Ethiop. J. Biol. Sci. **13(2): 87-98, 2014**
 ISSN: 1819-8678

SINGLE CELL PROTEIN EXTRACTION FROM ORANGE WASTES USING *ASPERGILLUS NIGER* ISOLATE NO. 5 UNDER SOLID STATE FERMENTATION

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ABSTRACT: An attempt was made to optimize protein extraction from sweet orange wastes (SOW) and orange pulp wastes (OPW) using *Aspergillus niger* isolate No. 5 by solid state fermentation. Maximum protein enrichment of 52.48% and 46.50% were made using the substrates of SOW and OPW, respectively, on the seventh day of incubation at 30°C. The single cell protein quantified from the fungus grown on malt extract broth enriched with SOW and OPW were 23.14% and 21.35%, respectively; whereas the protein extracted from the growth of the fungus on potato dextrose culture broth enriched with SOW and OPW were 23.60% and 21.48%, respectively. The highest production of intracellular protein (70.46%) was obtained from OPW in comparison with that of SOW in soluble protein as intracellular protein (65.31%). It was clearly indicated that the standardization and determination method of protein using SOW and OPW wastes by *A. niger* isolate No. 5 has produced the maximum protein enrichment under solid state fermentation.

Key words/phrases: *Aspergillus niger* isolate, Orange wastes, Protein enrichment, Standardization of protein, Solid state fermentation.

INTRODUCTION

The world citrus production was estimated to reach 68 million metric tonnes (MT) (FAO, 2014) of which only 45% was processed to yield juice, essential oils and other byproducts (FAO, 2003) indicating that a large amount of citrus wastes is disposed as citrus pulp wastes that causes both economic and environmental problems such as high transportation cost, lack of dumping site and accumulation of high organic content material (Tripodo *et al*., 2004; Pourbafrani *et al*., 2010). This necessitates the focus on alternative ways of treating and transforming these wastes to animal feed by using different microorganisms such as *Chaetomium*, *Aspergillus*, *Fusarium* and *Penicillium* species with different solid state fermentation (SSF) (Singhania *et al*., 2009; Biniyam Yalemtesfa *et al*., 2010; Nasseri *et al*., 2011; Tesfaye Alemu, 2013).

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Biniyam Yalemtesfa *et al*. (2010) and Tesfaye Alemu (2013) reported that the sweet orange waste (SOW) and orange pulp waste (OPW) are cheap raw materials suitable for the production of protein for food and animal feed. However, optimization of protein production of these wastes under different environmental and nutritional conditions under solid state fermentation has not been undertaken. Therefore, the objective of this study was to use *Aspergillus niger* No. 5 to optimize the production of protein under different environmental conditions under solid state fermentation.

MATERIALS AND METHODS

Preparation of substrates

Fresh-squeezed SOW and OPW wastes were obtained and purchased from local juice shops in Addis Ababa city. Some orange samples were collected from orange growing areas of Nazareth, Metahara, Hawassa and Zeway areas of Ethiopia. The samples were sun dried for five days and ground to fine particle size using mortar and pestle (locally made of metal) and stored in plastic bags and kept at 4°C. The samples of orange peel and skin were cut into small pieces of 1-1.5 cm and sterilized at 121°C for 15 minutes. The *Aspergillus niger* isolate No. 5 was obtained from Mycology laboratory. The *Aspergillus niger* isolate was used for inoculating 10 g of SOW and OPW. After seven days of growth of fungal culture on the substrates, 2 g of growth was taken and homogenized with buffers for standardization and determination of the amount of protein produced using *Aspergillus niger* isolate No. 5 (Tesfaye Alemu, 2013).

Inoculum preparation

Spore suspension of *Aspergillus niger* isolate No. 5 was obtained by growing the fungal isolate on malt extract agar and PDA at 30°C for 7 days. A single disc was taken by 5 mm cork borer aseptically from young mycelial growth of each plate into 20 ml sterile distilled water and mixed well by adding four drops of Tween 80. The number of spores was counted by microscopic observations using Heamocytometer (Neubauer counting chamber). The spore count of the suspension was found to be 10^6 spores/ml (Tesfaye Alemu, 2013; Getachew Gashaw *et al*., 2014). The suspension was made fresh for every experiment.

Solid state fermentation (SSF)

Five grams of SOW and OPW adjusted to 60% moisture content (Tesfaye Alemu, 2013) with 0.05 g of $(NH_4)_2SO_4$ was replicated in 250 ml Erlenmeyer flask and autoclaved at 121°C for 15 min. The substrate was inoculated with 2 ml of spore suspension (10^6 spores/ml) of *A. niger* isolate. The culture was maintained in stationary conditions at 30°C for seven days (Tesfaye Alemu, 2013). Samples were taken every day from each substrate that was inoculated by *A. niger* isolate No. 5. For this experiment, the samples were analyzed after 3, 5 and 7 days of incubation of SOW and OPW which was inoculated by *A. niger* isolate No. 5 (Singhania *et al*., 2009; Tesfaye Alemu, 2013). The soluble protein content and total nitrogen were measured by Folin method (Lowry *et al*., 1951) and modified Kjeldhal method (Sahlemedhin Sertsu and Taye Bekele, 2000).

Effect of moisture content, temperature and pH on protein enrichment

The standardization and optimization of moisture content, temperature and pH for the determination and production of maximum yield were according to Tesfaye Alemu (2013).

Effect of particle size on protein enrichment of substrate

The effects of using different size of particles less than 0.71 mm and greater than 0.71 mm of substrates (SOW and OPW) on protein enrichment were evaluated and optimized at 60% moisture content, pH 7 and 30°C (Ravindra *et al*., 2009; Tesfaye Alemu, 2013).

Time course of different fermentation culture broths on protein enrichment

The 60% moisture levels of 10 g SOW and OPW in 250 ml Erlenmeyer flask were autoclaved at 121°C for 15 minutes. After incubation, 10 g of SOW and OPW was inoculated with 2 ml of spore suspensions of *A. niger* isolate. The culture broths of Czapeck Dox's, malt extract and potato dextrose were incubated in stationary conditions at 30°C for 3, 5 and 7 days (Tesfaye Alemu, 2013). The effect of incubation period on the production of protein was evaluated and determined by *A. niger* isolate No. 5 under solid state fermentation. Samples were taken every day from each organism, and soluble protein content and total nitrogen were measured by Folin method (Lowry *et al*., 1951), and modified Kjeldhal method (Sahlemedhin Sertsu and Taye Bekele, 2000).

Methods of standardization and determination of protein extraction

Analysis of intracellular and extracellular protein

Aspergillus niger isolate was grown on three fermentation culture broths (malt extract, potato dextrose and Czapeck Dox's broths) for the production of mycelial biomass and determination of protein after seven days incubation at 30°C (Tesfaye Alemu, 2013). The amount of extracellular protein (soluble protein) secreted into the medium (broth) was measured by Folin method (Lowry *et al*., 1951). The *A. niger* isolate was grown in malt extract broth for seven days. Extracellular protein was measured from the culture filtrates of the fungal isolate. Sample from the filtrate was taken by Eppendorf tubes and centrifuged at 10000 rpm for 30 minutes. The supernatant obtained was used for standardization and determination/ estimation of protein content (Tesfaye Alemu, 2013).

Similarly, the dried mycelial mats (0.3-0.8 g) of *A. niger* isolate was first ground by mortar and mixed with 5 ml of 1 N sodium hydroxide (NaOH). Then, it was heated at 90°C for 10 minutes in a water bath. Samples were taken from treated solution in Eppendorf tubes and were centrifuged at 10000 rpm for 30 minutes. The supernatant was used for standardization and determination/estimation of soluble protein by Folin method (Lowry *et al*., 1951).

RESULTS

Effect of particle size on protein enrichment

The result showed that the amount of protein yield increased with an increase in particle size of substrates of SOW and OPW. The maximum protein enrichment of 24.04% (SWO) and 22.76% (OPW) was obtained with greater than 0.71 mm mesh size while the optimum protein enrichment of 22.73% (SOW) and 22.02% (OPW) were attained at 0.71 mm mesh size (Fig. 1).

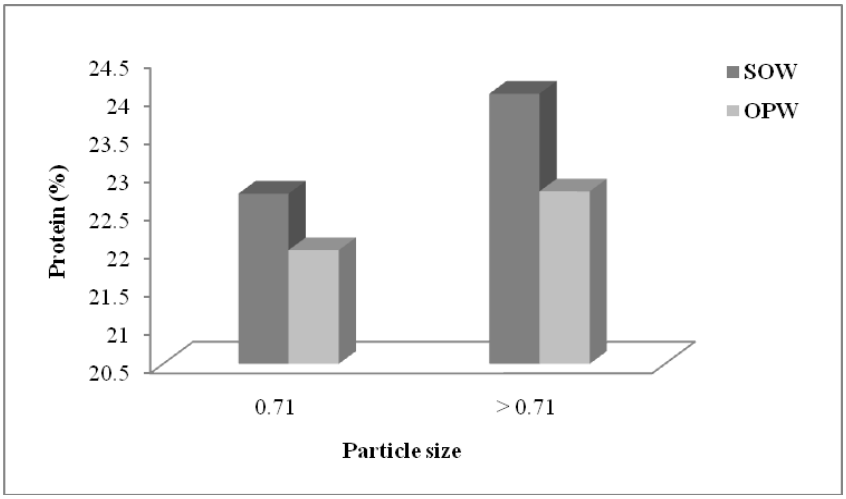

Fig. 1. Effect of particle size on protein enrichment of sweet orange waste (SOW) and orange pulp waste (OPW) by *A. niger* isolate No. 5.

Time course of different fermentation culture broths on protein enrichment

Maximum protein enrichment of 52.48% and 46.50% were observed using SOW and OPW, respectively, on the seventh day after incubation at 30°C. The lowest microbial protein enrichment of 15.6% and 15.64% were recorded, respectively, with SOW and OPW substrates on the third day after incubation at 30°C (Fig. 2).

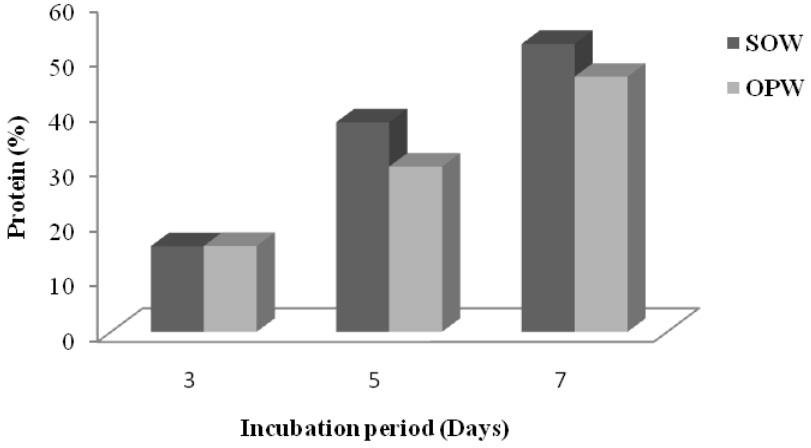

Fig. 2. Effect of incubation period on protein enrichment of sweet orange waste (SOW) and orange pulp waste (OPW) by *A. niger* isolate No. 5.

Effect of mycelial biomass production on standardization and determination of protein enrichment

Aspergillus niger isolate produced maximum mycelial biomass on SOW substrate (0.93 g and 0.80 g) after seven days of incubation on malt extract broth and potato dextrose culture broths, respectively. Similarly, the optimum mycelia biomass was produced on substrate OPW (0.8 g and 0.7 g) after seven days of incubation on potato dextrose and malt extract culture broths, respectively. The lowest mycelial biomass was obtained on substrates SOW (0.69 g) and OPW (0.59 g) on Czapeck Dox's culture broth (Fig. 3).

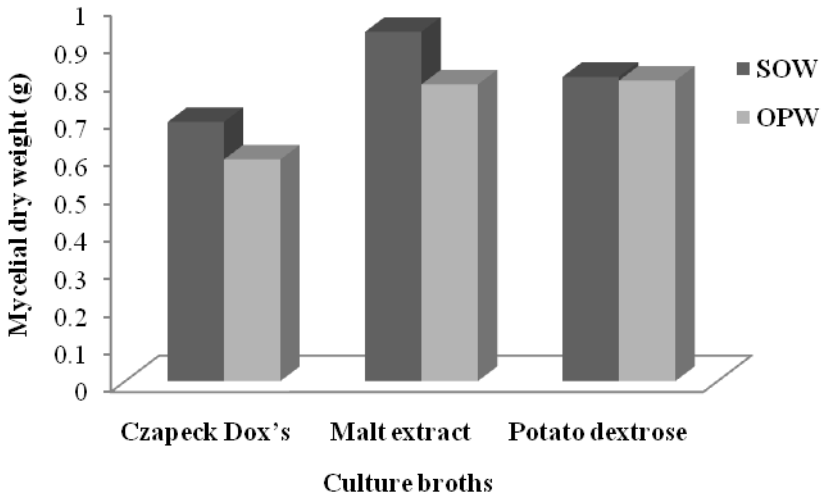

Fig. 3. The effect of different culture broths on mycelial biomass of sweet orange waste (SOW) and orange pulp waste (OPW) by *A. niger* isolate No. 5.

Effect of different culture broths on protein enrichment

The maximum protein of the biomass quantified indicated that the biomass produced on malt extract and potato dextrose culture broths consisted of 23.14% (SOW) and 21.60% (OPW) protein enrichment, respectively. The optimum amount of protein 20.06% (SOW) and 18.78% (OPW) were obtained by the fungal isolate grown on Czapeck Dox's broth culture (Fig. 4).

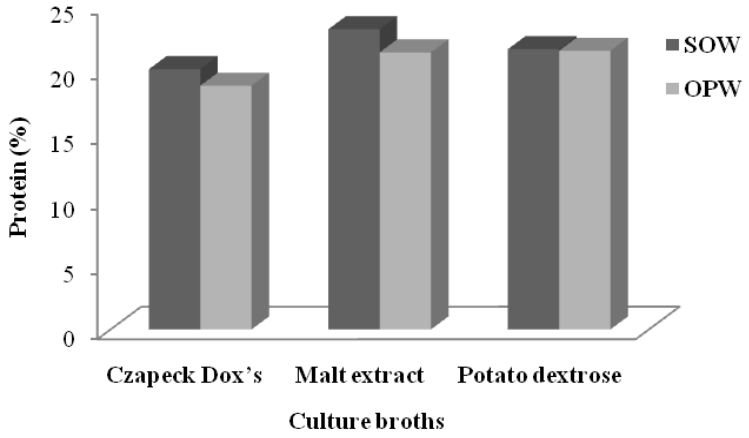

Fig. 4. Effect of different culture broths on protein enrichment of sweet orange waste (SOW) and orange pulp waste (OPW) by *A. niger* isolate No. 5.

The amount of extracellular and intracellular proteins produced using SOW and OPW substrates by *A. niger* isolate No. 5

The maximum amount of soluble protein (extracellular protein) obtained by *Aspergillus niger* isolate No. 5 using culture broth of SOW and OPW were 23.14% and 21.60%, respectively. The highest and optimum production of protein 70.46% and 65.31% were obtained using OPW and SOW substrates by *A. niger* isolate No. 5, respectively (Fig. 5).

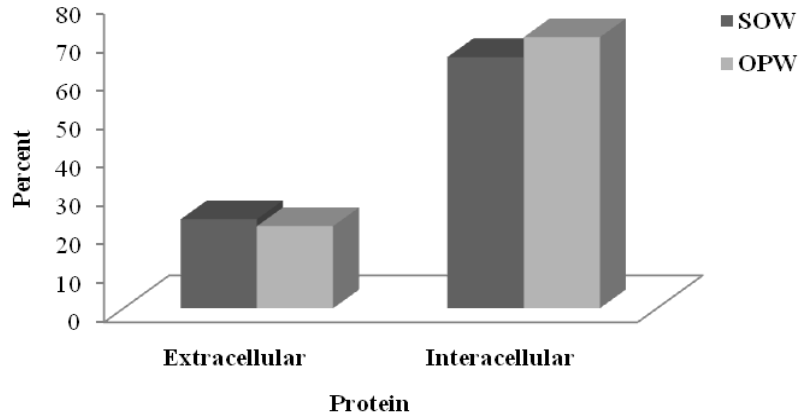

Fig. 5. The amount of extracellular and intracellular proteins produced using sweet orange waste (SOW) and orange pulp waste (OPW) by *A. niger* isolate No. 5.

Optimization and determination of protein extraction

Among the various buffers, 1.0 N NaOH extracted the maximum protein level (12.00 g) and 12.02 g/100 g of starting medium of dry matter using SOW and OPW substrates by *A. niger* isolate No. 5, respectively. The extraction that was done by Na_2HPO_4 buffer (pH 7) resulted in the lowest amount of protein using 11.3 and 9.56 g/100 g of starting medium dry matter, substrates SOW and OPW, respectively (Fig. 6).

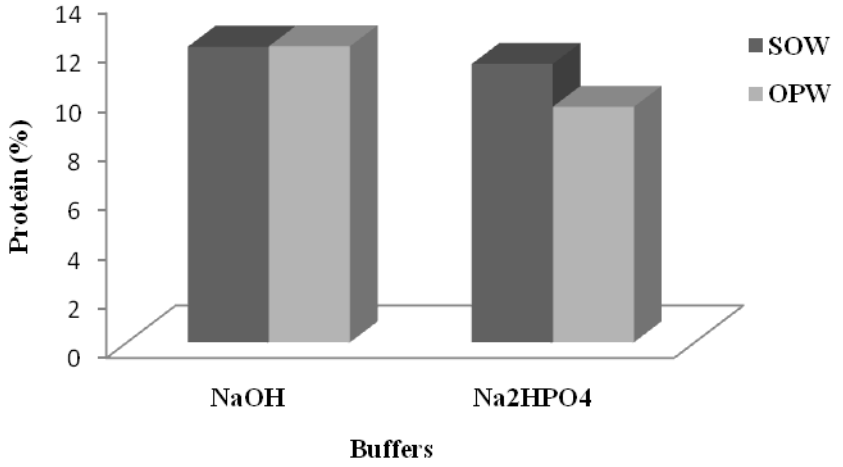

Fig. 6. Standardization and determination of protein extraction method using sweet orange waste (SOW) and orange pulp waste (OPW) by *A. niger* isolate No. 5.

DISCUSSION

In this experiment, optimum conditions for the production of high amount of protein enrichment was found effective at 60% moisture content; 30°C temperature; pH 7.0; 10^6 spores/ml inoculum concentration; $(NH_4)_2SO_4$ (inorganic nitrogen supplements) and particle size equal to or greater than 0.71 mm using SOW and OPW substrates by *A. niger* No. 5. The standardization and optimization of this experiment was done according to Tesfaye Alemu (2013). The result of this experiment has shown that the maximum protein enrichment of 24.04% SOW and 22.76% OPW were obtained with greater than 0.71 mm mesh size. Subsequently, the optimum protein enrichment of 22.73% (SOW) and 22.02% (OPW) was attained at 0.71 mm mesh size *A. niger* No. 5. However, Ravinder *et al*. (2006) have obtained protein when *A. niger* MTCC 1846 was grown on de-oiled rice

bran (ADOB) of various particle sizes ranging from 20-70 mm mesh size. Protein yield increased with the increase in particle size. The minimum protein yield was found at 20 mm mesh size (14.50%) while maximum protein (18.70%) was obtained at 70 mm mesh size.

The maximum protein enrichment of 52.48% and 46.50% were observed using the substrates of SOW and OPW, respectively, on the seventh day, after incubation at 30°C by *A. niger* No. 5. A similar result was obtained by Biniyam Yalemtesfa (2007) using *A. nige*r (KA-06) who found 34.2% of protein content enriched on the fourth day. Similarly, Oshoma and Ikenebomeh (2005) obtained maximum protein on the sixth day from *A. niger* that was grown on rice bran. The result of this experiment indicated that increasing the time of incubation for seven days increased the amount of microbial protein. Similar result was obtained by Daubresse *et al*. (1987) that the increase in protein was due to high fungal protein formed in the course of fermentation. *Aspergillus niger* isolate No. 5 produced maximum mycelial biomass on substrates SOW (0.93 g) and OPW (0.80 g) after seven days of incubation on malt extract broth and potato dextrose culture broths, respectively.

The maximum protein of the biomass quantified indicated that the biomass produced on malt extract and potato dextrose culture broths consisted of 23.14% (SOW) and 21.60% (OPW) protein enrichment, respectively. The crude protein content of dry biomass matter was also found as 42.29% by *A. niger* (KA-06) (Biniyam Yalemtesfa, 2007). Results of microbial protein enrichment overall yields of 12.6% was obtained using *A. niger* to ferment cassava peels (Obadina *et al*., 2006). However, John (1980) has grown *A. niger*, *Fusarium oxysporum* and *Fusarium manilforme* in a synthetic medium and obtained protein contents of 39.1-58.2% of biomass dry matter. Czajkowska and Ilnicka-Olenjniczak (1988) reported protein content of 34.8% using *A. oryzae*. Moo-Young *et al*. (1983) suggested that the difference in biomass protein content could also be attributed to the variation in the proportion of substrate utilized for energy purpose and synthesis of intracellular biomass protein.

The optimum amount of secretion of soluble protein (extracellular protein) of 23.14% (SOW) and 21.35% (OPW) was produced by *A. niger* isolate No. 5. However, the maximum production of intracellular protein of 70.46% and 65.31% were obtained using substrates of OPW and SOW, respectively. Similarly, the product, PRUTEEN can be added to animal feed as a supplement and contains 72% crude protein and has a high content of

vitamins (Nasseri *et al*., 2011). Similarly, *Aspergillus niger* (KA-06) has shown relatively higher amount of secretion of protein which was found to be 65.03% of its total protein as intracellular protein (Biniyam Yalemtesfa, 2007).

Among the tested buffers, NaOH (0.1 N) at pH 7.0 extracted the maximum protein level of 12.00 g and 12.02 g/100 g of starting medium dry matter using substrates SOW and OPW, respectively. However, the use of SOW and OPW substrates that were extracted by phosphate buffer resulted in the lowest amount of protein (11.3 and 9.56 g/100 g) of starting medium of dry matter. Similar result was obtained by Biniyam Yalemtesfa (2007), NaOH (0.1 N) who extracted the highest protein of 11 g/100 g of starting medium of dry matter using the orange pulp wastes from *A. niger* (KA-06).

It can be concluded from this study that sodium hydroxide extraction buffer at pH 7.0 is the most efficient among the buffers used for the extraction of protein using SOW and OPW substrates by *A. niger* isolate No. 5. In this experiment, *A. niger* isolate No. 5 was made to use the fiber of orange wastes to produce the microbial protein and it was shown that it is responsible for the reduction in crude fiber content because of fermentation and biodegradation processes of SOW and OPW wastes. The production of microbial proteins under solid state fermentation (SSF) of orange waste products is one of the most promising approaches for increasing the availability of proteins. The microbial conversions of orange wastes into protein enrichment are an innovative practical approach for protein supplementation of animal feed. Therefore, the production of a large amount and of best quality protein for animal feed using the orange wastes in solid state fermentation is very important in order to overcome shortage and insufficient protein for animal feed in the country.

ACKNOWLEDGEMENTS

I am grateful to Publication and Research Office and Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University for funding this project and providing laboratory facilities. I wish to express my sincere thanks to Dr. Amare Gessesse (Institute of Biotechnology, Addis Ababa University) for critical evaluation and valuable suggestions on this manuscript.

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