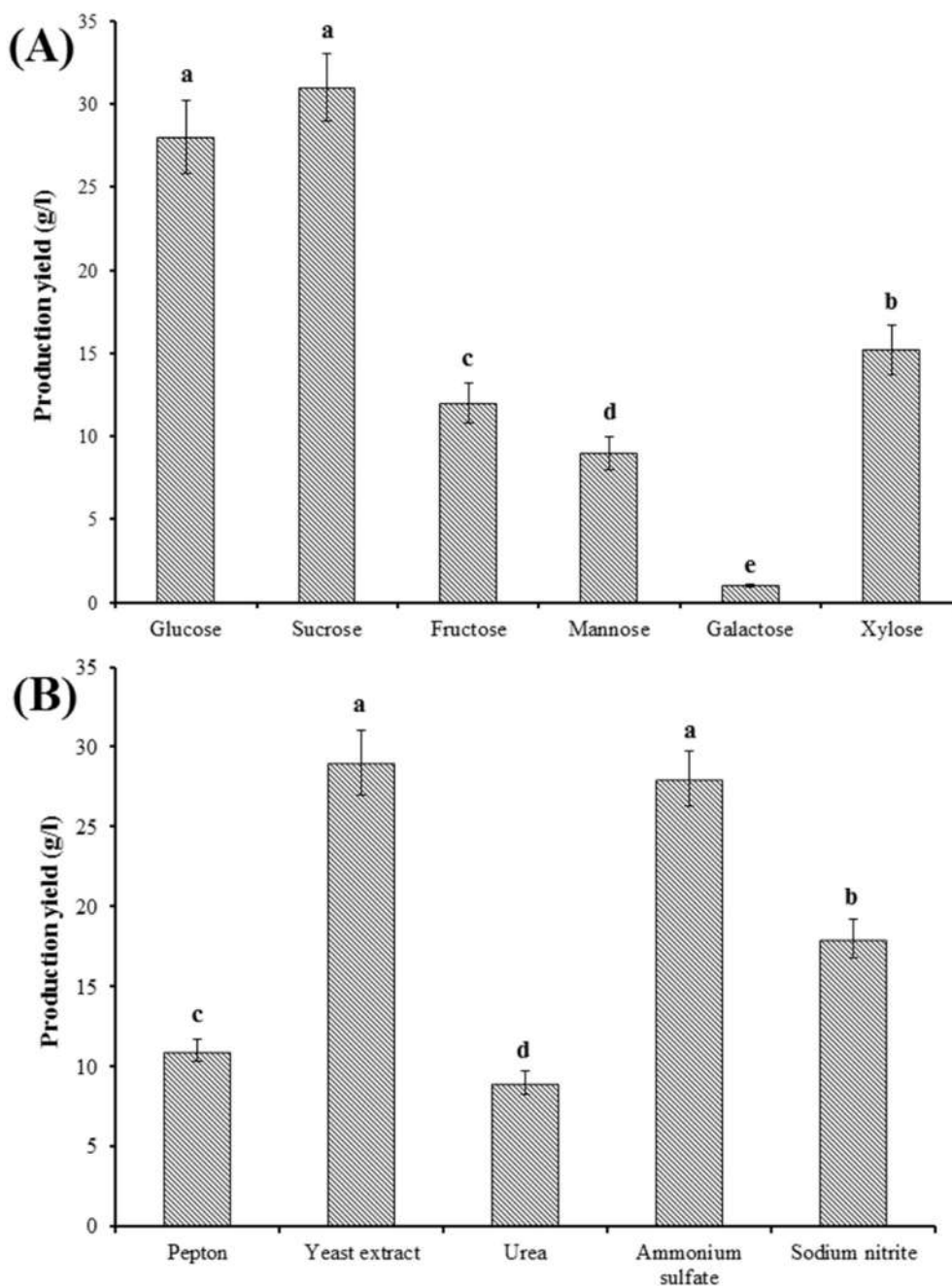


Fig. 2. Effect of different carbon (glucose, sucrose, fructose, mannose, galactose and xylose) and nitrogen (peptone, yeast extract, urea, ammonium sulfate and sodium nitrite) sources on pullulan production by *A. pullulans* MG271838: (A) for selection of the best carbon source: 50 g/L carbon source (variable) and 2.0 g/L yeast extract; (B) for selection of the best nitrogen source: 2.0 g/L nitrogen source (variable) and 50 g/L sucrose. Other components were constant: 5.0 g K_2HPO_4 ; 0.2 g $MgSO_4 \cdot 7H_2O$; 1.0 g NaCl and 0.01 g $FeSO_4$ per liter of deionized water in pH of 6.5. ^{a-c}Different letters in each figure indicate significant differences ($p < 0.05$).

glucopyranoside bonds, respectively [26,61]. Therefore, FTIR spectroscopy confirmed the chemical structure of pullulan.

3.9. XRD analysis

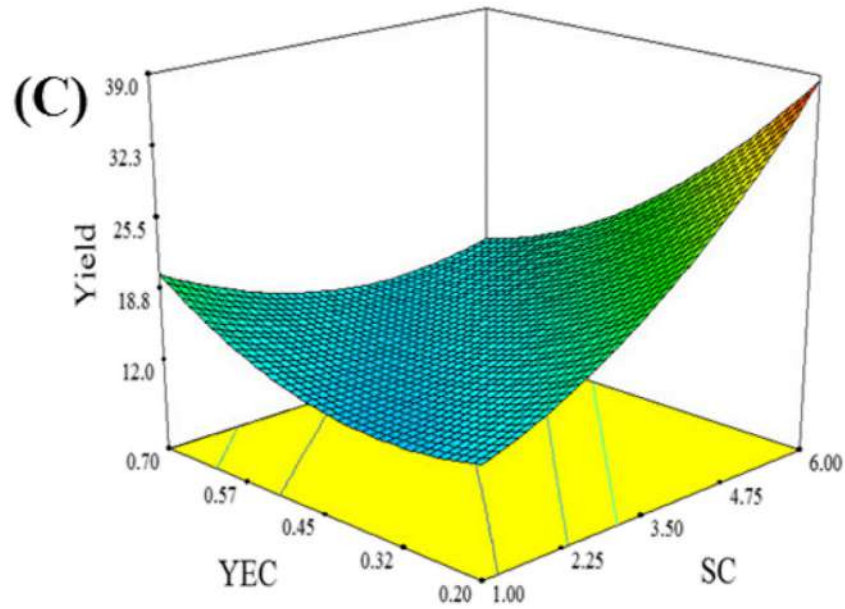
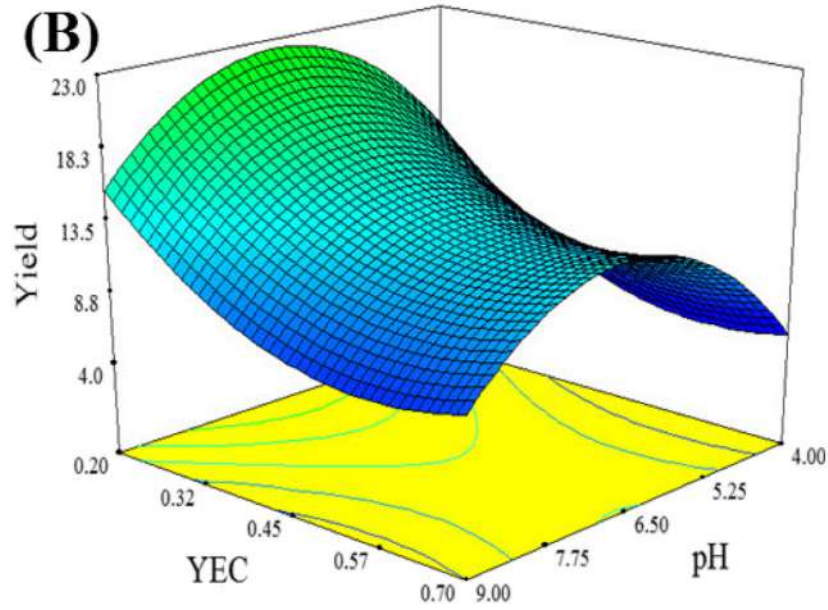
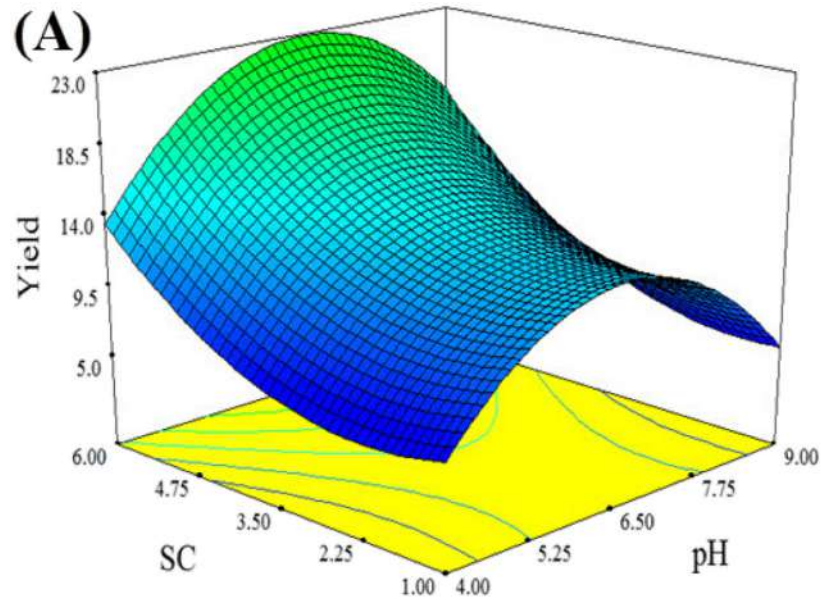
The XRD pattern of obtained pullulan under optimal production conditions (pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v) is present in Fig. 6B. As can be seen, a broad peak is exist at $2\theta = 20^\circ$ and so, the pullulan has an amorphous

structure. This observation is similar to the reported results by Trovatti et al. [62] and Sugumaran et al. [49].

3.10. Thermo gravimetric analysis

The thermal stability of pullulan is a key factor in its applications. In this step, the thermal properties of the exopolysaccharide were evaluated by TGA. Fig. 6C showed that the thermal decomposition of pullulan extracted under optimal production conditions was started at about 300 °C. This result was close to data reported by

Fig. 3. 3-D response surface showing the effect of pH, sucrose concentration (SC: %w/v) and yeast extract concentration (YEC: %w/v) on the pullulan production yield: (A) effect of SC and pH on the production yield with YEC of 0.45%w/v; (B) effect of YEC and pH on the production yield with SC of 3.5%w/v; (C) effect of YEC and SC on the production yield with pH of 6.5.



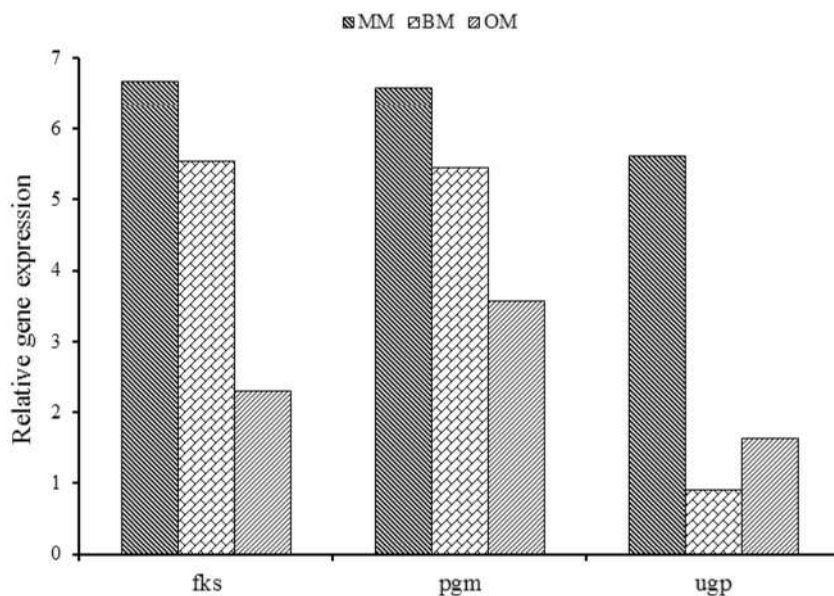


Fig. 4. The expression of fks, pgm and ugp genes of *A. pullulans* MC271838 separated from the medium with minimum production yield of pullulan (MM: pH of 4, sucrose concentration of 3.5%w/v and yeast extract concentration of 0.7%w/v), basic medium (BM: pH of 6.5, sucrose concentration of 5%w/v and yeast extract concentration of 0.2%w/v) and optimal medium (OM: pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v).

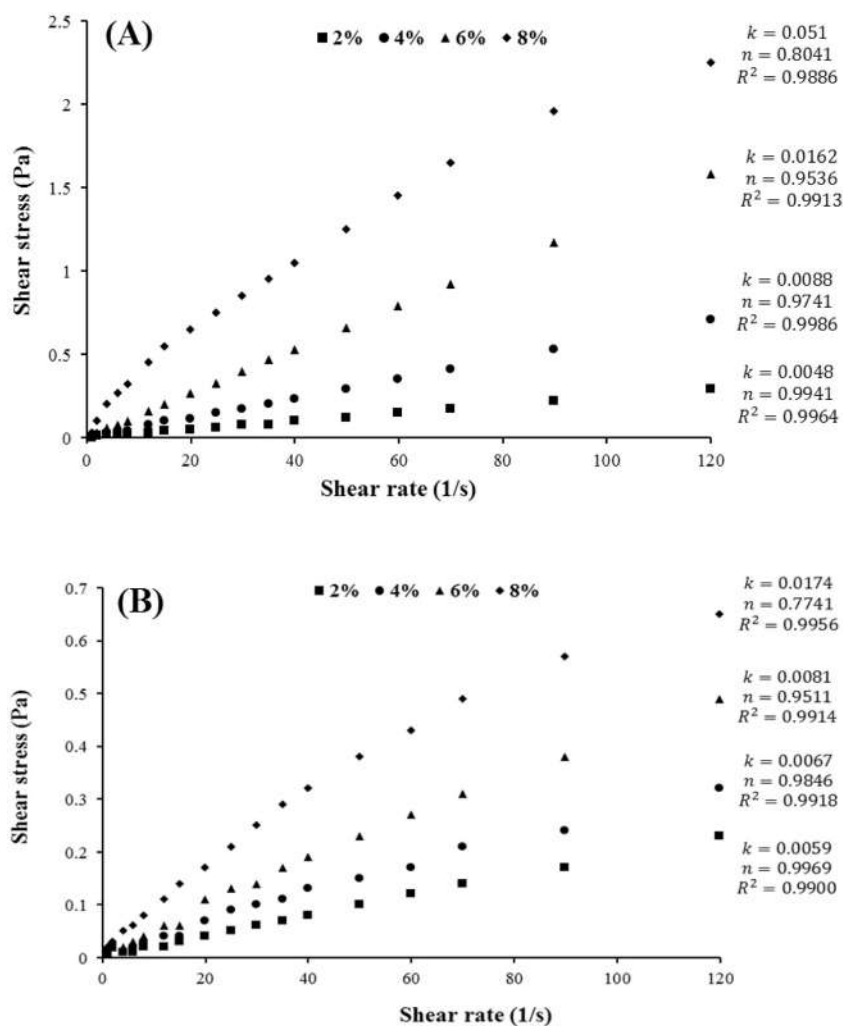


Fig. 5. The flow behavior of the solutions of (A) obtained pullulan under optimum condition (pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v) and (B) commercial pullulan in concentrations of 2, 4, 6 and 8%w/v. n and k for various concentrations are flow consistency index and flow behavior index, respectively.

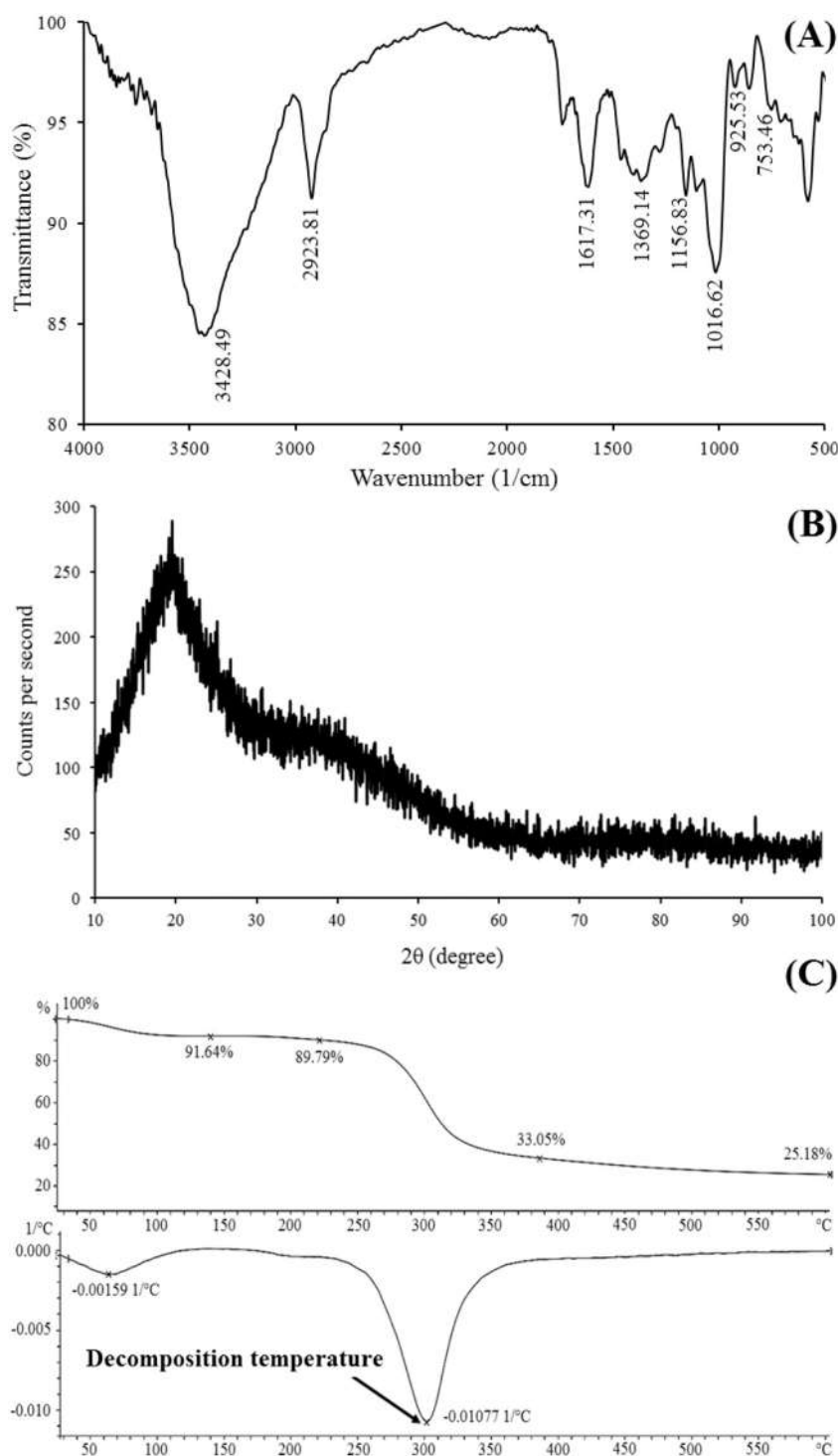


Fig. 6. (A) FTIR spectrum of obtained pullulan; (B) XRD pattern of obtained pullulan; (C) thermo gravimetric analysis of obtained pullulan. All the figures are related to the pullulan obtained in optimal conditions: pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v.

Sugumaran et al. [49] and Oğuzhan and Yanglar [63]. These researchers reported that the thermal decomposition of pullulan was started at 250 °C and 250–280 °C, respectively.

4. Conclusion

In this study, 20 isolates with the ability to produce pullulan were obtained from many leaf samples and strain with maximum production yield was selected. The molecular analysis showed that the selected isolate was a new strain of *Aureobasidium pullulans*

MG271838. In the next step, the medium conditions for pullulan production by the selected strain were optimized by Box-Behnken response surface experimental design with three variables in three levels. The results indicated that the maximum production yield (37.55 ± 0.45 g/l) was obtained in pH of 6.76, sucrose concentration of 6%w/v and yeast extract concentration of 0.2%w/v. XRD pattern and thermogravimetric analysis indicated that the obtained pullulan had an amorphous structure and high thermal stability, respectively. Also, FTIR spectroscopy confirmed the chemical structure of pullulan in the obtained supernatant.

Table 4

The results of analysis of variance (ANOVA) for regression model of pullulan production yield.

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	901.67	9	100.19	27.58	0.001
Linear	339.17	3	113.07	31.12	0.001
Square	326.62	3	108.87	29.97	0.001
Interaction	235.88	3	78.63	21.64	0.003
Residual error	18.16	5	3.63		
Lack-of-fit	7.18	3	2.39	0.44	0.752
Pure error	10.99	2	5.49		
Total	919.83	14			
R ²	0.9803				
Adj R ²	0.9447				
Pred R ²	0.8483				

Table 5

Assignments of FTIR wavenumbers in the range 4000–500 cm⁻¹ of pullulan obtained under optimum production conditions (OCP) and commercial pullulan (CP).

FTIR wavenumber (cm ⁻¹)		Assignments
OCP	CP	
3428.49	3422.33	O-H
2923.81	2920.98	C-H
1617.31	1625.87	O-C-O
1369.14	1465.13	C-O-H
1156.83	1166.68	C-O-C
1016.62	1111.66	C-O
925.53	993.98	glucopyranoside

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References

- [1] T.J. Pollock, L. Thorne, R.W. Armentrout, Isolation of new *Aureobasidium* strains that produce high-molecular-weight pullulan with reduced pigmentation, *Appl. Environ. Microbiol.* 58 (1992) 877–883.
- [2] P. Lotrakul, P. Deenarn, S. Prasongsuk, H. Punnapayak, Isolation of *Aureobasidium pullulans* from bathroom surfaces and their antifungal activity against some *Aspergilli*, *African J. Microbiol. Res.* 3 (2009) 253–257.
- [3] R. Gaur, R. Singh, M. Gupta, M.K. Gaur, *Aureobasidium pullulans*, an economically important polymorphic yeast with special reference to pullulan, *African J. Biotechnol.* 9 (2010) 7989–7997.
- [4] B. Bhadra, R.S. Rao, P.K. Singh, P.K. Sarkar, S. Shivaji, Yeasts and yeast-like fungi associated with tree bark: diversity and identification of yeasts producing extracellular endoxylanases, *Curr. Microbiol.* 56 (2008) 489–494.
- [5] R. Singh, R. Gaur, S. Bansal, F. Jamal, P.K. Pandey, S. Tiwari, S. Sarsaiya, S. Mishra, N. Chaturvedi, D.P. Singh, M.K. Gaur, F.G.V. Arasu, Production of pullulan from a high yielding strain of *Aureobasidium pullulans* in non-stirred flask-type fermentation system, *J. Microbiol. Biotechnol. Res.* 7 (2017) 26–32.
- [6] S. Prasongsuk, M.A. Berhow, C.A. Dunlap, D. Weisleder, T.D. Leathers, D.E. Eveleigh, H. Punnapayak, Pullulan production by tropical isolates of *Aureobasidium pullulans*, *J. Ind. Microbiol. Biotechnol.* 34 (2007) 55–61.
- [7] E. Zhang, T. Tanaka, M. Tajima, R. Tsuboi, A. Nishikawa, T. Sugita, Characterization of the skin fungal microbiota in patients with atopic dermatitis and in healthy subjects, *Microbiol. Immunol.* 55 (2011) 625–632.
- [8] M.S. Deshpande, R.L. Deopurkar, V.B. Rale, Beta-galactosidase from *Aureobasidium pullulans*, *Appl. Microbiol.* 9 (1989) 21–24.
- [9] J.O. Rich, T.D. Leathers, A.M. Anderson, K.M. Bischoff, P. Manitchotpit, Laccases from *Aureobasidium pullulans*, *Enzym. Microb. Technol.* 53 (2013) 33–37.
- [10] X. Zou, Y. Zhou, S.T. Yang, Production of polymeric acid and malic acid by *Aureobasidium pullulans* fermentation and acid hydrolysis, *Biotechnol. Bioeng.* 110 (2013) 2105–2113.
- [11] X. Chen, Q.Q. Wang, N.N. Liu, G.L. Liu, Z. Chi, Z.M. Chi, A glycosyltransferase gene responsible for pullulan biosynthesis in *Aureobasidium melanogenum* P16, *Int. J. Biol. Macromol.* 95 (2017) 539–549.
- [12] R.S. Singh, G.K. Saini, Pullulan-hyperproducing color variant strain of *Aureobasidium pullulans* FB-1 newly isolated from phylloplane of *Ficus sp.*, *Bioresour. Technol.* 99 (2008) 3896–3899.
- [13] V.D. Prajapati, G.K. Jani, S.M. Khanda, Pullulan: an exopolysaccharide and its various applications, *Carbohydr. Polym.* 95 (2013) 540–549.
- [14] R.S. Singh, N. Kaur, J.F. Kennedy, Pullulan production from agro-industrial waste and its applications in food industry: a review, *Carbohydr. Polym.* 217 (2019) 46–57.
- [15] K.I. Shingel, Current knowledge on biosynthesis, biological activity, and chemical modification of the exopolysaccharide, pullulan, *Carbohydr. Res.* 339 (2004) 447–460.
- [16] T.D. Leathers, Biotechnological production and applications of pullulan, *Appl. Microbiol. Biotechnol.* 62 (2003) 468–473.
- [17] X. Duan, Z. Chi, L. Wang, X. Wang, Influence of different sugars on pullulan production and activities of α -phosphoglucose mutase, UDPG-pyrophosphorylase and glucosyltransferase involved in pullulan synthesis in *Aureobasidium pullulans* Y68, *Carbohydr. Polym.* 73 (2008) 587–593.
- [18] K.C. Cheng, A. Demirci, J.M. Catchmark, Pullulan: biosynthesis, production, and applications, *Appl. Microbiol. Biotechnol.* 92 (2011) 29–44.
- [19] X. Duan, Z. Chi, H. Li, L. Gao, High pullulan yield is related to low UDP-glucose level and high pullulan-related synthases activity in *Aureobasidium pullulans* Y68, *Ann. Microbiol.* 57 (2007) 243–248.
- [20] R.S. Singh, G.K. Saini, J.F. Kennedy, Pullulan: microbial sources, production and applications, *Carbohydr. Polym.* 73 (2008) 515–531.
- [21] S.F.G. Oskouie, F. Tabandeh, B. Yakhchali, F. Eftekhari, Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*, *Biochem. Eng. J.* 39 (2008) 37–42.
- [22] A.R. Choudhury, M.S. Bhattacharyya, G.S. Prasad, Application of response surface methodology to understand the interaction of media components during pullulan production by *Aureobasidium pullulans* RBF-4A3, *Biocatal. Agric. Biotechnol.* 1 (2012) 232–237.
- [23] L. Jiang, Optimization of fermentation conditions for pullulan production by *Aureobasidium pullulan* using response surface methodology, *Carbohydr. Polym.* 79 (2010) 414–417.
- [24] A.R. Choudhury, P. Saluja, G.S. Prasad, Pullulan production by an osmotolerant *Aureobasidium pullulans* RBF-4A3 isolated from flowers of *Caesulia axillaris*, *Carbohydr. Polym.* 83 (2011) 1547–1552.
- [25] R. Singh, R. Gaur, S. Tiwari, M.K. Gaur, Production of pullulan by a thermotolerant *aureobasidium pullulans* strain in non-stirred fed batch fermentation process, *Brazilian J. Microbiol.* 43 (2012) 1042–1050.
- [26] S. Wu, J. Chen, S. Pan, Optimization of fermentation conditions for the production of pullulan by a new strain of *Aureobasidium pullulans* isolated from sea mud and its characterization, *Carbohydr. Polym.* 87 (2012) 1696–1700.
- [27] H. Moubasher, S.S. Wahsh, N.A. El-Kassem, Isolation of *Aureobasidium pullulans* and the effect of different conditions for pullulanase and pullulan production, *Microbiol. Res.* 82 (2013) 155–161.
- [28] C. An, S. Jian Ma, W. Jiao Xue, C. Liu, H. Ding, Comparative study of different molecular weight pullulan productions by *Aureobasidium pullulans* CGMCC No.11602, *3 Biotech* 9 (2019) 156.
- [29] J. Yang, Y. Zhang, S. Zhao, Q. Zhou, X. Xin, L. Chen, Statistical optimization of medium for pullulan production by *Aureobasidium pullulans* NCP52016 using fructose and soybean meal hydrolysates, *Molecules* 23 (2018) 1–16.
- [30] R.S. Singh, N. Kaur, Understanding response surface optimization of medium composition for pullulan production from de-oiled rice bran by *Aureobasidium pullulans*, *Food Sci. Biotechnol.* (2019) 1–14.
- [31] K.R. Sugumaran, V. Ponnusami, Conventional optimization of aqueous extraction of pullulan in solid-state fermentation of cassava bagasse and Asian palm kernel, *Biocatal. Agric. Biotechnol.* 10 (2017) 204–208.
- [32] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* 25 (1997) 3389–3402.
- [33] J.D. Thompson, T.J. Gibson, F. Plewniak, J. Jeanmougin, D.G. Higgins, Optimization of microwave assisted extraction of pectin from sour orange peel and its physicochemical properties author, *Nucleic Acids Res.* 25 (1997) 4876–4882.
- [34] R. Gaur, R. Singh, Optimization of physico-chemical and nutritional parameters for pullulan production by a mutant of thermotolerant *Aureobasidium pullulans* in fed batch fermentation process, *African J. Biotechnol.* 9 (2010) 7322–7330.
- [35] S.L.C. Ferreira, R.E. Bruns, H.S. Ferreira, G.D. Matos, J.M. David, G.C. Brandão, E.G.P. da Silva, L.A. Portugal, P.S. dos Reis, A.S. Souza, W.N.L. dos Santos, Box-Behnken design: an alternative for the optimization of analytical methods, *Anal. Chim. Acta* 597 (2007) 179–186.
- [36] X.-M. Ju, D.-H. Wang, G.-C. Zhang, D. Cao, G.-Y. Wei, Efficient pullulan production by bioconversion using *Aureobasidium pullulans* as the whole-cell catalyst, *Appl. Microbiol. Biotechnol.* 99 (2015) 211–220.
- [37] M. Kazemi, F. Khodaiyan, M. Labbafi, S. Saied Hosseini, M. Hojati, Pistachio green hull pectin: optimization of microwave-assisted extraction and evaluation of its physico-chemical, structural and functional properties, *Food Chem.* 271 (2019) 663–672.
- [38] S.S. Hosseini, F. Khodaiyan, M. Kazemi, Z. Najari, Optimization and characterization of pectin extracted from sour orange peel by ultrasound assisted method, *Int. J. Biol. Macromol.* 125 (2019) 621–629.
- [39] M. Kazemi, F. Khodaiyan, S.S. Hosseini, Utilization of food processing wastes of eggplant as a high potential pectin source and characterization of extracted pectin, *Food Chem.* 294 (2019) 339–346.
- [40] R. Gaur, R. Singh, S. Tiwari, S.K. Yadav, N.S. Daramwal, Optimization of physico-chemical and nutritional parameters for a novel pullulan-producing fungus, *Eurotium chevalieri*, *J. Appl. Microbiol.* 109 (2010) 1035–1043.
- [41] F. Youssef, T. Roukas, C.G. Biliaderis, Pullulan production by a non-pigmented strain of *Aureobasidium pullulans* using batch and fed-batch culture, *Process Biochem.* 34 (1999) 355–366.
- [42] L.H. Gibson, R.W. Coughlin, Optimization of high molecular weight pullulan production by *Aureobasidium pullulans* in batch fermentations, *Biotechnol. Prog.* 18 (2002) 675–678.

- [43] K.C. Cheng, A. Demirci, J. Catchmark, Effect of temperature, carbon source, yeast extract, and pH on pullulan production by *Aureobasidium pullulans*, Am. Soc. Agric. Biol. Eng. (2009), 096033 American Society of Agricultural and Biological Engineers (ASABE) with ASABE Paper No.
- [44] K. Thirumavalavan, T.R.M. and R. Dhanasekar, Pullulan production from coconut by-products by *Aureobasidium pullulans*, African J. Biotechnol. 8 (2009) 254–258.
- [45] Y. Goksungur, P. Uzunogullari, S. Dagbagli, Optimization of pullulan production from hydrolysed potato starch waste by response surface methodology, Carbohydr. Polym. 83 (2011) 1330–1337.
- [46] R. Singh, R. Guar, S. Bansal, P. Biswas, P.k. Pandey, F. Jamal, S. Tiwari, M.K. Guar, Production of pullulan from a high yielding strain of *Aureobasidium pullulans* isolated from Jabalpur region of Madhya Pradesh in central India, J. Chem. Pharm. Res. 8 (2016) 126–132.
- [47] M.S. Bitaraf, F. Khodaiyan, M.A. Mohammadifar, S.M. Mousavi, Application of response surface methodology to improve fermentation time and rheological properties of probiotic yogurt containing *Lactobacillus reuteri*, Food Bioprocess Technol. 5 (2012) 1394–1401.
- [48] M. Kazemi, F. Khodaiyan, S.S. Hosseini, Eggplant peel as a high potential source of high methylated pectin: ultrasonic extraction optimization and characterization, LWT Food Sci. Technol. 105 (2019) 182–189.
- [49] K.R. Sugumaran, P. Shobana, P. Mohan Balaji, V. Ponnusami, D. Gowdhaman, Statistical optimization of pullulan production from Asian palm kernel and evaluation of its properties, Int. J. Biol. Macromol. 66 (2014) 229–235.
- [50] K.C. Cheng, A. Demirci, J.M. Catchmark, Evaluation of medium composition and fermentation parameters on pullulan production by *Aureobasidium pullulans*, Food Sci. Technol. Int. 17 (2011) 99–109.
- [51] L. Sheng, G. Zhu, Q. Tong, Comparative proteomic analysis of *Aureobasidium pullulans* in the presence of high and low levels of nitrogen source, J. Agric. Food Chem. 62 (2014) 10529–10534.
- [52] Z. Chi, S. Zhao, Optimization of medium and cultivation conditions for pullulan production by a new pullulan-producing yeast strain, Enzym. Microb. Technol. 33 (2003) 206–211.
- [53] A. Lazaridou, T. Roukas, C.G. Biliaderis, H. Vaikousi, Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation, Enzym. Microb. Technol. 31 (2002) 122–132.
- [54] O. Ates, Systems biology of microbial exopolysaccharides production, Front. Bioeng. Biotechnol. 3 (2015) 200.
- [55] D.J. McClements, Principles, Practices, and Techniques, 2005.
- [56] B. Pasandide, F. Khodaiyan, Z. Mousavi, S.S. Hosseini, Pectin extraction from citron peel: optimization by Box–Behnken response surface design, Food Sci. Biotechnol. 27 (2018) 997–1005.
- [57] R. Teran Hilares, J. Resende, C.A. Orsi, M.A. Ahmed, T.M. Lacerda, S.S. da Silva, J.C. Santos, Exopolysaccharide (pullulan) production from sugarcane bagasse hydrolysate aiming to favor the development of biorefineries, Int. J. Biol. Macromol. 127 (2019) 169–177.
- [58] S.W. Cui, Y.H. Chang, Emulsifying and structural properties of pectin enzymatically extracted from pumpkin, LWT Food Sci. Technol. 58 (2014) 396–403.
- [59] B.M. Yapo, C. Robert, I. Etienne, B. Wathelet, M. Paquot, Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts, Food Chem. 100 (2007) 1356–1364.
- [60] Y. Matsumura, C. Satake, M. Egami, T. Mori, Interaction of gum Arabic, maltodextrin and pullulan with lipids in emulsions, Biosci. Biotechnol. Biochem. 64 (2000) 1827–1835, <https://doi.org/10.1271/bbb.64.1827>.
- [61] D. Wang, X. Ju, D. Zhou, G. Wei, Efficient production of pullulan using rice hull hydrolysate by adaptive laboratory evolution of *Aureobasidium pullulans*, Bioresour. Technol. 164 (2014) 12–19.
- [62] E. Trovatti, S.C.M. Fernandes, L. Rubatat, D. da S. Perez, C.S.R. Freire, A.J.D. Silvestre, C.P. Neto, Pullulan-nanofibrillated cellulose composite films with improved thermal and mechanical properties, Compos. Sci. Technol. 72 (2012) 1556–1561.
- [63] P. Ögüzhan, F. Yangilar, Pullulan: production and usage in food industry, African J. Food Sci. Technol. 4 (2013) 2141–2145.