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Research article

Enhancement of protein contents of rice bran for animal feed by solid state fermentation

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Abstract
The present study was conducted to focus on the output of single cell protein by solid state fermentation on rice bran. *Candida utilis* and *Rhizopus oligosporus* has been used in this study for the fermentation of rice bran for the nutritional enhancement which then can be used for cattle feed. A number of parameters such as incubation temperature , moisture, substrate ratio, inoculums age, incubation period and inoculums size were studied to evaluate the improvement of single cell protein (SCP) production. Analysis of variance test (ANOVA) was applied to compare the replicate values. Results showed that the maximum crude protein was obtained when the inoculums size 10% (v/v) and 48 hours old culture was practiced. A fermentation period of 60 hours, the incubation temperature of 30°C and moisture level of 500 ml in growth medium was found to get maximum protein yield and viable count of yeast and mold. After complete optimization of fermentation parameters, a batch of rice bran fermented with *Candida utilis* and *Rhizopus oligosporus* at optimized conditions, moved over a maximum crude protein yield, i.e., 33.14% as compared to 7.22% of unfermented rice bran. Significant values at optimized conditions revealed that there is a considerable improvement in nutritional contents of rice bran through solid state fermentation for animal feed.
Key words: Single cell protein; *Candida utilis*; *Rhizopus oligosporus*; solid state fermentation.

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Introduction

Protein is derived from the Greek word "Proteios" which means the beginning. The epithet was given because they are the foremost among the natural polymers that are important for the development and sustenance of life. Proteins are present in all living tissues as the building block components of the physical structure. Proteins are an important dietary component of the supply of nitrogen as well as sulfur. In addition, they are structural parts of the physical structure that provide mechanical support to the body and are necessary for all life processes and are important for proper maturation and growth of whole living beings including human and animals. Its deficiency may lead to a number of disorders in an individual, as high population in this cosmos, especially those existing along the poverty line is suffering from malnutrition [1, 2].

Thither is a great divergence in the demand of protein rich food so its supply to ever increasing world population. Single cell protein (SCP) is an alternative non conventional protein source. Single cell protein may be utilized straight off as human food and it may be applied in animal feed to partially replace the currently used protein rich soybean meal and fish proteins and even cereals, which can be derived from human use of goods and services [3, 28]. The intention behind the single cell protein production is to fulfill the short protein supply and partly in the commercial growth in the economic advantages derived by the substitution of microbial protein from the conventional protein sources used in animal feed [4]. Microorganisms can utilize waste materials for example, *Candida utilis* and *Rhizopus oligosporus* to enhance the protein content of rice bran. SCP production has the potential to fulfill the feeding requirement of the increasing world population at cheaper rates. As a source of protein, it is very important in solving protein shortage problem for animal feed [3, 4].

Candida utilis has been used in the fermentation process for the production of single cell proteins which is used for the nutritional enhancement of the product. It has been immensely evident that for the success of the aquaculture industry, it is now important to develop low cost, high quality protein food stuffs. Fish meal is the major source of protein in the aquaculture feeds and it mainly consists of 250-400g/kg of the formulated feed. Alternative food stuffs should be developed to replace the costly protein sources [5, 6]. In urban areas the people are health conscious and consume large quantity of fruits and cereal crops, e.g. rice and straw, these traditional food sources generate large amounts of waste such as rice bran, wheat bran and fruit peels. *Rhizopus oligosporous* can be employed as microorganisms to ferment these substrates to feed supplement for the animal feed including domestic animals and cattle and also for human consumption [7, 28].

Solid state fermentation is a best suited method because it involves the growth of microorganisms specially yeast and fungi on the solid material in the absence of free floating water. The wide range of solid materials used in solid state fermentation can be of two types, inert synthetic materials and non inert organic materials. The former acts as an attachment place for the fungus whereas later one also functions as a source of nutrients, due to which it is called a support substrate. Filamentous fungi are suitable for SSF and the hyphal growth of fungi, and their good tolerance to low water action and high osmotic pressure conditions make fungi an efficient microorganism in the natural micro flora for bioconversion of solid substrates like Rice bran [8]. And also yeast has some specific characteristics as they have larger size (easier to harvest) low nucleic acid content and ability to grow at acidic pH. And yeast has accepted in the long history of its uses in the fermentation process.

SSF is cost effective due to use of simple growth and production media comprising agro-industrial residues, uses a small quantity of water which lets go a negligible and low amount of effluent, thus reducing pollution problems. Solid state fermentation is a simple process of fermentation as it uses low volume equipment; provide concentrated products, aeration process (Availability of atmospheric oxygen to the substrates) since oxygen deficiency does not happen as there is increased diffusion rate of oxygen into the moistened solid substrate supporting the growth of aerial mycelium [9, 10]. In present research work, the nutritional contents of rice bran was significantly improved by optimizing a number of diffrerent parameters of incubation like temperature, inoculum age and size, moisture, and incubation period through solid state fermentation.

Materials and Methods

This research was carried out for the production of single cell protein through solid state fermentation of rice bran by *Rhizopus oligosporus* and *Candida utilis* for animal feed. Experimental work was carried out at the Pakistan Council of Scientific & Industrial Research, Laboratories Complex, Lahore (PCSIR).

Substrate and Culture

Rice bran was used as substrate for production of single cell protein through solid state fermentation, which was brought from the local grocery store and was screened to remove any impurities and dirt and kept in a clean polythene bag in the laboratory until used. The strain of *Rhizopus oligosporus* and *Candida utilis* were provided by Microbiology Laboratory, Food and Biotechnology Research Center, PCSIR, Laboratory Complex Lahore, Pakistan. These strains were cultured on a potato dextrose agar (PDA) (Oxoid) for 48 hours at 30°C. The cultures were then preserved at 4°C and further shifting on the PDA slant at the interval of 15 days to keep the viable.

Inoculum preparation

For the production of 1 kg batch of single cell protein, 250 ml flasks were used and inoculum was prepared separately consisting of 1g glucose, 0.2g Potato starch, 0.125g Diammonium phosphate (DAP) dissolved in 50ml water. The media was autoclaved at 121°C for 15 minutes. When the temperature of autoclaved media reached to room temperature; one flask was inoculated with *Candida utilis* and the other one with *Rhizopus oligosporus.* Then the inoculated flasks were kept in water shaker bath at 100 rpm at 30°C for 48 hours.

Fermentation of growth media

To achieve the purpose of production of SCP through the solid state fermentation the growth media comprises of 1kg substrate (Rice bran) was mixed with 20g Diammonium phosphate, Chloramphenicol 0.4g, in the diluent (Tap water) (e.g. 450 ml water in case of rice bran) with the moisture level to optimize the effects of enhancement of protein production. After that 50ml *Candida utilis* and 50ml *Rhizopus oligosporus* inoculum were added, respectively, and were mixed homogeneously throughout the substrate. The substrate was taken in the plastic bag, and the plastic bag was pierced with a syringe for aeration and the holes were about 1cm distance apart. After that, weight the bag with moisture; the labeled batch was put in incubator at 30°C for 48 hours.

Proximate Analysis of rice bran

Proximate composition of dried fermented and dried unfermented food sample were determined in respect of entire proteins, fats, fiber, amino acid, ash and moisture content as the percentage composition of the product. The crude protein was determined by Kjeldhal method, while others were studies by standard procedure [11].

Proximate analysis of substrate before fermentation

Before performing the experiments the proximate analysis of rice bran was performed to assess the nutritional value of the unfermented rice bran, so that the results can be compared with the fermented rice bran whose nutritional value was improved after optimizing the parameters affecting the production of single cell protein. Unfermented rice bran whose protein content was 7.22% and had moisture percentage 11.03%, ash percentage 6.22%, fat percentage 3.35%, fiber percentage 11.21% **(Table 01)**.

Optimization of production parameters

To optimize the process of solid state fermentation process, several experiments were performed in which various factors like size of inoculum, age of inoculum, incubation temperature, moisture level, incubation period were studied as they are important fermentation parameters which affects the process of solid state fermentation. So these parameters were used to improve the growth of *Candida utilis and Rhizophus oligosporus* on the inoculated rice bran batches. And thus the nutritional value of the rice bran was improved by applying the maximum single cell protein yield after the agitation procedure. Various parameters of production such as fermentation period (24, 36, 48, 60, 72 hours), inoculum size (5, 10, 15, 20, 25%) v/v, incubation temperature (20, 25, 30, 35, 40°C). Inoculum age (24, 48, 60, 72, 84 hours), and different moisture level (40, 45, 50, 55, 60%) w/v were optimized by *Rhizopus oligosporus and Candida utilis* in solid state fermentation using rice bran as substrate.

Statistical Analysis

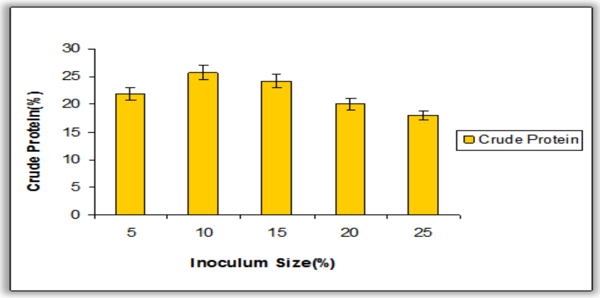
The data was subjected to statistical analysis for the determination of significance by using ANOVA.

Results and Discussions

Beneficial results were obtained by using strains of *Rhizopus oligosporus* and *Candida utilis.* Optimized conditions for the single cell protein production were incubation temperature 30°C, incubation period 60 hours, moisture level 500ml and age of inoculum was 48 hours, inoculum size was 10% optimized. Different inoculum sizes ranging from 5 to 25% (v/v) were used in the fermentation medium. Maximum crude protein was obtained at 10% (v/v) of fermentation medium, 25.74% was the maximum crude protein and maximum yeast and mold count was 4.5×10^5 CFU/g at 10% (v/v) of inoculum size **(Figure 1)**. The minimum crude protein, 18.01% and lowest yeast and mold count 1.2×10^2 cfu/g were obtained when 25% size of inoculum was used. Rajoka *et al*., [12] studied the kinetics of batch Single cell protein production from rice polishing with *candida utilis* in continuously aerated tank reactors and obtained maximum yield of biomass when they used 10% (v/v) inoculum size in rice bran substrate. Oshoma and Ikenebomeh, [13] studied the production of single cell protein from rice bran by using *Aspergillus niger,* and they used 2% (v/v) inoculum size to obtain the maximum yield of protein. Fungal spores loading of 108 to the orange waste medium produces the protein content of 39.65% of *Chaetominum sp.* and 30.47% of *A. niger* [14]. Ravinder [15] obtained maximum biomass of Aspergillus oryzae while using 3% (v/v) inoculum size on the deoiled rice bran which is in accordance with the outcomes of the research study. The divergence in the maximum yield while using different inoculum sizes may lie in the fact that different microorganisms, substrates and fermentation techniques were employed. Inoculum size of 7.5% (v/v) was suitable for the maximum output of crude protein from Candida utilis [16]. Fruit wastes extracts also yield a maximum cell biomass of *Candida utilis* with 4% inoculum size [17]. The issue of agitation speed and aeration rate of the Candida utilis biomass and the intracellular protein content was examined C. *utilis* inoculum of 10^6 cells /ml (7.8% v/v) was cultured in 1.5 L pineapple waste material in a 2-L fermentor for 30 hours at 30°C [18].

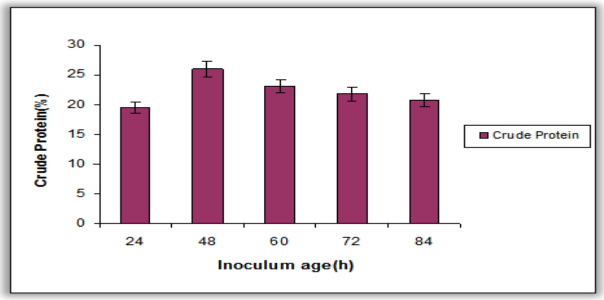
In case of the effect of age of inoculum, our results shpwed that 48 hours old culture used for the agitation of the rice bran, and maximum growth of yeast and mold cell 3.85×105 CFU/g and maximum crude protein 26.02% was obtained. While the minimal grade of crude protein was obtained when the sample was inoculated with 24 hours inoculum it gives 19.45% crude protein and 2.2×102 CFU/g yeast and mold development **(Figure 2)**. In other study, microorganisms cultured under the same conditions with difference in composition [19]. Rapidly growing microorganisms contain a high content of carbohydrates, lipid or less protein resulted when microorganisms cease to grow. Therefore microorganisms can be managed according to need [20]. Fermentation for the production of single cell protein from rice polishing was carried out in the 50-L fermentor using thirty liter medium under optimized culture conditions. The medium was steamed in situ in the case of the fermentor. The medium was inoculated with a vegetative inoculum of 24 hours old culture [21].

Figure 1: Effect of different inoculum size on single cell protein production from rice bran using *Candida utilis* and *Rhizopus oligosporus* under solid state fermentation.



Note: Bars represent the standard deviation among three replicates, which differ significantly at $p \leq 0.05$.

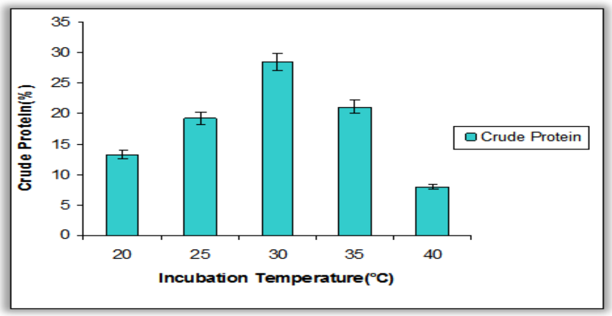
Figure 2: Effect of different ages of inoculums on the production of single cell protein from rice bran using *Candida utilis* and *Rhizopus oligosporus* under solid state fermentation.



Note: Bars represent the standard deviation among three replicates, which differ significantly at $p \leq 0.05$.

Maximum crude protein and cell development was obtained when the culture medium was kept at 30°C. At this temperature the crude protein obtained was 26.46% and the total viable count was 3.5×10^{5} CFU/g. As this is the appropriate temperature for the development of the yeast cells. Minimum grade of crude protein, 7.98% was obtained from fermented rice bran when the fermented culture was incubated at 40°C **(Figure 3)**. Similar outcomes were obtained by Adoki [22], as he was working on factors affecting the yeast protein and protein yield production from orange, banana and plantain wastes processing residues using Candida species. The incubation temperature of 25°C produces maximum protein for *Chaetomium sp.* and *A. niger*. The production of SCP by *C. utilis* at 25-35°C revealed that there was a sudden increase from 25-35°C in the protein production. It was likewise discovered that protein production decreases as temperature increases up to 35°C which was significant temperature for the development of human microbial pathogens at 37°C. This temperature is suitable for animal feed. Roma and Ooi [23] also noted the consequences during the investigation of effect of agitation and aeration rate for the production of C. *utilis* growing on pineapple waste material while the temperature was 30°C at 900 RPM. Rajoka [10] concluded that the end result of the fermentation process is affected by temperature of fermentation medium. Fermentation process for the production of SCP by *C. utilis* was carried out at different temperatures (20-45°C) using stirrer fermentors. Maximum crude protein was obtained at 35°C. After this there was a decrease in protein production as temperature decreases [24]. Lemmel [25] observed that more carbon was consumed at 32°C, 34°C than 27°C for the production of *Candida utilis* and *S. fibuliger* on potato processing waste water.

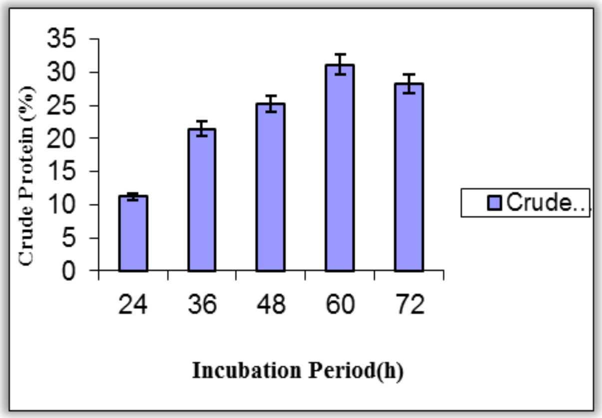
Figure 3: Effect of incubation temperature on the production of single cell protein from rice bran using *Candida utilis* and *Rhizopus oligosporus* under solid state fermentation.



Note: Bars represent the standard deviation among three replicates, which differ significantly at $p \leq 0.05$.

The effect of different incubation periods was studied in the solid state fermentation process during this research work **(Figure 4)**. A set of experiments was performed to evaluate the impact of on the overall yeast and fungi growth and the protein content enhancement to increase the nutritive value of rice bran. All the fermentation media were inoculated with 10% initial inoculum and incubation period at 30°C for 24 to 72 hours. The results showed that maximum growth and crude protein was obtained when the inoculated substrate was incubated for 60 hrs maximum crude protein after this period was 31.16% and yeast and mold count was 3.8×10^{6}CFU/g. After 60 hours again the total protein content decreases may be due to the production of poisonous metabolites and sporulation which may kill the yeast and fungi. The minimum yield of protein and cells was obtained at 24 hours. The crude protein obtained was 11.2%, yeast and mold count was 1.8×10^{1} CFU/g. The nutrient evaluation of fermented rice bran as poultry feed was carried out. The effect of temperature of rice bran by *Aspergillus niger* was observed. Incubation time of 72 hours of fermented rice bran contained the high nutritive value [26]. Munawar [17] optimized fermentation process for 69 hours for setting maximum yield of *Candidia utilis.* Ravinder [15] studied the effect of the fermentation period in the production of mutant strain of *Rhizopus oligospous* SCP from deoiled rice bran maximum protein was obtained at 72 hrs [27] also observed that 72 hrs is the optimum condition of the fermentation period for single cell protein production by *Candida sp.*

Figure 4: Effect of different incubation periods in the production of single cell protein from rice bran using *Candida utilis* and *Rhizopus oligosporus* under solid state fermentation.

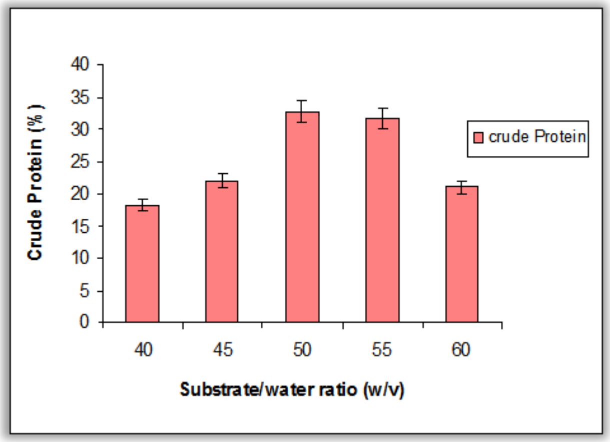


Note: Bars represent the standard deviation among three replicates, which differ significantly at p≤0. 05.

The effect of moisture was studied for SCP production and it was found that a maximum crude protein was obtained when 50% (w/v) moisture level was used. The crude protein obtained on this condition was 32.74% and yeast and mold count was 4.5×10^6 CFU/g. The minimum crude protein, 18.22% and yeast and mold count was 3.1×10^3 CFU/g using moisture level of 40% (w/v) **(Figure 5)**.

A batch of I kilogram rice bran was processed through the SSF process to examine the process parameters at relatively large scale. All the optimized fermentation conditions applied were of optimized conditions **(Table 1)**. As a result of proximate analysis of fermented and non-fermented rice bran, moisture percentage 3.45%, ash percentage 6.98%, fat percentage 3.26%, fiber percentage 5.81%, crude fiber percentage 33.14% of fermented rice bran was obtained so compositional analysis of fermented rice bran indicates that protein content and nutritive quality of rice bran was improved by using *Candida utilis* and *Rhizopus oligosporus* as compared to unfermented rice bran whose protein content was 7.22% and had moisture percentage 11.03%, ash percentage 6.22%, fat percentage 3.35%, fiber percentage 11.21%. And also the amino acid profile of non fermented and fermented rice bran was analyzed by the thin layer chromatography (TLC) method. It was found that fermented rice bran had high amino acid content than non fermented rice bran.

Figure 5: Effect of different moisture level in the production of single cell protein from rice bran using *Candida utilis* and *Rhizopus oligosporus* under the solid state fermentation.



Note: Bars represent the standard deviation among three replicates, which differ significantly at p≤0. 05.

Figure 6: Amino acid profile of unfermented and fermented rice bran analyzed by thin layer chromatography (TLC), Unfermented rice bran (1), fermented rice bran (2).

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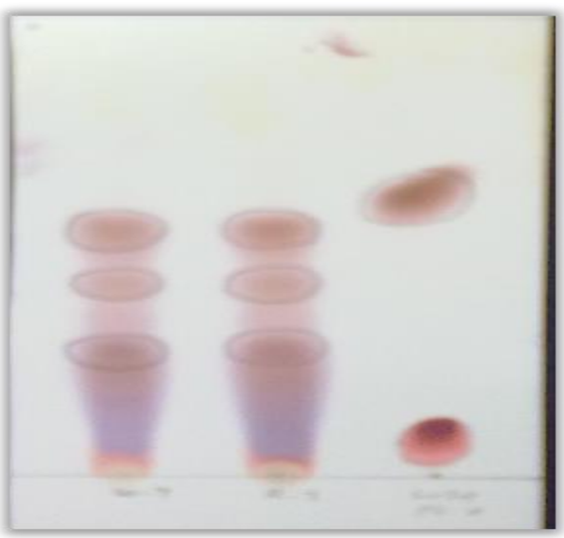

Table 1: Comparison of Proximate analysis of fermented and unfermented rice bran at optimum conditions

Rice Bran	Moisture (%)	Ash (%)	Crude fat (%)	Crude fibers (%)	Crude protein (%)
Non-fermented	11.03% ±0.55	6.22% ±0.31	3.35% ±0.16	11.21% ±0.56	7.22% ±0.36
Fermented	3.45% ±0.17	6.98% ±0.34	3.26% ±0.16	5.81% ±0.29	33.14% ±1.65

Conclusion

It is concluded that to meet the protein requirements of the increasing population there is a need to enhance the domestic fowl and livestock output. There is great need for the alternative, unconventional protein sources. Also, it is important that these sources should be cheaply and easily available in Pakistan. Rice bran is frequently available source in Pakistan and it is used to produce single cell protein from rice polishing using *Candida utilis*. it is likewise employed in the poultry feed as by nutrient evaluation of fermented rice bran. The stake in the production of single cell protein has been increased because these microbes (Fungi and Yeasts) can ferment variable type of waste materials so it plays role in the conservation of the environment. Besides, it is resolved that the mixed culturing of microorganisms is a really secure method for winning over the carbohydrate wastes into high yields using solid state fermentation process. The fermentation process for rice bran was also very much improved by optimizing the parameters of incubation temperature, inoculum age, inoculums size, moisture, and incubation period.

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