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Original Research Article

Protein Enrichment of Olive Cake Substrate by Solid State Fermentation of *Lentinus edodes*

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HIGHLIGHTS

- Solid-state fermentation technique can be used for protein enrichment of the olive cake substrate (OCS).
- The nutritional value of olive cake substrate (OCS) was improved upon fungal treatment.
- *Lentinus edodes* fungi enhanced the protein content in experimental OCS.

ABSTRACT

Solid-state fermentation technique can be used for protein enrichment of the olive cake substrate (OCS). Among microorganisms, mushrooms, in particular, white-rot fungi , the genus *Lentinus* is known for its ability to digest the lignin and also the most effective producers of lignocellulosic enzymes. Hence, the objective of this work is to evaluate the effect of *Lentinus edodes* on protein content of agro by-product namely, olive cake substrate. To do so, solid state fermentation was performed at 25ºC in different conditions including various nitrogen sources, inoculum size, fermentation time, and moisture content using glass bottle as bioreactor. Protein extraction was carried out at 4ºC. The results showed significantly increasing protein content of OCS.

Introduction

During the last years, there has been an increasing interest in learning more about efficient exploitation of agro-industrial residues in the aim to provide an alternative approach in order to significantly reduce production costs and dealing with many ecological pollutions (Singhania, Soccol et al. 2008). During the industrial processing of olive oil in Iran, a solid residue known as olive cake substrate (OCS), which is annually a considerable waste material is produced. OCS is an additional product of olive manufacturing and is discharged as an agro-industrial waste, and the greatest amount of OCS is incinerated, which has caused many serious environmental problems (Alcaide and Nefzaoui, 1996; Baçaoui et al., 2001; Homapour et al., 2016).

In fact, OCS contains nutritional elements, such as proteins and carbohydrates, which allow the use of OCS as an enriched media that promotes the growth of a filamentous organism (Homapour et al., 2016). Therefore, efforts should be made to develop the potential for use of OCS in order to decrease the environmental pollution and also to produce valuable compounds in Iran.

For using of valuable OCS, it is necessary to increase protein content and this will be achieved when solid-state fermentation of filamentous fungi is used as a relatively low-cost and suitable technology (Cordova et al., 1998; Hölker et al., 2004; Krishna, 2005; Fadel and El-Ghonemy, 2015).

Solid-state fermentation (SSF) technique has gained a new attention for the upgrading of lignocellulosic residues in the recent years, mainly due to its advantages

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over submerged fermentation (Pandey et al., 1988; Toca-Herrera et al., 2007). Fadel et al. (2015) recommended SSF technique for protein enrichment of OCS for industrial purposes (Fadel and El-Ghonemy, 2015).

However, available information is insufficient for use of OCS as a medium for solid-state fermentation. The enhancement of the total protein is directly affected by the fermentation conditions in which the mycelium is grown (Rashad et al., 2011; Fadel and El-Ghonemy, 2015) Hence, the goals of this research are to assess the use of OCS as a substrate for *L. edodes* mycelium and to optimize the fermentation process. In this research, different experiments refer to the fermentation conditions that may improve the amount of total protein employed. Among microorganisms, mushrooms, in particular and white-rot fungi belonging to the genus *Lentinus* were selectively evaluated for its ability to digest the lignin and also the most effective producers of lignocellulosic enzymes (Forrester et al., 1990; Mishra et al., 1990; Rahi et al., 2009).

Lentinus edodes (*L.edodes*) (shiitake mushroom) is one of the most famous medicinal macrofungus. Modern pharmaceutical research shows that *L. edodes* has several physiological and health effects and seem to be powerful against many different types of cancer (Bisen et al., 2010).

The objective of this study is to evaluate the effect of *L. edodes* on protein content of agro by-product (OCS). For this proposes, different fermentation conditions in solid-state fermentation were investigated. One-factor-at-a time approach was used to improve protein content of substrate (OCS). By doing so, the positive effects of different conditions such as nitrogen source, humidity, inoculum size, and incubation time for the sustainable production were studied by solid-state fermentation in 250 mL glass bottle (bioreactor).

Materials and Methods

OCS substrate

Olive cake substrate was obtained from Fadak garden located in Qom. The samples of olive cake substrate (OCS), resulted from a continuous process of olive oil extraction, were collected and packed in polyethylene bags and kept in the dark place at -20°C until required for analysis.

Microorganism and media

L. edodes (strain D.P.B 319) was obtained from the Fermentation Laboratory, School of Pharmacy, Shahid Beheshti University (Tehran, Iran). The stock culture was maintained on malt extract agar (MEA) at 4°C and was sub-cultured monthly. The cultures were inoculated with mycelia and incubated at 25°C for 14 days.

Nitrogen source: Two different nitrogen sources including Molasses, Rice bran were used.

Seed culture on wheat by solid-state culture

Wheat grains (Mahpasandepars, Qom, Iran) were used as substrate and culture support for spawn preparation. The spawn medium was prepared by mixing 60 g of the grain (The grain was washed and soaked in sufficient distilled water for 24h, then the excess water was removed).

The medium was packaged (60 g of wheat grains in 250 mL glass bottle (bioreactor)) and autoclaved at 121°C for 45 min. Then, the substrate was inoculated with 5 agar disks (5 mm diameter). The culture was incubated at 25°C in the absence of light for a period of 14 days.

Solid-state fermentation

After preparation of spawn, the olive cake substrate was sterilized. Based on different conditions, each flask was filled with 45g of OCS and inoculated. Unless otherwise specified, all experiments were done in triplicates.

Sample preparation

To analyze the total protein content, all samples (fermented OCS) were freeze dried using Benchtop freeze drier (ALPHA 1-2 / LD Plus - Christ, Germany) and grinded using blender (Asan Toos Shargh, Model 1000) and also kept in freezer (-20°C) until they were required for analysis.

The determination of protein content

Determination of protein content was carried out using the Bradford method (Bradford 1976). Four mL of phosphate buffer (pH 7.6) was added to 500 mg of fermented OCS and shaken for 5 min followed by filtration using filter paper (Whatman 2). After 15 min, about 10 µL of the supernatant was added to 200 µL of Coomassie Brilliant Blue (CBB). The mixture was left for 2 minutes and added into a 96-well plate separately and the absorbance was read at 595 nm (SECOMAM CE, France). Protein content was calculated using a Bovine serum albumin (BSA) solution as the standard. The results were expressed as milligram of protein per gram of the fermented OCS. Finally, the results were represented in percentage form.

Fermentation condition

Humidity levels were determined between 50-70 %. Inoculum size was considered between 5-25 % (w/w) and fermentation time was determined between 5-35 days were used.

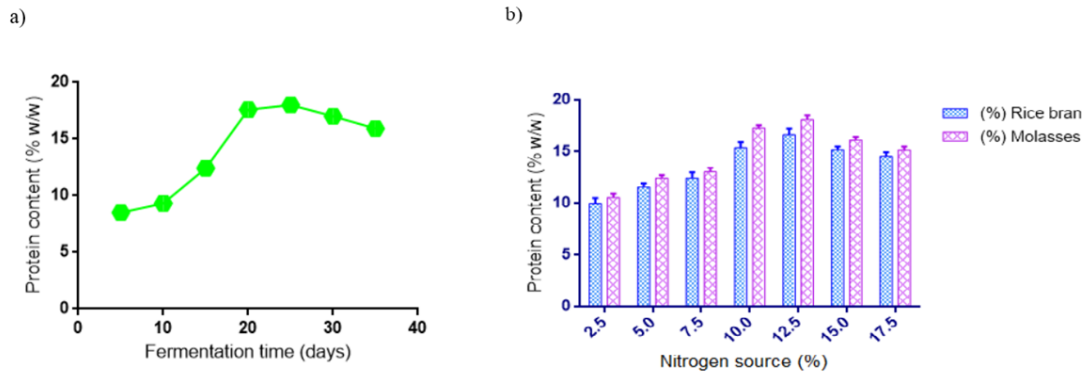

Figure 1. Change in the protein contents of olive cake substrate (OCS) by *L. edodes*: (a) effect of fermentation time (days) and (b) effect of different levels of two different forms nitrogen source (%) in growth medium. T = 25°C, substrate amount 45 g, inoculum size 15 % (w/w) and pH 5.5.

Statistical Analysis

The data represents the mean of three independent trials. Moreover, results were reported as mean and standard deviation.

Results and Discussion

Effect of fermentation time

Solid-state fermentation was carried out to increase the amount of protein. The optimum incubation time was evaluated by assessing the amount of protein content at different periods. Time course fermentation was carried out for 35 days. Maximum protein content was obtained after 25 days fermentation (total protein 18 % w/w). (Fig. 1a).

In this research the effect of fermentation time, nitrogen source, moisture content, and inoculum size was analyzed for *L.edodes* regarding enhanced production of total protein. The results obtained showing further fermentation after 30 days did not lead to an increase in total protein and the productions were declined. This suggests that reduction in total protein yield after prolonged fermentation time could be due to hydrolysis of proteins by *L. edodes* proteases. Indeed, attention to the fermentation time is a significant factor in protein production (Han et al., 2005; Pal and Khanum, 2010).

Effect of nitrogen source

Two different nitrogen sources at different concentrations (2.5-17.5 % w/w) were also used to observe the effect on total protein production. As shown in Fig. 1b, there was a significant difference between the two different forms of nitrogen sources for total protein production in Molasses and Rice bran under solid-state fermentation. The highest protein content was recorded in OCS treated bioreactor with 12.5 % (w/w) Molasses as a nitrogen source, and the lowest protein content was observed in OCS treated with 2.5 % (w/w) Rice bran.

Nitrogen source has a significant importance in production of total protein. The effect of two different forms of nitrogen source including Molasses and Rice bran on total protein production was determined by the addition of different concentrations of the two forms of nitrogen (Molasses and Rice bran). Each nitrogen source was mixed with the OCS medium. After fermentation, fermented OCS was collected for analysis. Both of these nitrogen sources increased the total protein production. The highest protein content was obtained on media containing 12.5 % (w/w) Molasses, an increase of around 18.2 % was also seen when compared with the unfermented OCS containing 8 % (w/w) of protein. Meanwhile using Rice bran affected protein content of OCS less than Molasses. Similar results have been reported (Haapala et al., 1994; Levin et al., 2010; Huang and Zhang, 2011).

Effect of moisture content

To evaluate the effect of moisture content on total protein production by *L. edodes*, OCS flasks was moistened with distilled water from 50-70 % (v/w) (Pandey et al., 1988; Raimbault, 1998; Pandey et al., 1999; Pandey, 2001; Shen et al., 2008).

The results clearly demonstrate that the protein content reached to its maximum amount (16.5 % w/w) at 65 % moisture level and also, further increase in moisture content caused a gradual decrease in the amount protein content (Fig. 2a).

Moisture content has important roles in the solid-state fermentations. In this work, solid-state fermentation of *L. edodes* on OCS for total protein production resulted in protein content of up to 16.5 % at 65 % moisture level (comparable to 8 %: unfermented OCS). However, further

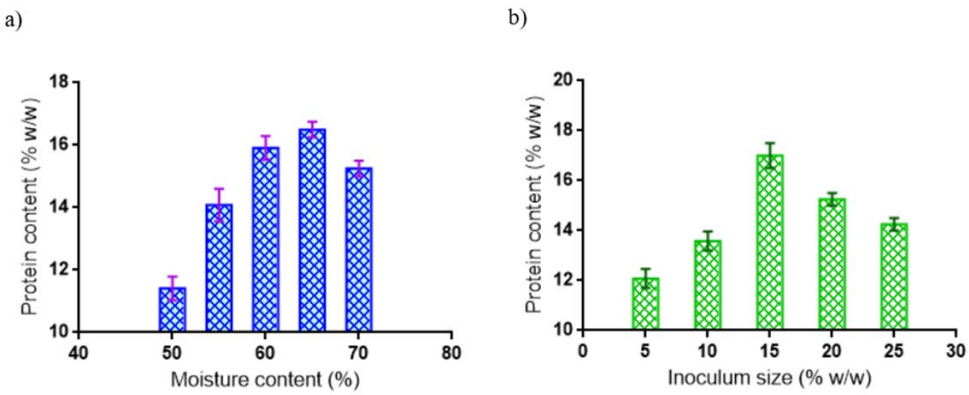

Figure 2. Change in the protein contents of olive cake substrate (OCS) by *L. edodes*: (a) effect of moisture content and (b) effect of inoculum size. Incubation time 30 days, T = 25°C, substrate amount 45 g, and pH 5.5.

increasing in moisture content decreased the total protein. Many researchers believe that 60- 70% initial moisture was the optimum for protein production from fungi on solid substrate (Gervais and Molin, 2003; Krishna, 2005; Pal and Khanum, 2010). Insufficient moisture content can result in a reduction of the *L. edodes* growth and metabolism due to the decrease in the available OCS nutrients, air, and also high concentration of metabolites which severely inhibit fermentation process.

It was also worth noting that the fungal sporulation was also induced by insufficient moisture content in response to undesirable conditions of process, whereas high moisture content can result in a significant increase in bacterial contamination (Raimbault, 1998; Krishna, 2005).

Effect of inoculum size

Different inoculum sizes i.e. 5- 25 % (w/w) were used to demonstrate their effect on increase in the protein content. The obtained results (Fig. 2, a) revealed that the total protein was increased by an increase in the inoculum size and attained the maximum total protein (17 % w/w) with 15 % (w/w) of inoculum, after 25 days of solid-state fermentation. Further increase in the inoculation size did not lead to an increase in the total protein (Fig. 2b).

Protein content of the OCS was increased by increasing the inoculum size. However, extended fermentation time did not lead to an increase in the total protein. It has been recommended that higher inoculum size has an inhibitory effect on production of biomass. Indeed, it may be correlated to the fact that nutrients and oxygen are consumed more quickly due to greater inoculum size. In contrast, the fermentation starting time was long due to lower inoculum size (Raimbault, 1998; Pandey et al., 1999; Krishna, 2005; Lakhtar and Roussos, 2016).

Conclusion

Fermentation process is an alternative method for production of valuable compounds. However, limitations and difficulties of production methods restrict the large-scale manufacturing of nutraceuticals and valuable products. Solid-state fermentation via microbial cultivation has been advanced as a new approach for production of value-added products from agricultural residue (Pandey, 2001). As a result, in this study, our data confirm that solid-state fermentation is a powerful technique for development of these compounds. In summary, our results demonstrated that, *L. edodes* can produce 18.5 % (w/w) total protein in presence of 15 % (w/w) of inoculum, which is higher than other studies. Considering all experiments studied, *L.edodes* is more efficient in solid-state culture and shows higher performance when used for the enhancement of the total protein.

In this work, solid-state fermentation by *L. edodes* fungi resulted in enhancement of the protein content in experimental OCS. According to the obtained results, it can be suggested that the use of OCS by *L.edodes* will be a promising approach for conversion of agro by-products to higher quality compounds, enhancing their application by industrial processes.

Finally, in this investigation, the results demonstrated that *L. edodes* is capable of enhancement of total protein and this ability was dependent on culture media and fermentation conditions. Various experimental conditions were used for solid-state cultivation of *L.edodes* and production of total protein. Molasses was shown to be superior in terms of nitrogen source. Also, it was concluded that fermentation conditions including nitrogen source, moisture content, inoculum size, and incubation time significantly influenced on total protein enhancement in

solid-state culture.

Meanwhile, the protein content of fermented OCS increased during the fermentation process at different conditions in OCS. In conclusion, this study can be a basic attention for solid-sate fermentation and great value for production of total protein and nutritional attributes of fermented OCS efficiently by *L. edodes* with effective lignocellulosic enzymes.

On the other hand, we need to further research the obtained information regarding feasibility of study involving important factors. Indeed, the use of OCS in solid-state fermentation and total protein production should be evaluated in protein content, improved nutritional value of the OCS, and also sustainable production of enzyme, protein, and essential amino acids.

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Competing Interests

The authors declare that there are no conflicts of interest.

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