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USING CENTRIFUGE AND ULTRAFILTRATION AS DOWNSTREAM PROCESSES FOR THE CONCENTRATION OF MICROBIAL β-MANNANASE FERMENTATION MEDIA

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Abstract

Enzymes are protein-based catalyzers which organize the specific chemical reactions. Therefore, they could be used for lots of industrial applications. For the industrial grade, enzymes were extracted by different physical or chemical methods from the plant or animal tissues formerly. But these techniques are very hard to apply and provide enough enzymes for the industry. The production of the microbial enzymes could be done by a series of operations, which can be divided into two groups (upstream and downstream processes). Production of bulk or partial concentrated enzyme solutions are easier than the production of pure enzyme extracts. In this study, the effects of centrifuge and ultrafiltration on the production of concentrated β-mannanase enzyme extract from fermented carob pod medium by recombinant Aspergillus sojae were investigated. For this purpose, combination of time (5, 10, and 15 min) and speed (7000, 10000, and 15000 rpm) were tested for centrifuge assays. Then, ultrafiltration processes were performed with 10kDa and 30kDa membranes by using 1000 ml fermented media. Because the enzyme molecular weight was 50-60 kDa. Results showed that the centrifuge was not statistically important at β-mannanase enzyme purification in carob medium in point of enzyme activity. But both ultrafiltration membranes were help to improve the specific enzyme activity from 2176.65 U/mg (initial) to 2582.92 U/mg for 10kDa and 2718.89 U/mg for 30kDa ($p<0.05$). It was obviously seen from the results that 100ml concentrated enzyme extract was collected from the retentate.

Key words: *Centrifuge, Enzyme, Ultrafiltration, β-mannanase.*

INTRODUCTION

The chemical reactions are catalyzed in vivo or in vitro by the proteins which are called enzymes. Different extraction strategies for enzyme purification from animal or plant cell tissues were used in the past. But today industrial enzyme requirements could not be adequate by traditional methods from the animal or plant cell tissues.

Therefore, new biotechnological methods have been used to produce industrial enzymes by an economical and environmental ways. Amylases, pectinases, hemi-cellulases, invertases or etc. are the most produced industrial enzymes.

They have lots of different usage all over the world. β-mannanase is also one of the important industrial enzyme.

It has been used for the detergent formulas, pharmaceutical applications, paper and pulp production, animal feeds, instant coffee production, manno-oligosaccharides production etc. (Van Zyl et al., 2010; Lu et al., 2014).

The production of value-added products by biotechnological processes has been increasing day by day. But the downstream processing is also important for the purified value-added products to use for specific reactions.

There are lots of different ways to get purified value added products from fermented medium. But it is hard and not economical for some of the industrial usage.

So, partial purified or concentrated fermented medium could be used for general industrial applications.

Centrifuge, membrane filtration and other processes have been generally used to produce bulk enzyme solutions.

For this purpose, centrifuge and ultrafiltration were performed to get partial purified/concentrated bulk β-mannanase enzyme solution from fermented media by recombinant *Aspergillus sojae* in this research.

MATERIALS AND METHODS

Microorganism and medium

Recombinant *Aspergillus sojae* from Prof. Dr. Z.B. Ogel Laboratory (METU, Ankara, Turkey) (Duruksu et al., 2009) was used for β-mannanase production. Microorganism was sub-cultured on PDA (Potato Dextrose Agar) at 30°C for 4-5 days bimonthly and stored at 4°C (Ozturk et al., 2010).

Fermentation medium and fed-batch β-mannanase fermentation

Fed-batch fermentations were performed in a stirred tank bioreactor with addition microparticle agent (talcum). Carob pod extract was prepared as explained by Turhan et al., (2010). And fermentation media was prepared by enhancing 4 °Bx carob pod extract with 4 g/L of yeast extract, 0.5 g/L of $MgSO_4.7H_2O$, and 1 g/L of K_2HPO_4 (Ozturk et al. 2010). Then, fermentation was started with %1 inoculation of spore suspension (prepared with sterile salt-tween solution to be 10^7 spores/ml). The fermentation conditions were 400 rpm, no pH control, 1 lpm aeration, and 30 ºC. Whole fermentations were performed for 9 days. 10ºBx carob pod extract was also prepared to feed the media twice when the sugar concentration was under 5 g/L.

Concentration of fermented medium by centrifuge

Three different reverse speeds (7000 rpm, 10000 rpm, and 15000 rpm) and time (5 min, 10 min, and 15 min) were combined to concentrate the fermented medium. All processes were performed triplicated. After the centrifuge process, samples were taken from the supernatant. Enzyme activity, residual sugar and total protein analyses were performed to calculate the purification coefficient.

Concentration of fermented medium by ultrafiltration

Sartocon Slice 200 model (Sartorius Stedim Biotech, Goettingen, Germany) ultrafiltration system was used to ultrafiltrate fermented medium. For this purpose, 10 kDa and 30 kDa ultrafilters were used. Ultrafiltration process were started with 1000 ml fermented medium and stopped when the retentate was 100 ml. All processes were performed triplicated. Then permeate and retentate samples were analysed to calculate enzyme activity, residual sugar and total protein.

Analysis

Enzyme activity was determined by DNSA method and calculated from the mannose standard curve (Puchart et al., 2004, Ozturk et al., 2010). DNSA method was also used to determine the residual sugar as described by Miller (1959). Total protein analyse was performed with Thermo Scientific Coomassie (Bradford) Protein Assay Kit. The supernatants and other samples were treated with SDS-PAGE protocols to carry out molecular weight of β-mannanase enzyme (Karaoglan et al., 2016). Data were subjected to analysis of variance using the General Linear Models procedure of the Statistical Analyses System software (Version 7, SAS Institute Inc., Cary, NC). Differences among the mean values of the various treatments were calculated and the significance was defined at $p<0.05$ (Yatmaz et al., 2016).

RESULTS AND DISCUSSIONS

Concentration of fermented medium by centrifuge

Centrifuge processes were used not only for concentration of β-mannanase but also for filtration of filamentous fungi from fermented medium. So, different reverse speed-time combinations were performed to carry out the effect of centrifuge process on concentration of β-mannanase enzyme. Samples were taken from the supernatant and results were given in Table 1.

Table 1. Specific activity and purification coefficient for centrifuge assays

Sample	Specific β-mannanase act. (U/mg)		Purification coefficient
	Initial	After process	
7000 rpm-5 min	2149.86	2030.25^{b}	0.94^{b}
7000 rpm-10 min	2149.86	2006.44^{b}	0.93^{b}
7000 rpm-15 min	2149.86	2208.04^{ab}	1.03^{ab}
10000 rpm-5 min	2149.86	2180.70^{ab}	1.01^{ab}
10000 rpm-10 min	2149.86	2177.50^{ab}	1.01^{ab}
10000 rpm-15 min	2149.86	2041.01^{b}	0.95^{b}
14000 rpm- 5 min	2149.86	2173.58^{ab}	1.01^{ab}
14000 rpm- 10 min	2149.86	2342.59^{a}	1.09^{a}
14000 rpm- 15 min	2149.86	2364.98^{a}	1.10^{a}

*The differences between the values were statistically significant ($p<0.05$)

It was obviously seen from the results that 14000 rpm and its time combinations were always given the higher specific activities than initial fermented medium.

The highest specific activity value was 2364.98 U/mg for 14000 rpm-15 min. It was also given the highest purification coefficient to be 1.10.

The results showed that the centrifuge process was not statistically significant for concentration process.

So, the centrifuge process could just be used to separate filamentous fungi from fermented medium instead of filter paper.

But for this aim, continuous centrifuge system should be chosen to filter higher amounts of fermented medium.

Concentration of fermented medium by ultrafiltration

Before the ultrafiltration processes SDS-PAGE analyse were performed to determine the molecular weight of the enzyme. So, ultrafiltration cartridge with 10 kDa and 30 kDa cut-off value was chosen for the ultrafiltration processes.

1000 ml fermented medium were used for each ultrafiltration processes. The ultrafiltration was ended when the retentate was concentrated to be 100 ml. So, 900 ml permeate and 100 ml retentate was taken from the 1000 ml fermented medium. Then total protein, total enzyme activity, specific enzyme activity, and purification coefficients were calculated to carry out the best ultrafiltration process for concentration of β-mannanase. The results were given in Table 2.

Table 2. Specific activity and purification coefficient for ultrafiltration assays

Samples	Total protein (mg)*	Total enzyme activity (U)*	Specific activity (U/mg)*	Volume (ml)	Purification coefficient*
Initial	178.02[a]	387480.80[a]	2176.65[b]	1000	1[b]
10 kDa UF retentate	38.38[b]	99132.81[b]	2582.92[a]	100	1.19[a]
10 kDa UF permeate	0[c]	0[c]	0[c]	900	0[c]
30 kDa retentate	35.91[b]	97642.61[b]	2718.89[a]	100	1.25[a]
30 kDa permeate	0[c]	0[c]	0[c]	900	0[c]

*The differences between the values were statistically significant (p<0.05)

The initial specific enzyme activity was 2176.65 U/mg for fermented medium.

The highest specific activity value was calculated to be 2718.89 U/mg for 30 kDa retentate. It was also given the highest purification coefficient (1.25).

And, there were no protein and enzyme activity determined in the permeate solutions for 10 kDa and 30 kDa. So, the β-mannanase concentration by ultrafiltration processes was carefully done by 10 kDa or 30 kDa ultrafiltration cartridges. And, ultrafiltration results were statistically significant for concentration processes ($p<0.05$).

The SDS-PAGE analyse was also performed again to carry out the ultrafiltration performance.

The results were given in Fig 1.

It was obviously seen from the Figure 1 that β-mannanase molecular weight was 50-60 kDa. There wasn't seen any band for the initial of the fermentation.

The end of the fermentation β-mannanase protein band was appeared. Then the ultrafiltration processes were performed with different cartridges, and the β-mannanase protein band became bigger and clearer.

Because the protein was concentrated from 1000 ml fermented medium to 100 ml concentrated bulk enzyme.

So, the same amount of the samples of retentate samples was given the bigger and clearer band from the initial.

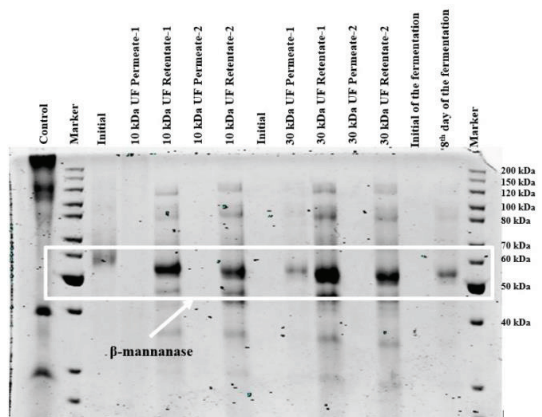

Figure 1. SDS-PAGE analyse for UF and fermentation

CONCLUSIONS

The production of value-added products by biotechnological processes has been increasing day by day. There are lots of different ways to get purified value added products from fermented medium. But it is generally hard and not economical for some of the industrial usage. So, partial purified or concentrated fermented medium could be used for general industrial applications. Consequently, the results showed that the centrifuge process were not statistically significant for concentration of β-mannanase ($p<0.05$). It could be just used for filtration of filamentous fungi from the fermented medium. But ultrafiltration process was statistically significant for β-mannanase concentration ($p<0.05$), and there was not any protein residue in the permeate. So, the results showed that the best ultrafiltration process was 30 kDa, and it could be used to concentrate the β-mannanase from the fermented medium.

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