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Determination of the Amount of Protein and Amino Acids Extracted from the Microbial Protein (SCP) of Lignocellulosic Wastes

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Abstract: With the increasing world population, the use of lignocellulosic wastes for production of microbial protein as animal feed becomes a necessity of our time. In order to verify the most productive protein, the amount of protein and amino acid extracted from Single Cell Protein (SCP) needs to be determined by an effective method. In this study Microbial protein was produced by treatment of wheat straw with *Pleurotus florida;* with heat at 100°C and NaOH 2% as substrate by solid state fermentation. Concentration of protein was 62.8% per 100 g of dried microbial protein. Then the extracted protein hydrolyzed with HCl 6 Normal for 48 h under 110°C temperature condition. Then the amino acids analyzed by using A-200 Amino Nova analyzer. The results of this study indicated that the ratio of essential amino acids to total amino acids was 65.6%. The concentration of essnyial amino acids were: Lysine = 9.5, histidine=19.8, threonine=0.6, valine = 6.6, methionine = 2.1, isoleucine = 7.3, leucine = 6.8, phenylalanine = 4.3 and arginine = 8.3 g/100 g of extracted protein that indicated the obtained microbial protein can be a good or suitable substitute in the food program of animal feed.

Key words: Single cell protein, *Pleurotus*, white rot fungi, wheat straw, animal feed

INTRODUCTION

The accumulation of lignocellulosic wastes around the world and the efficient capture of their bioconversion potential have become issues that are yet to get resolved (Kumar *et al*., 2008). Developing countries have a great opportunity to use their natural resources (Zadrazil *et al*., 1995). Despite the many studies in microbial protein production to date, future research is still needed especially into the improvement of the lignocellulolytic power of microorganisms aiming for selective delignification (Villas-Boas *et al*., 2001). Fungi, bacteria and seaweed cultivated on a large scale, can be used as feed. These micro-organisms are very attractive feedstuffs because they can be cultivated on agro-industrial wastes with production of large amounts of cells rich in proteins that commonly contain all the essential amino acids. In addition to favorably having high vitamin and mineral levels (Dorado *et al*., 1999; Kuhad *et al*., 1997). Further, growing microbes on lignocellulosic wastes win furnish all the hydrolytic enzymes often added in the preparation of feeds and also makes the minerals more available for absorption by the animal (Villas-Boas *et al*., 2001).

Lignocellulosic materials such as wood and straw are renewable resources. These vast resources are the potential source of Single Cell Protein (SCP) (Vassal, 2002). Lignocelluloses are generally composed of 30-56% cellulose, 10-24% more hemicelluloses, 3-30% lignin and 3-7.2% protein. The major limitations of straw as an animal feed are its low digestibility and low protein content.

Close associations of cellulose with lignin prevents full microbial digestion of straw in the rumen (Fazaeli, 2007). During microbial process for conversion of lignocellusic wastes into feed at least one of the three objectives must be reached: an increase in the protein level, an increase in digestibility and an increase in the essential amino acids (Kamara and Zadrazil, 1988).

Various physical and chemical treatments are available to improve the nutritive value and digestibility of these agricultural wastes (Fazaeli, 2007).

Delignification of cereal straws by white rot fungi is of great interest and has been investigated to improve the digestibility of wood or straw for animal feed (Agosin *et al*., 1999; Valmaseda *et al*., 1991; Vares *et al*., 1995). *Pleurotus* have the ability to grow on a wide range of unfermented plant wastes (Bano and Srinivasan, 1962; Hadar *et al*., 1993). This is of particular relevance as the

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biological value of food protein depend on its amino acids profile. The suitability of an organism for commercial protein production should be based on its protein content, determined as accurately as possible and its amino acid composition (Vega *et al*., 2005). In addition to the efficiency of converting substrate carbon and inorganic nitrogen into organic nitrogenous compounds. Moreover, the quality of the product should be tested in animal trials (Christias, 1975). The present investigation was conducted to examine the protein and essential amino acids composition of microbial protein that was produced by *Pleurotus florida* on treated wheat straw as a substrate. This microbial protein was found nutritionally beneficial as a dietary protein where contains essential amino acids for which proper growth and good function of immunity system of their body.

MATERIALS AND METHOD

Cultures and maintenance: This study was carried out from 2008 to 2009 at the Alzahra University, Iran. *Pleurotus florida* were provided kindly by Dr. Mohammadi Goltapeh, E., Tarbiat Modarres University in the year of 2008. The cultures were maintained by sub-culturing Potato Dextrose Agar (PDA) slants at 25°C. After 7-9 days, they were separated and washed with distilled water. The spours were then added to the treated wheat.

Inoculation development: Inoculants for culture were produced on boiled wheat grains supplemented with 0.2% calcium carbonate and 1.2% calcium sulfate for adjusting the pH and avoid them sticking to each other. Cultures were incubated at 25°C for three weeks in a dark place and these grains with mycelium were used as inoculant.

SSF substrate preparation: Wheat straws were cut into 2 cm pieces and treated with 2% NaOH and sterilized at 100°C for 30 min. The straws were washed with distilled water and dried in the oven under 80°C then 30 g of treated dried straws as a substrate in 1000 mL conical flasks moistened with Mandel's culture with 0.3 g L^{-1} urea [$CaCl_2$ 0.3 g, Urea 0.3 g $(NH_4)_2$ SO_4 1.4 g, KH_2 PO_4 2 g $ZnSO_4$. 7 H_2O 1.4 mg $MnSO_4$. H_2O 1.6 mg, $FeSO_4$. 7 H_2O 5.0 mg/$CoCl_2$ 2.0 mg L^{-1}]. The flasks were autoclaved at 100°C for 15 min and then inoculated with wheat grain based inocula with *P. florida*. The cultures were incubated at 25°C in a dark room for 4 weeks until the mycelium of the fungi is fully grown. The mixture was then dried at 60°C for 24 h and turned into a very fine powder.

Protein extraction: Protein extraction was performed by preparation of the buffer composed of: Tris, HCl pH = 8, Glycerol, SDS, 2-Mercaptoethanol and distilled water. Microbial protein to the amount of 0.5 g was added into this buffer and boiled for 7~18 min. After cooling down, the solution was centrifuged at 14000 g, then supernatant of the solution was taken and added to acetone at -20°C to precipitate. Protein later was solved in Tris HCl, pH = 8 and result is concluded based on Bradford method.

Amino acid analysis: To analyze the obtained amino acids, the protein needed to be dried first. This was done by adding the protein contained in Ttris solution into the pure acetone 99% at -20°C. The produced protein was then collected for analysis of its amino acids.

Methods: To analyze the amino acids, following 3 steps need to be taken as: (1) Acid hydrolysis, (2) Derivetization and (3) Separation.

The protein was hydrolyzed by HCl 6 Normal at 110°C for 48 h and then the German made Amino Nova-A200 analyzer was used for derivetization and separation steps. The same process was repeated for 3 times and the chromatograms obtained were compared against the standard solution. Thirty two bit control software amino control including optimized programs was used as controlling software and also Acquisition and Analysis software amino peak was used as chromatography software (www.aminoNova.de/document).

RESULTS AND DISCUSSION

Wheat straw pretreated with heat at 100°C, NaOH 2% and inoculated with *Pleurotus florida* and after that it has been used for protein analysis by using Bradford method. Protein concentration of microbial protein was determined. Result of protein analysis confirmed that this product had 62.8% protein per 100 g of dried microbial protein.

Results of essential amino acids analysis of microbial protein of dried treated wheat straw with *Pleurotus florida* obtained by this study is shown in Table 1 which indicates the amount of Threonine (Thr), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Phenylalamine (Phe), Histidine (His), Lysine (Lys) and Arginine (Arg) in g/100 g extracted protein of microbial protein.

Table 1: The amount of essential amino acids in SCP in this study g/100 g protein

Essential amino acid	Average
Thr (T)	0.64
Val (V)	6.68
Met (M)	2.11
Ile (I)	7.32
Leu (L)	6.82
Phe (F)	4.37
His (H)	19.88
Lys (K)	9.55
Arg (R)	8.30

Table 2: Comparison of microbial protein with fungi, egg and cake oil of cotton seed g/100 g protein

Essential amino acid	*Pleurotus* sp. (a)	Egg (a)	Cotton seed (b)	This study
Thr (T)	4.2	4.9	3.2	0.6
Val (V)	4.6	7.3	4.5	6.6
Met (M)	1.2	4.1	1.3	2.1
Ile (I)	5.8	8.0	3.2	7.3
Leu (L)	4.4	9.2	5.9	6.8
Phe (F)	2.0	6.3	5.4	4.3
His (H)	2.1	2.1	2.6	19.8
Lys (K)	5.0	7.2	4.1	9.5
Arg (R)	6.7	6.4	11.1	8.3

a: data adapted from Bano and Srivasan (1962) b: Data adapted from Smith, K.J

As shown Table 1 results were further compared against the obtained value from the other researches and found compatible Table 2. The highlight of the research in hand is the fact that by the new and innovative methods, it was managed to obtain a better result in comparison to the previous researches and indicates that obtained amino acids by this research are almost equal to that of egg and more than that of fruit of *Pleurotus* sp. in g/100 g protein (Bano and Srivasan, 1962).

To improve the nutritious value of the cereal straws and lignocellulosic wastes by the use of white rot fungi, the selected fungi, substrate and its environmental conditions need to be optimized (Zadrazil, 1985).

The decisive factors in characterization of a preferred type of micro-organism are its spreading speed and its saprophytic power to produce more protein (Zadrazil and Brunnert, 1981).

In fact, the higher the percentage of protein in cereal straws after treatment by fungi, will the better nutrition value of the animal feed (Jwanny *et al*., 1995). The effects of increasing dietary crude protein level on nitrogen retention and intestinal supply of amino acids were studied in lambs fed diets based on alkaline-treated wheat straw that shown flows of all essential amino acids increased with increasing crude protein (Willims *et al*., 1991). So in this research NaOH 2% used as alkaline treatment to get better results. Protein is very essential for growth and repair of the damaged tissues. Moreover, it is very critical for the development of the immunity system at the beginning of the growth. Various researches have indicated that feeding laying hens with high percentage of protein can lead to production of the body organs and Timus and Bruce glands in new born babies of domestic animals and birds (Novak *et al*., 2006). The increasing of dietary protein density is effective in improving broiler performances (Khadem *et al*., 2006).

An ideal protein is the one which has a good balance of Essential amino acids and can provide necessary nutrition for the animals. This protein should in the meantime contain Sufficient Nitrogen for production of the non essential amino acids by itself (Christias, 1975; Keshavarz and Austc, 2004).

In this study the microbial protein produced by *Pleurotus fiorida* contains a lot of essential amino acids which provide a good quality animal feed.

This becomes important when we know that human and animal bodies can not produce any essential amino acids and should receive them through food stock. Essential amino acids are composed of : Threonine (T), Valine (V), Methionine (M), Isoleucine (I), Leucine (L), phenylalanine (F), Histidine (H), Lysine (K) and Arginine (R), each of which plays a major role in growth, repair of the damaged tissues and correct performance of the Immunity system (Osborne, 1974).

With simple comparison between the essential amino acids obtained from this research with what is available in egg and cotton seed cake oil, we can understand that a suitable percentage of essential amino acids exist in our SCP. The optimum amount of protein in the animal feed depends on their type and age (Shapiro, 1968). As the animals get older; they need less protein and more energy. For producing soft meat in domestic animals, necessary attention must be paid to the percentage of Lysine in their feed (Latshow, 1976).

In fact, increasing the percentage of lysine in animal feed has been recently turned into focal point for many researchers. Therefore finding various biotechnological methods has become strategically very important.

Here, the best technique for production of portion and analysis of the amino acids in the microbial protein was used. Accurate analysis of amino acids depends very much on extraction of protein without pollution and additional compounds such as fat and various salts in the sample. Several advantages have been cited in the use of spores rather than vegetative cells for inoculums. They can serve as a biocatalyst in bio conversion reactions because they are often able to carry out the same reactions as the corresponding mycelium. Other advantages include convenience and greater flexibility in the coordination of inoculums preparation. The appropriate enzyme systems must be produced before the fungus begin to utilize the substrate and grow (Krishna, 2005).

In this study, the protein was extracted most efficiently. Treating the cereal straws with heat causes the phenolic compounds to be released. It has also a positive effect in producing lignocellulosic Enzymes (Kutlu, 2000).

While treating with NaOH 1% and 2%, the researchers found out that the higher the concentration of NaOH, the more protein is produced. Lignin after pretreatment by NaOH gets hydrolyzed and as the result more suitable carbohydrates is made available for the fungi. Lignin is a three-dimensional phenyl propanoids polymer. Biological delignification by micro-organisms under solid state fermentation produce lignolytic enzymes. White rot fungi, particularly *Pleurotus* sp. can use the wheat straw as the substrates (Krishna, 2005).

As the amount of the nitrogen in the cereal straws is low in value, we need to supply adequate amounts of nitrogen for the growth of micro organisms (Carrilo *et al*., 2005). In this connection, the use of Mendel's culture will serve as a nutrition source as well as providing adequate humidity for solid state fermentation in addition to making available for the fungi the other essential minerals and metals with only 3% g L^{-1} concentration. Various researches have shown that adding nitrogen source to the solid state fermentation will produce more biomass (Dias and Sundestol, 1986). It is needless to say that the effect of nitrogen source on the enzyme activity of the fungi depends on the type of micro-organism. These show that the amount of produced protein in the culture has a reverse relationship with the density of Urea. This is because the culture has become more alkaline (Kutlu, 2000).

Therefore using heat at 100°C, NaOH 2% and Urea 3 g L^{-1} is the best condition at which the most and best quality protein was getting produced. Following these experiences, the researchers could produce more microbial protein from micro-organisms with higher nutritious quality than before. The chemical composition of treated with *Pleurotus ostreatus* increased the CP content of the wheat straw (Fazaeli, 2006; Zadrazil *et al*., 1996). In corporation of cotton seed powder 3% mixed with rice straw as the substrate, while culturing *Pleurotus florida* enhanced mushroom yield and increasing crude protein and essential amino acids.The total protein content showed 37.2% in dried edible mushroom. (Shashirekha *et al*., 2004). In this study, a comparison was made between each set of results. The amount of essential amino acids obtained through this study against previous researches was tabulated as fallows in Table 3 that indicates our result is almost the same as other microbial Proteins treated by *Cellulomonas* sp., Alcaligenes and Alfa Alfa (Han, 1974) in g/100 g microbial protein.

Table 3: Comparison amongst several microbial proteins

Essential Amino acid	*Cellulomonas* sp.	*Alcaligenes faecalis*	*Alfa alfa*	*Pleurotus florida* This study
Thr (T)	4.37	4.46	5.12	0.64
Val (V)	6.79	7.01	6.70	6.68
Met (M)	1.69	2.70	1.96	2.10
Ile (I)	4.20	5.40	5.54	7.32
Leu (L)	8.66	7.64	8.43	6.82
Phe (F)	3.69	4.11	5.75	4.37
His (H)	2.96	2.53	2.53	19.88
Lys (K)	8.00	9.92	6.92	9.55
Arg (R)	6.18	4.85	6.82	8.30

As shown Table 3 various amounts of proteins and Essential amino acids can be produced by the micro-organisms with the lignocellulosic wastes as the substrates (Han, 1974).

It also shows the importance of their elements in the animal feed. In fact those that are low in comparison with International standards need to be improved both in quality and quantity aspects to answer the need of the animals for a proper food.

Nowadays obtaining adequate information regarding the nutrition quality of the animal feed has become a necessity of life. Animals need nutritious feed for proper growth and these affect the reaction of their immunity system and the volume of the antibodies and proper performance of Bruce and Timus glands in comparison to the other organs. The essential point in this connection is to make sure having a suitable amount of protein at the beginning of the growth is guaranteed. In developed countries, the interest is mostly for live stock feeding industry because farm animals generally provide sufficient amount of essential amino acids for human diets (Ufaz and Galili, 2008). Various studies have shown that for obtaining proper performance of above organs, we need to ensure that the amino acids in their feed are more than amount (Osborne, 1974). Also mixing of date waste and rice straw as the substrate ror growing *Pleurotus ostreatus* had shown suitable source of protein (27.44%) of dried fruting bodies that containing amounts of essential amino acids (Jwanny *et al*., 1995).

Lysine is one of the essential amino acids which are important for growth (Prochaska and Carey, 1996). Due to this fact it needs to be added in the animal feed adequately in order to improve hemoglatination and increase the M and G immunoglobulin (Coleman, 2003). The need for dietary Lysine is greater for breast meat yield than for growth rate (Dozier *et al*., 2008). In this study the amount of lysine obtained was 9.55 g/100 g of extracted protein which is very ideal and optimal.

Also argentine is one of the essential amino acids which has an assistive role in the activity of macrophages for phagocytosis, which kills bacteria and factor of intercellular pathogens. In this study the amount of

Arginine obtained was 8.3 g/100 g protein which is an optimal value. However, with the antagonistic effect between lysine and arginine amino acids (Hurso, 1995), the result obtained indicates a good balance between them. It must be mentioned that the antagonistic effects of amino acids are of rare occurrence and has been observed where high level of amino acids have been consumed (Dozier *et al*., 2008).

As the other achievement of the current research and study in hand is the high percentage of the histidine in the microbial protein which has been 19.88 g/100 g protein. Histidine is one of those essential amino acids which play a major role in the growth and repair of the tissues at the beginning of growth and in producing the myelin sheet in the nerves cells. More over Histidine from metabolic point of view is a producing requirement for histamine and carnosine and in addition is a good Source of carbon atoms in purine synthesis. Histidine is also playing an important role in the production of white and red blood cells and in releasing heavy metals from the body and has great roles in active site of many Enzymes (Osborne, 1974).

In this study the amount of Histidine has been the highest amongst the other amino acids obtained and we may in the future succeed in its extraction and purification. This will surely be an important step in the medical science.

Valine is another essential amino acid which is produced from the pyruvic acid and by the micro-organisms. Valine is an aliphatic and hydrophobic amino acid which will cause the proteins to be held beside each other (Dozier *et al*., 2008).

As in the structure of immunoglobulin high amounts of Valine and thereonine have been used, then the lack of any of these two will negatively affect the response of the immunity system. Further with proper amounts of leucine, Valine and Isoleucine the antagonistic affects on the performance of immunity system get reduced (Peganova and Eder, 2002; Peganova, 2003). This conditions can exist while amount of Isoleucine in the feed is lower than the expected and proper percentage of this three amino acids in the produced microbial protein have no negative effects on bodies (Burnham, 1992). Others have shown by increasing the amount of Threonine in the food portion of the laying hens they have increased the weight of the produced eggs (Ishibashi, 1998; Rangeel-lugo, 1994). Further it was found that feeding laying hens with low protein food portion can produce eggs with lower protein and visa versa (Novak *et al*., 2006). They further found out that sulfur contained amino acids such as Cysteine and Methionine can produce eggs with the stronger shells and improve the production of feather on the laying hens (Kalinowski *et al*., 2003). The role of sulfur amino acids and optimal intakes for physiological substrates such as glutathione are currently considerable interst in human and animal health (Ball *et al*., 2006).

In the study in hand, the amount of Valine = 6.68, leucine = 6.82, isoleucine = 7.32, Threonine = 0.64 and methionine = 2.1 g per 100 g of protein have shown that we have obtained an ideal and optimal set of results at last.

Looking at the amount of the protein and amino acids obtained in this study, one can proudly claim that microbial protein is a valuable source of animal feed and can replace the current enriched feed stuck available.

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