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Investigation the Exopolysaccharide Production Potential of Newly Isolated *Trametes versicolor* during Repeated-Batch Fermentation

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Abstract

White rot fungi can produce exopolysaccharides (EPS) and these EPSs have the potential to be used in various applications. *Trametes versicolor*, a white rot fungus, can also produce high amount of EPS. EPS production varies depending on fermentation method, production conditions, nutrient sources (especially glucose concentration) in the medium, and also the strain used. Therefore, in this study, EPS production ability of *T. versicolor* strain collected from Hatay/Turkey was firstly investigated during the repeated-batch fermentation (RBF) process. *T. versicolor* was incubated under RBF condition. After investigating the EPS production in different media during repeated-batch process, the effect of medium retention time on EPS production was determined. Then; the effects of agitation, temperature, pH, amount of pellets used and amount of glucose on EPS production were determined. The results of the study showed that both production conditions and glucose concentration affect the EPS production of this strain during the RBF process.

Keywords: Exopolysaccharide; Repeated-batch fermentation; *Trametes versicolor*; White rot fungus.

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Yeni İzole Edilmiş *Trametes versicolor*'un Tekrarlı-Kesikli Fermentasyon Sürecinde Ekzopolisakkarit Üretme Potansiyelinin Araştırılması

Öz

Beyaz çürükçül funguslar ekzopolisakkarit (EPS) üretebilir ve bu EPS'lerin çeşitli uygulamalarda kullanılma potansiyeli vardır. Beyaz çürükçül fungus olan *Trametes versicolor* da yüksek miktarda EPS üretebilir. EPS üretimi fermentasyon metodu, üretim koşulları, ortamdaki besin kaynakları (özellikle glukoz konsantrasyonu) ve ayrıca kullanılan suşa bağlı olarak değişir. Bu nedenle, bu çalışmada öncelikle Hatay/Türkiye'den toplanan *T. versicolor* suşunun EPS üretim yeteneği tekrarlı-kesikli fermentasyon (TKF) sürecinde araştırılmıştır. *T. versicolor*, TKF koşulunda inkübe edildi. Tekrarli-kesikli süreç sırasında farklı ortamlarda EPS üretimi araştırıldıktan sonra, besiyeri alıkonma süresinin EPS üretimi üzerindeki etkisi belirlendi. Daha sonra da çalkalama, sıcaklık, pH, kullanılan pelet miktarı ve glukoz konsantrasyonun EPS üretimi üzerindeki etkileri belirlendi. Çalışmanın sonuçları, hem üretim koşullarının hem de glukoz konsantrasyonun bu suşun TKF sürecinde EPS üretimini etkilediğini göstermiştir.

Anahtar Kelimeler: Beyaz çürükçül fungus; Ekzopolisakkarit; Tekrarlı-kesikli fermentasyon; *Trametes versicolor*.

1. Introduction

Exopolysaccharides (EPS) are polysaccharides secreted by microorganism into the extracellular medium. The biological activities of EPSs (long chain and molecular weight polymers composed of especially sugar units) including anti-inflammatory, antioxidative, antitumoral and antidiabetic have led to their increasing interest in various fields and especially in medicine [1, 2].

White rot fungi are efficient EPS producers. It was reported that the EPS from *Pleurotus pulmonarius* has potential antioxidant capacity and therefore, it can be used for functional food and medicine production [3]. Similarly, EPS from *Pleurotus eryngii* have antitumoral and antioxidative activities [1]. *Trametes versicolor* is a biotechnologically important medicinal white rot fungus. It can be used in bioremediation and for producing various enzymes such as laccase and peroxidases. Another important characteristic of this fungus is its ability to produce EPS [4-6]. The anti-inflammatory and prebiotic potential of crude EPS obtained from submerged culture liquid of *T. versicolor* was also reported [7].

EPS production potential varies depending on microorganism and even the strain used, physical conditions of the microorganism such as agitation, pH and temperature, and also inorganic/organic sources in the growth medium [8].

Fermentation method is also important for EPS production. Repeated-batch fermentation (RBF) method is an alternative method to batch method, and it allows maintenance long term activity of fungal pellets for a long period of time [9]. This method can also be used for production of metabolites and it improves the productivity of fungal products [10-13]. In this method, the mycelia of the fungus are in free pellet form and free pellets are self-immobilized mycelia [10, 14]. It is possible to store and reuse these pellets and retain their long-term activity [10]. For example, it is an easy and simple process for production of high amounts of laccase enzyme by whole fungal pellets. RBF process was reported as the efficient method for obtaining high amount of EPS [2]. This method provides an easy operation for EPS production by free and immobilized pellets of *Ganoderma lucidum* [15, 16]. Highly stable *Ganoderma pfeifferi* pellets can be used in extended fermentation cycles for production of EPS efficiently [14]. It is possible to produce EPS with antioxidant activity by immobilized Chinese medicinal mushroom *Cordyceps militaris* [17]. However, there are limited studies on EPS production under RBF conditions [13, 15, 16] and according to our literature knowledge, there is no study on EPS production potential of repeated-batch culture of *T. versicolor* and this strain. The aim of this study is to investigate the EPS production potential of newly isolated *T. versicolor* pellets during RBF process.

2. Materials and Methods

2.1. Fungus

The white rot fungus *Trametes versicolor* was used in this study. This fungus was isolated as a pure culture after being collected from Hatay/Turkey and stored in Biotechnology laboratory in Inonu University. It was maintained at 4 °C on Sabouraud dextrose agar (SDA) plates.

2.2. Production of Stock Inoculum and Pellets

T. versicolor cultured on SDA plates was inoculated on slant SDA and incubated statically at 30 °C for 5 days. After incubation, distilled water was added, and a conidial suspension of the culture was prepared. Four mL of this suspension was transferred into 250 mL flask containing 100 mL Sabouraud dextrose broth (SDB) and the culture was incubated at 30 °C and 150 rpm for 5 days. After incubation, the whole culture was gently homogenized and 1.5 mL of this homogenized culture inoculated into fresh SDB. This culture was incubated and then homogenized in the same conditions as above. This homogenized culture was used as stock

inoculum culture. For fungal pellet production, 7.5 mL from this stock inoculum culture was inoculated into 600 mL SDB/1000 mL flask. It was incubated 30 °C and 150 rpm for 5 days and the pellets obtained [18].

2.3. Repeated-Batch Fermentation with Fungal Pellets

The fungal pellets prepared as stated above were harvested and used for RBF studies. Appropriate amounts of the prepared pellets were transferred to fresh medium to be used in RBF studies and incubated. After incubation, the culture was filtered under sterile condition, and fresh medium was added onto the pellets remaining in the flask [18].

2.4. Selecting the Most Appropriate Culture Medium for EPS Production

Firstly, the RBF studies were conducted in two different culture media. These media were

A) Commercial SDB medium (g/L): Peptone 10, glucose 20

B) Complex medium (CM) (g/L): KH_2PO_4 0.5, $MgSO_4.7H_2O$ 0.5, peptone 2, yeast extract 2, glucose 22.

In RBF experiments, the pellets were, firstly, transferred into 100 ml medium/250 mL. After incubation for appropriate time, the culture was filtered without removing the pellets. The medium was discharged and was exchanged with fresh medium. In these experiments the pellets were repeatedly used in these two media for 3 times with medium retention time of 1 h, 2 h, 24 h or 72 h and then, the most appropriate medium and retention time were selected.

2.5. Optimization the EPS Production of Repeated-Batch Cultures

These studies were performed in the most appropriate medium. After the most appropriate medium selected, studies were conducted to determine the optimum conditions for exopolysaccharide production in this medium. The medium and medium retention time used in these studies were the Complex medium and 24 h, respectively.

2.5.1. Effect of Agitation on EPS Production of Repeated-Batch Cultures

Effect of agitation on EPS production of pellets during RBF was investigated within the agitation rate of 0-200 rpm. The temperature and pellet amount used were 30 °C and 20 g, respectively.

2.5.2. Effect of Incubation Temperature on EPS Production of Repeated-Batch Cultures

To test the effect of incubation temperature on EPS production of pellets during RBF, the cultures were incubated within the temperature range of 20-40 °C. The agitation rate and pellet amount used were 150 rpm and 20 g, respectively.

2.5.3. Effect of Initial pH on EPS Production of Repeated-Batch Cultures

Effect of initial pH on EPS production of pellets during RBF was investigated within the pH range of 3.0-7.0. The incubation temperature, agitation rate and pellet amount used were 30 °C, 150 rpm and 20 g, respectively.

2.5.4. Effect of Pellet Amount on EPS Production of Repeated-Batch Cultures

Different pellet amounts (5, 10, 20 and 30 g) were tested to determine the effect of pellet amount on EPS production during RBF studies. The incubation temperature, agitation rate and initial pH used were 30 °C, 150 rpm and 7.0, respectively.

2.5.5. Effect of Glucose Concentration on EPS Production of Repeated-Batch Cultures

To determine the effect of glucose concentration on EPS production 0, 11, 22, 44, 88 g/L of glucose (final concentrations) were used, and RBF were conducted under the optimum culture parameters detected.

2.6. Isolation of Exopolysaccharide

To isolate the EPS from culture broth of repeated-batch cultures, the culture was centrifuged at 9000 rpm for 15 min. to separate the supernatant from pellets. The supernatant obtained was mixed with cold ethanol and kept overnight at 4 °C. Then, it was filtered using filter paper (pre-dried at 65 °C for overnight/pre-weighed) and the filter paper with EPS was kept again at 65 °C for overnight, placed in desiccator and dried to a constant weight. After that, the dry weight of the EPS was calculated [14, 19]. The macroscopic image of EPS obtained under optimal condition during RBF is shown in Fig. 1.

Figure 1: Macroscopic image of EPS.

3. Results and Discussion

Both the isolation of new microorganisms/strains and increasing the capacity of EPS production attract the attention of researchers [17, 20]. *Trametes versicolor* is one of the most frequently studied medicinal mushroom [7]. Therefore, in this study, the EPS production ability of *T. versicolor* strain collected and isolated from Hatay/Turkey was investigated under repeated-batch fermentation conditions. This fermentation method has various advantages such as storage and reuse of the pellets and use of fungal pellets for a long period of time with long-term activity [9, 10]. The effects of various production conditions and glucose concentration on EPS production of this strain was also tested.

3.1. Selecting the Most Appropriate Culture Medium

EPS production by *T. versicolor* pellets under RBF was investigated in two different media (SDB and CM). These pellets were used for 3 times with various medium retention times of 1 h, 2 h, 24 h and 72 h. Low amounts of EPS could be produced at the retention times of 1 h and 2 h. On the other hand, when the medium retention time of 24 h was used, the amounts of EPS detected at third cycle were 2.14±0.36 and 2.75±0.33 g/L in SDB and CM media, respectively (Table 1).

Table 1: EPS production (g/L) by *T. versicolor* pellets in SDB and CM media at 24 h and 72 h medium retention times.

	Medium Retention Time			
	24 h		72 h	
Number of Times Pellets Used	**SDB**	**CM**	**SDB**	**CM**
First cycle	1.88±0.16	1.67±0.07	2.02±0.10	1.74±0.23
Second cycle	1.80±0.27	3.06±0.17	1.13±0.23	1.99±0.32
Third cycle	2.14±0.36	2.75±0.33	1.32±0.31	2.41±0.10

When 1 h and 2 h medium retention times used the cumulative total EPS amounts obtained after three cycles were below 2.5 g/L. However, when medium retention of 24 h and 72 h were used for SDB cultures, the cumulative total EPS amounts after three cycles were 5.82 and 4.47 g/L, respectively. These values were 7.48 and 6.14 g/L for CM cultures (Fig. 2). The fungal pellets could produce low amounts of EPS at short retention times. However, when medium retention times of 24 h and 72 h were conducted, higher EPS amounts were obtained by the pellets (Fig. 2). As a result of the studies, CM medium and 24 h medium retention time were selected and used throughout the studies.

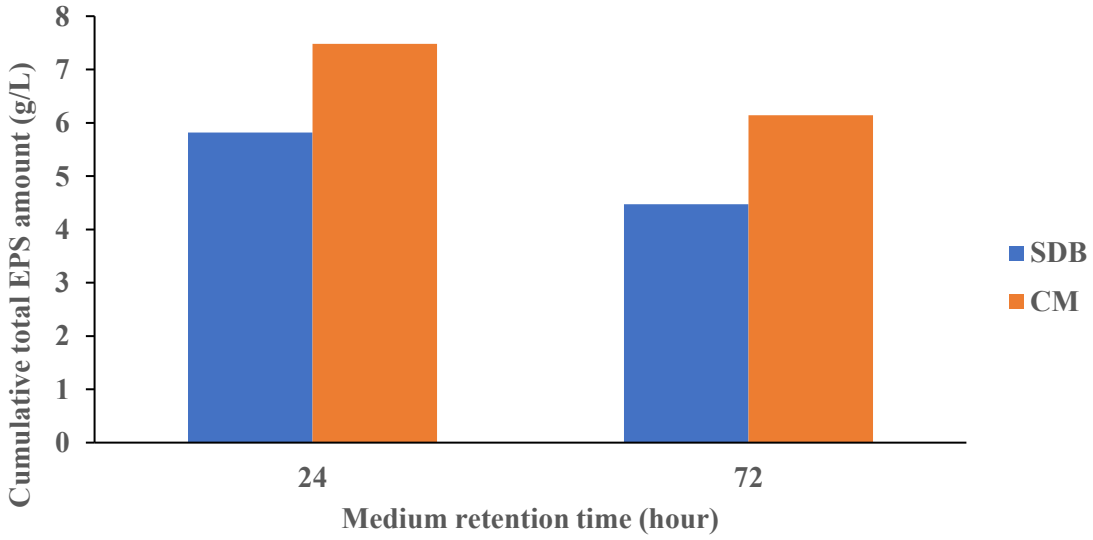

Figure 2: EPS production (g/L) by *T. versicolor* pellets at 24 h and 72 h medium retention times after three cycles in SDB and CM media.

3.2. Effect of Agitation Rate on EPS Production of Pellets During Repeated-Batch Fermentation

Agitation and aeration ensure good mixing of liquid, solid and gas phases in the medium. However, high agitation rates can also cause the pellets to break down. Therefore, the effect of agitation on EPS production during RBF mode was tested. Agitation affected the EPS production of the pellets. As can be seen from Table 2, the EPS production of the RB cultures at each cycle remained low when the culture was mixed at low agitation speed such as 50 ppm or without mixing. However, higher levels of EPS could be produced at 100 (2.15±0.19 at third cycle) and 150 rpm (3.06±0.17 at second cycle) and the production decreased at 200 rpm.

Table 2: Effect of agitation on EPS (g/L) production of pellets during RBF.

	Number of Times Pellets Used		
Agitation (rpm)	**1**	**2**	**3**
0	0.34±0.13	0.62±0.05	0.63±0.06
50	0.19±0.09	0.64±0.15	0.78±0.09
100	0.65±0.04	1.81±0.52	2.15±0.19
150	1.67±0.07	3.06±0.17	2.75±0.33
200	0.83±0.30	1.20±0.28	1.79±0.26

The cumulative total EPS amounts at 0, 50, 100, 150 and 200 rpm after three cycles were 1.59, 1.61, 4.61, 7.48 and 3.82 g/L, respectively (Fig.3). Oxygen transfer rate is reduced at low agitation and therefore, it affects the cell growth and metabolism. On the other hand, high agitation rate can cause shear stress and lead the cell (pellet) damage [2, 10]. In this study, the best agitation rate for EPS production was determined as 150 rpm. It was reported that 150 rpm is the best agitation rate for EPS production of white rot fungal cultures [21].

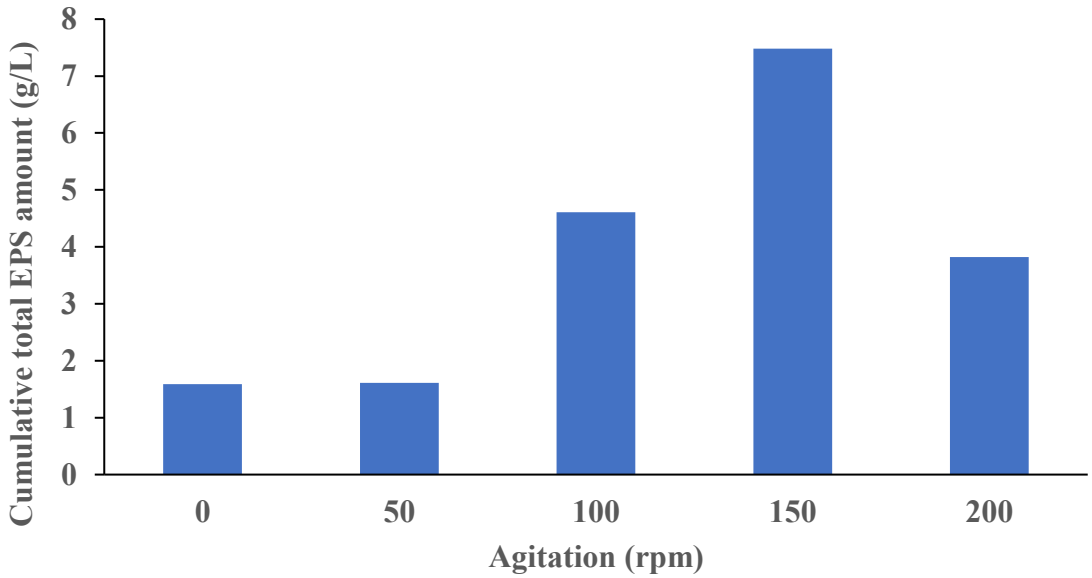

Figure 3: The cumulative total EPS amounts obtained at various agitation speeds after three cycles.

3.3. Effect of Temperature on EPS Production of Pellets During Repeated-Batch Fermentation

EPS production by pellets was carried out at various temperatures such as 20-40 °C and 150 rpm. The pellets were able to produce EPS at all temperatures tested. However, more EPS was produced between 20-30 °C than between 35 and 40 °C. 30 °C was detected as the best temperature for EPS production and the EPS amounts obtained at second and third cycles were 3.06±0.17 and 2.75±0.33, respectively (Table 3).

Table 3: Effect of temperature on EPS (g/L) production of pellets during RBF.

Temperature (°C)	Number of Times Pellets Used		
	1	**2**	**3**
20	1.88±0.48	2.26±0.13	2.01±0.41
25	1.56±0.07	3.03±0.44	2.28±0.14
30	1.67±0.07	3.06±0.17	2.75±0.33
35	1.41±0.10	1.76±0.39	1.12±0.11
40	1.42±0.15	1.26±0.16	1.13±0.07

The cumulative total EPS amounts at 20, 25, 30, 35 and 40 °C after three cycles were 6.15, 6.87, 7.48, 4.29 and 3.81g/L, respectively (Fig. 4). Most fungi can produce EPS within a temperature range of 22-30 °C [22].

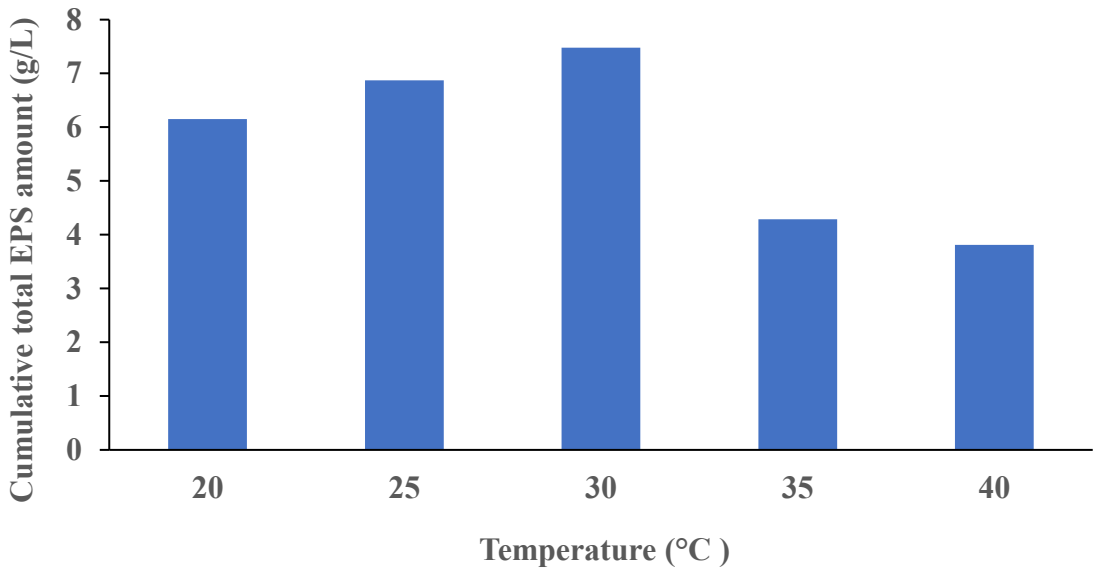

Figure 4: The cumulative total EPS amounts obtained at various temperatures after three cycles.

3.4. Effect of pH on EPS Production of Pellets During Repeated-Batch Fermentation

The effect of initial pH values (pH 3.0-7.0) on EPS production activity of the pellets was also tested with 20 g wet pellet amounts at 30 °C and 150 rpm. All initial pH values supported the EPS production of the pellets. At pH 3.0, the EPS amounts were 2.49±0.32 g/L and 3.18±0.38 g/L at second and third cycles. When initial pH 7.0 was used, it was 3.58±0.46 g/L at the third cycle (Table 4).

Table 4: Effect of pH on EPS (g/L) production of pellets during RBF.

	Number of Times Pellets Used		
pH	**1**	**2**	**3**
3	1.57±0.32	2.49±0.32	3.18±0.38
4	1.53±0.05	1.75±0.19	2.20±0.25
5	1.62±0.18	2.12±0.20	1.79±0.07
6	1.55±0.35	1.58±0.04	2.08±0.11
7	1.51±0.11	1.99±0.35	3.58±0.46

The cumulative total EPS amounts detected after three cycles for pH values of 3, 4, 5, 6, and 7 were 7.24, 5.48, 5.53, 5.21 and 7.08 g/L, respectively (Fig. 5). It was reported that the best initial pH values for EPS production of shake flask cultures of *C. versicolor*, *Grifola umbellata* and *Grifola frondosa* are pH 5.5, pH 5.0 and 5.5, respectively [23-25]. *Aspergillus* sp. DHE6 produces the highest amount of EPS at pH 6.0 under submerged fermentation conditions [26]. *L. edodes* can produce high amount of EPS at initial pH of 4.5 under submerged culture condition [27].

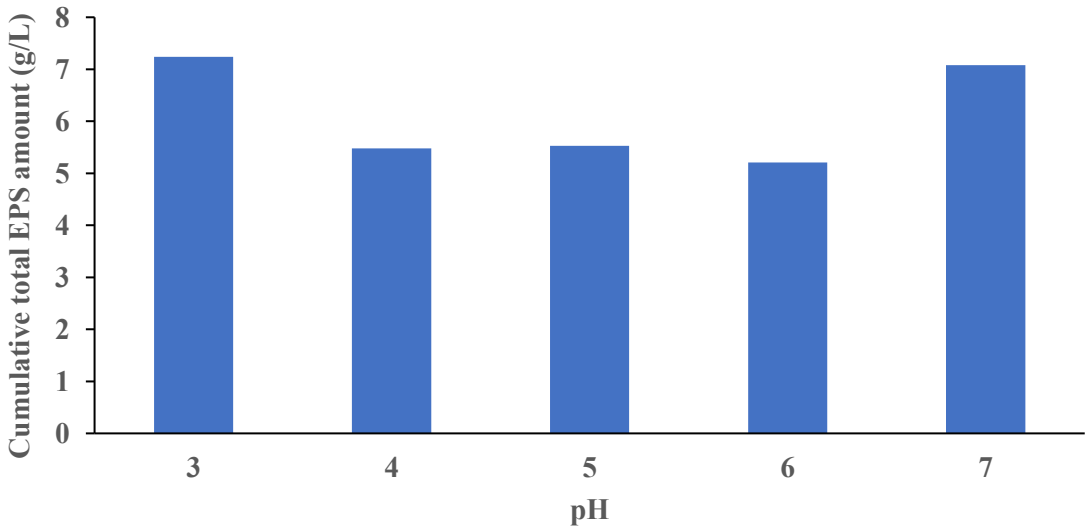

Figure 5: The cumulative total EPS amounts obtained at various pH values after three cycles.

3.5. Effect of Pellet Amounts on EPS Production of Pellets During Repeated-Batch Fermentation

It was reported that an increase in pellet amounts makes a positive effect on activity of pellets during RBF [10, 28]. In our study, pellets were used for three times under optimum conditions (Table 5).

Table 5: Effect of pellet amounts on EPS (g/L) production.

Pellet Amounts	**Number of Times Pellets Used**		
(g/100 mL)	**1**	**2**	**3**
5	1.59±0.10	1.80±0.05	2.67±0.24
10	1.74±0.13	2.11±0.32	2.59±0.08
20	1.67±0.07	3.06±0.17	2.75±0.33
30	1.44±0.27	2.98±0.39	2.79±0.24

When 5 and 10 g pellet amounts used, the cumulative total EPS amounts after three cycles were 6.06 and 6.44 g/L, respectively. However, these values were 7.48 and 7.21 g/L for pellet amounts of 20 and 30 g/L (Fig. 6). The results showed that higher pellet amounts positively affected the EPS production.

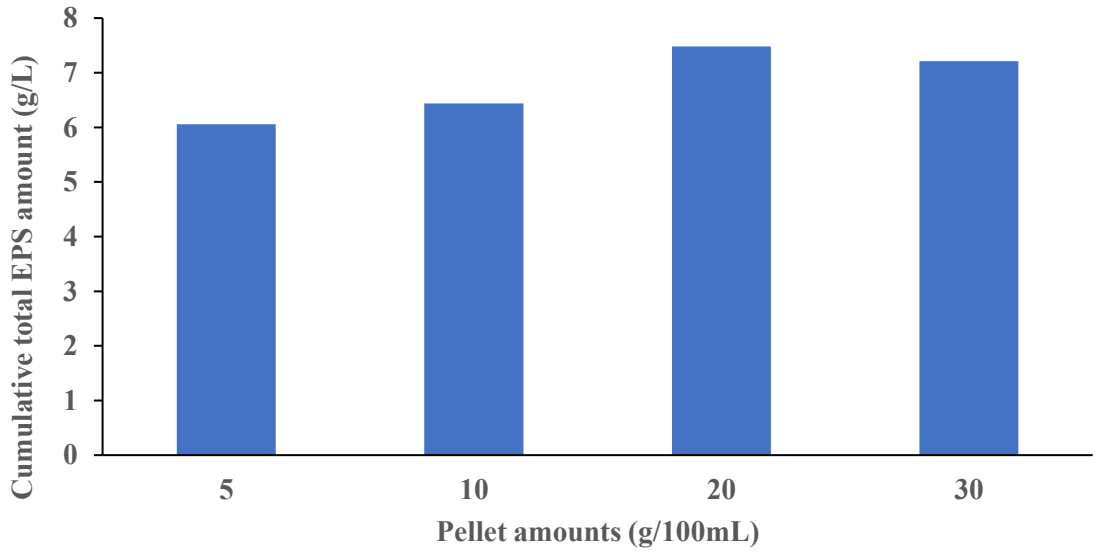

Figure 6: The cumulative total EPS amounts obtained by various pellets amounts after three cycles.

3.6. Effect of Glucose Concentration on EPS Production During Repeated-Batch Fermentation

Glucose is a principal carbon and energy source for most of the microorganisms. Therefore, the effect of glucose amount on EPS production potential of the pellets was tested. As shown in Table 6, an increase in the glucose amount had a positive effect on the EPS production potential of the pellets and EPS production activity increased at each cycle.

Table 6: Effect of glucose concentration on EPS (g/L) production of pellets during RBF.

Glucose (g/L)	**Number of Times Pellets Used**		
	1	**2**	**3**
22	1.67±0.07	3.06±0.17	2.75±0.33
44	3.26±0.35	5.75±1.08	6.87±0.20
88	3.27±0.44	6.22±0.51	9.68±0.74

The cumulative total EPS amounts obtained after three cycles for the glucose concentrations of 22, 44, 88 g/L were 7.48, 15.88 and 19.17, respectively (Fig. 7). This shows the critical role of glucose for EPS production. Glucose is a favorable nutrient for EPS production of *G. formosanum* [29] and the best carbon source for EPS production of submerged cultures of *P. sajor-caju* [30]. α-phosphoglucomutase gene was reported as an important gene for EPS production of *G. lucidum* [31].

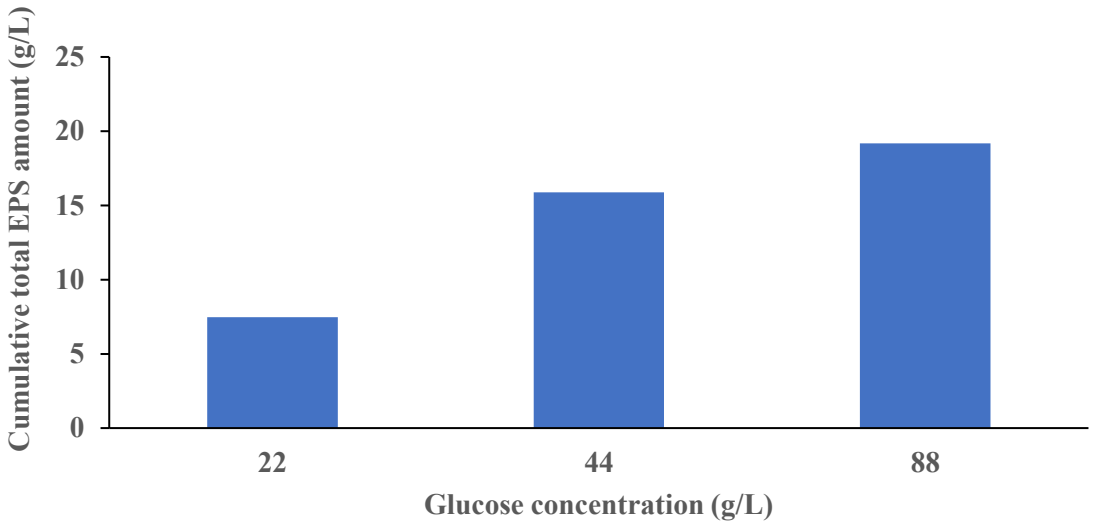

Figure 7: The cumulative total EPS amounts obtained at various glucose concentrations after three cycles.

4. Conclusion

This biotechnologically important *T. versicolor* strain used in our study was able to produce significant amounts of exopolysaccharide (EPS) under repeated-batch condition. The EPS production potential of this microorganism varied depending on the agitation speed, temperature, pellet amount, medium type and glucose concentration. It was observed that the concentration of glucose, which is a carbon and energy source, was very effective in the EPS production of these pellets in RBF process. Our results show that this strain and RBF method can be used for production of high amounts of EPS repeatedly.

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