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Full Length Research Paper

Solid substrate fermentation and conversion of orange waste in to fungal biomass using *Aspergillus niger* KA-06 and *Chaetomium* Spp KC-06

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Orange waste consisting of both peel and pulp is highly susceptible to hydrolysis by mixtures of cellulolytic and pectinolytic enzymes which give it potential as feedstock for biological conversion to value added products. Whereas the pulp is relatively rich in carbohydrate, the peel consists of 16.9% soluble sugars, 9.21% cellulose, 10.5% hemicellulose and 42.5% pectin as the most important components. Thus, *Aspergillus niger* (KA-06) and *Chaetomium* spp (KC-06) isolated from municipal waste were investigated for their ability to degrade orange waste under solid substrate fermentation (SSF). This study evaluated the influence of various fermentation parameters such as substrate concentration, initial moisture content, Nitrogen source supplementation, inoculum concentration, temperature and pH on the biodegradation of orange waste into protein enriched biomass using *A. niger* (KA-06) and *Chaetomium* spp (KC-06). The production of protein enriched biomass from orange waste as a substrate for the fermentation process was found to be optimized by moisture content of 40% and substrate concentration of 10 g with inoculum of 10^6 to 10^8spores/ml at 25°C for both *Chaetomium* spp (KC-06) and *A. niger* (KA-06). The maximum protein content of 39.64% was obtained with *Chaetomium* spp (KC-06) at inoculum load of 10^8 spores/ml. *A. niger* (KA-06) give a protein yield of 31.7% with inoculum load of 10^6 spores/ml. This research has demonstrated the potential for protein production from orange waste under solid substrate fermentation.

Keywords: *Aspergillus niger, Chaetomium* spp., orange waste, solid substrate fermentation.

INTRODUCTION

The global shortage of food and feed protein has prompted researchers to seek protein production improvements from both conventional and unconventional sources. One promising unconventional source is the mass cultivation of microbial biomass using renewable substrates which occur abundantly in nature. The use of fermentation processes to produce microbial biomass has several advantages over other unconventional processes that rely on agricultural by products, including the fact that fermentation processes are not subject to the variability of weather conditions and they can be controlled for product quantity and quality in virtually any geographic location (Czajkowska and Ilnicka-Olenjniczak, 1988; Rudravaram et al., 2006). In particular, solid substrate fermentation (SSF) offers numerous advantages for the production of proteins because of its high productivity, low cost media, less effort in down stream processing and products with added nutritional market value (Pandey, 1992a and Ashakumary et al., 1994). Attempts are thus being made to direct utilization of microbiological biosynthesis of protein by microscopic and filamentous fungi which, similar to yeasts, exhibit a fairly high growth rate, high protein biomass, high protein digestibility and interesting amino acid composition

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(Oshoma and Ikenebomeh, 2005). Although, the utilization of microscopic fungi for protein biosynthesis is attractive, their ability to form an enzymatic complex permitting transformation of various raw materials and different agricultural and industrial by-products into proteins is a favorable property of these organisms. Also favorable is the low nucleic acid content of fungal biomass (Rudravaram et al., 2006).

Much of the research is focused on the value-addition of agricultural products by SSF using agricultural residues generated every year (Soccol and Vandenberghe, 2003). Production of microbial proteins by fermentation of agricultural waste products is one of the most promising approaches for increasing the availability of protein. In addition, the utilization of these waste products provides alternative substrates and helps in solving pollution and waste disposal problems (Pandey et al., 1999). The nutrients of food wastes may be re-used in agriculture by composting or biotransformation of food wastes into animal feed. Vegetable and fruit processing wastes contain mainly starch, cellulose, soluble sugars and organic acids. Large and increasing quantity of citrus processing wastes are disposed of every year mainly in the form of citrus pulp, which is the semi solid by-product obtained after juice extraction (Plessas et al., 2007). The high cellulose and low protein content of orange waste (both peel and pulp) prevents them from being used as non-ruminant feedstuff while their high moisture content leads to difficulty in storage. Citrus (mainly orange) pulp, peel and membrane from oranges are highly susceptible to hydrolysis. In previous studies various microorganisms such as *Aspergillus niger* (De Gregorio et al., 2002) *Trichoderma viride* (Gregorio et al., 2002), *Chaetomium* spp (Karla et al., 1989) and *Neurospora sitophila* (Shojaosadati et al., 1999) used in submerged or solid-substrate fermentation processes have been proposed for biotransformation of orange wastes in to biomass, with or without chemical and enzymatic pre-treatment.

However, there is need to understand, characterize and standardize the optimal environmental conditions for high protein yield from orange waste under solid substrate fermentation process. Hence, this study was aimed at (i) to evaluate the possibility of reuse of orange waste in Ethiopia under solid substrate fermentation (ii) to investigate optimum growth conditions and (iii) to examine the potential of *A. niger* (KA 06) and *Chaetomium* spp. (KC 06) using various growth parameters for the production of enriched protein on orange waste.

MATERIALS AND METHODS

Sample collection and preparation of substrate

The fresh orange fruit wastes (with peel and pulp obtained after removal of the external part of the skin) were obtained from local fruit stalls in and around the city of Addis Ababa, Ethiopia.

The orange wastes were sun dried for few days. The dried orange wastes were ground to small particles of 1 - 2 cm in size using mortar and pestle stored in plastic bags at room temperature for use as a source of substrate for subsequent fermentation studies.

Isolation and identification of fungal microorganism

Fungal spp were isolated from decaying waste and composts of municipal solid waste disposal site of Addis Ababa (Koshe), Ethiopia. The Isolation of the fungal isolates was done by serial dilution method (Aneja, 1993) and plated on malt extract agar (MEA), Czapeck Dox's agar and potato dextrose agar (PDA). The plates were incubated at 30°C for four to six days and observed everyday for colony development. The fungal spp were identified according to their morphological, cultural characteristics. They were preliminarily identified according to their morphological physiolo-gical, biochemical cultural characteristics (Barnett and Hunters, 1998; Davis, 1969). Microscopic identification was on the basis of the structures bearing the spores. The fresh sub-cultured actively grown fungal isolates were picked with a sterile needle into a clean glass slide and observed under microscope. The stock cultures of the isolates were maintained on MEA and PDA slants at 4°C. For sub culturing the fungal isolates were maintained on agar slants for five days at 30°C and stored in a refrigerator. Among the isolates, two fungal isolates were identified as *A. niger* (KA-06)) and *Chaetomium spp* (KC-06) and were selected based on their protein production capability for this fermentation study. The cultures of *A. niger* (KA-06) and *Chaetomium* spp (KC-06) were stored on PDA slants for further evaluation and for fermentation studies on the production of protein enriched biomass from orange wastes.

Solid substrate fermentation inoculation and culture conditions

Spore suspension of *A. niger* (KA-06) and *Chaetomium* spp (KC-06) were made from spores of fungal isolates grown on malt extract agar at 30°C for five days for *Chaetomium* spp (KC-06) (KC-06) and four days for *A. niger* (KA-06). To make the suspension, a single fungal disc was taken aseptically with a 5 mm cork borer from each isolate and mixed in 20 ml sterile distilled water to which four drops of Tween80 was added. The total number of spores was determined by counting under microscope using haemocytometer (Neubauer counting chamber). The suspension was made fresh for each experiment. The experiment was designed to determine the suitable initial inoculums with optimized initial substrate. The effect of different inoculums size was studied by adding spore suspension of 10^4 to10^8 spores/ml. The SSF substrate consisted of 10 g of orange waste that was adjusted to 60% moisture content with 0.05 g of $(NH_4)_2SO_4$, autoclaved at 120°C for 15 min, and placed in 31 x 21 cm size plastic bags. All fermentation was done in triplicate unless otherwise stated. The substrate was inoculated with 2 ml of spore suspension (10^6 spores/ml) of *A. niger* (KA-06) or C*haetomium* spp (KC-06). The fermentation was maintained under stationary conditions at 30°C for six days. Samples were removed daily for analysis and to measure soluble protein and total nitrogen contents.

Effect of temperature, pH and moisture content on protein enrichment

Chaetomium spp *(*KC-06) and *A. niger* (KA-06) were grown at different temperatures to study the effect of temperature on protein enrichment of orange waste. Fermentation was done at 25^0, 30^0, 35^0 and 45^0C four days for *A. niger* (KA-06) and five days for *Chaetomium* spp (KC-06). The effect of initial pH on protein enrichment of orange waste by *Chaetomium* spp *(*KC-06) and *A. niger* (KA-06) was investigated within the range of pH 3.0 - 7.0. The

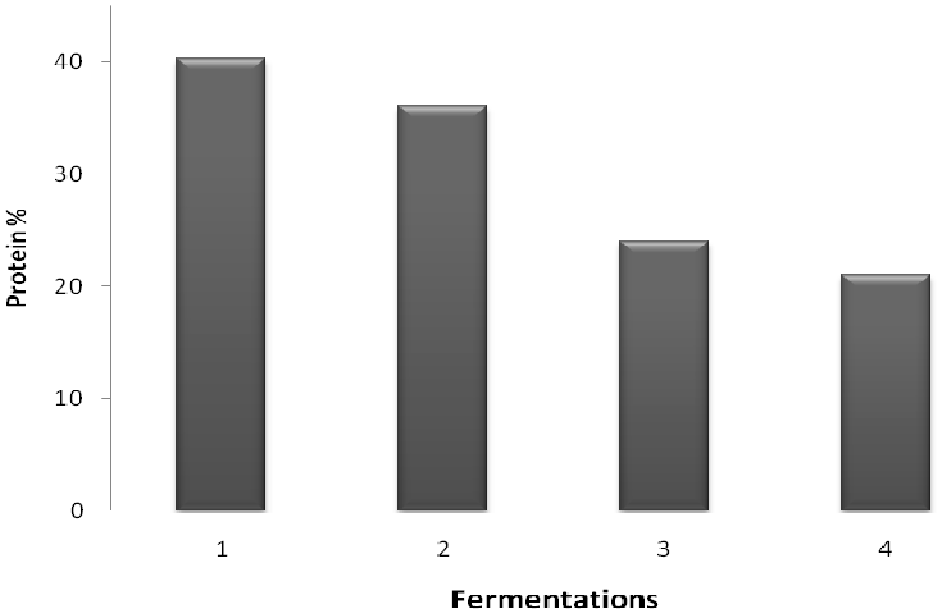

Figure 1. Comparison of yields of crude and soluble proteins produced from orange waste by *Chaetomium* spp KC-06 and *A. niger* KA-06, Fermentation 1 and 2 - Crude protein of *Chaetomium* Spp KC-06 and *A.niger* KA-06, Fermentation 3 and 4 - Soluble protein of *Chaetomium* Spp KC-06 and *A.niger* KA-06. The result for each fermentation is average of three replicates.

pH was adjusted either with 1N NaOH or 1N HCl to pH value of 3.0, 5.5, 7.0 and control (pH 5.0). The effect of moisture levels on protein yields was done over the range of 20 to 60%. The moisture level was adjusted with distilled water to obtain appropriate moisture content. The fermentation was carried out under the same conditions as described before.

Effect of substrate (initial load of orange waste) and nitrogen sources on protein enrichment

This experiment was designed to match substrate load and size of inoculum. To investigate substrate inhibition, substrate load was varied with inoculum size kept constant. The various substrate amounts of 10, 20 and 30 g/l were each placed in plastic bags of the same size (31 x 21 cm) and inoculated with the same amount of fungal spore suspension. Nitrogen supplementation was done by adding 1% (w/w) of compounds of $(NH_4)_2S0_4$, NH_4NO_3, $NaNO_3$, and a mixture of $(NH_4)_2SO_4$ and urea as nitrogen (N) sources in the fermentation media. The effect of these sources on protein enrichment was studied while other fermentation parameters are kept constant.

Sampling, extraction and analytical methods

Three extraction methods were compared to see which was the best method for protein extraction: Phosphate buffer (pH 7.0), NaOH (1N) and sonication (30 second cooling on ice between the sonication cycle, 10 seconds) with lysis buffer. Insoluble materials in the extracts were removed by centrifugation at 6000 rpm for 45 min. The protein yield in the extract was measured as reported by Lowry et al. (1951). Total nitrogen reported as crude protein was determined by modified modified Kjeldhal method (Sahlemedhin and Taye, 2000). One-way ANOVA was done and P values of <0.05 were considered significant. Stastical calculations were performed using the SPSS version 12.0 Stastical software.

RESULTS

Solid substrate fermentation of *Chaetomium* spp *(*KC-06) and *Aspergillus niger* (KA-06)

The study on mycelium production revealed that *A. niger* (KA-06) and *Chaetomium* spp (KC-6) produced maximum biomass on the sixth day. *A. niger* (KA-06) produced 0.75 gm/l of dry cell weight (DCW) while *Chaetomium* spp (KC-06) produced 0.85 gm/l of DCW, however, the statistical analysis showed no significant difference in biomass production between the two fungal isolates. The crude and soluble protein produced by *Chaetomium* spp (KC-06) and *A. niger* (KA-06) are shown in Figure 1. The result indicated that the mycelial biomass of *Chaetomium* spp (KC-06) produced more crude protein than *A. niger* (KA-06). Maximum protein enrichment was observed on the fifth day of fermentation by *Chaetomium* spp KC-06 and thereafter the protein yield declined. The optimum time for maximum protein production was determined to be four days for *A. niger* (KA-06) and five days for *Chaetomium* spp (KC-06). Of the three extraction methods of protein evaluated, the highest protein yields were obtained with 1 N NaOH with values of 13.65 gm for *Chaetomium* spp (KC-06) and 11 gm/100 gm of starting dry matter for *A. niger* (KA-06). In contrast, protein extraction using phosphate buffer (pH 7.0) yielded the lowest amount of protein with values of 9.1 gm for *Chae-*

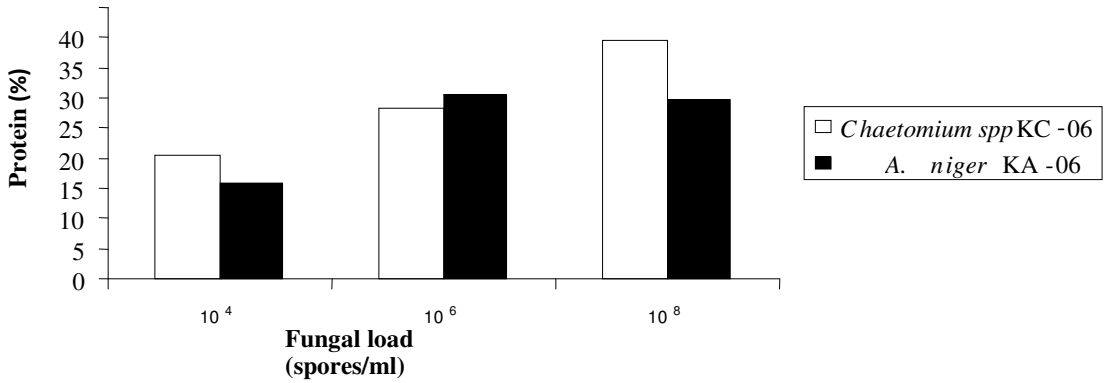

Figure 2. Effect of inoculum on soluble protein yield from orange waste of SSF by *A. niger* KA-06 and *Chaetomium* spp KC-06.

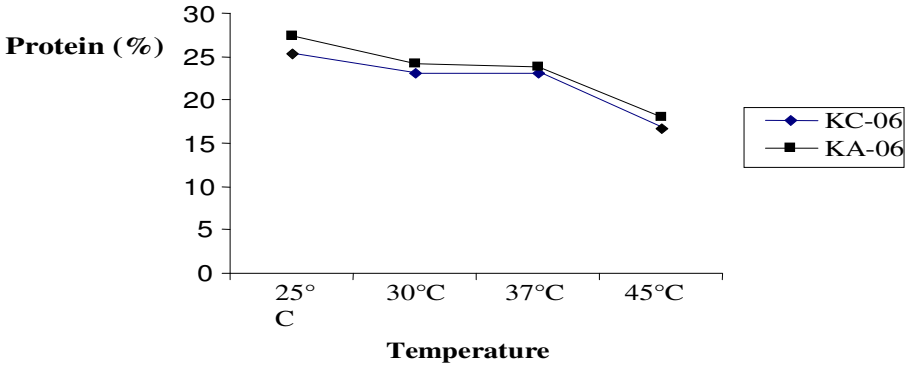

Figure 3. Effect of temperature on soluble protein production from orange waste fermented by *Chaetomium* spp *(*KC-06) and *A. niger* (KA-06).

tomium spp (KC-06) and 7.35 for *A. niger* (KA-06) gm/100 gm of starting dry matter.

Effect of inoculum, temperature and pH

As the fungal inoculum increased from 10^4 to 10^8 spores/ml, highly significant increase in protein yield was recorded for *Chaetomium* spp KC-06 (Figure 2). The highest protein content obtained for *Chaetomium* spp (KC-06) was 39.65% (w/w) whereas *A. niger* (KA-06) had protein yield of 30.47% (w/w). Increase in spore inoculum beyond 10^8 not only did not increase protein yields but rather led to very slight reduction in protein yields. Different incubation temperatures resulted in highly significant change (at 95% confidence interval) in the final protein content of the product. In this study both organisms, *Chaetomium* spp (KC-06) (25.28%) and *A. niger* (KA-06) (27.38%) produced high protein yield at 25°C (Figure 3). However, the protein content of the fermented product decreased as the incubation temperature increased. Generally, the effect of pH on the protein production was found to be statistically significant in this study. It was observed that neutral pH enabled maximum enrichment in the case of *A. niger* (KA-06) which attained its maximum protein content of 30.54% (w/w) with initial pH of 7.0 (Figure 4) whereas protein yield for *Chaetomium* spp *(*KC-06) was 29.39% (w/w) obtained with initial pH of 5.5. It was also observed that lower (acidic) initial pH (pH 3.0) adversely affected the final protein content.

Effect of moisture content and substrate concentration

The effect of different moisture levels on protein production from orange waste after four days for *A. niger* (KA-06) and five days for *Chaetomium* spp (KC-06) of incubation showed significant variation. The substrate with 40% moisture yielded maximum protein enrichment by both fungal isolates. *Chaetomium* spp (KC-06) and *A. niger* (KA-06) produced maximum total protein of 29.59% (w/w) and 31.72% (w/w), respectively, which are significantly higher than those obtained at other moisture levels

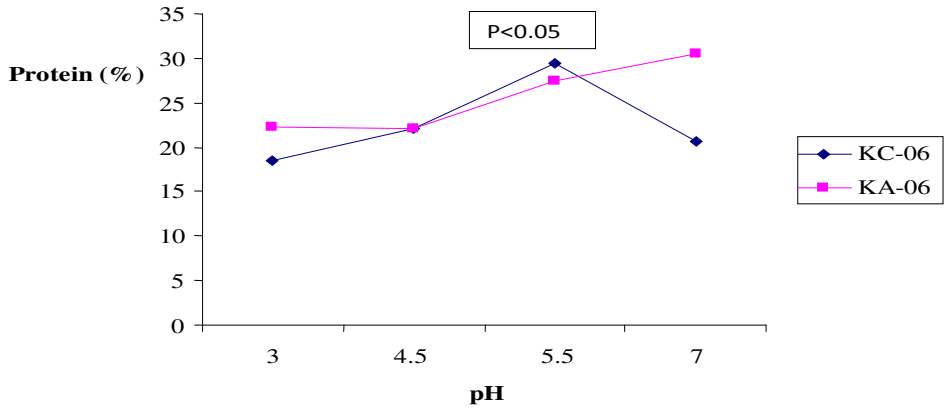

Figure 4. Effect of pH on soluble protein production from orange waste fermented by *A. niger* KA-06 and *Chaetomium* spp KC-06.

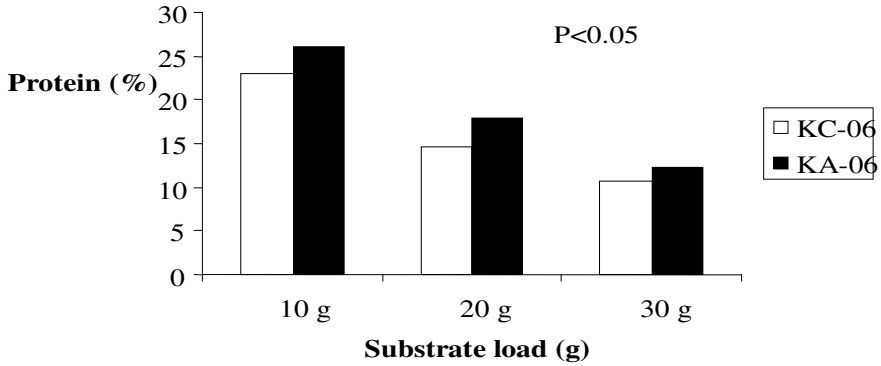

Figure 5. Effect of varying concentration of orange waste substrate load on protein production by *A. niger* KA-06 and *Chaetomium* spp KC-06.

at 5% level of significance. There was a gradual decrease in the protein content observed as the effect of moisture content increased from 40 to 60%. The results were found to be statically significant ($P < 0.05$). The effect of different substrate concentration on protein production from orange waste after four days for *A. niger* (KA-06) and five days for *Chaetomium* spp (KC-06) of incubation showed significant variation (Figure 5).

Effect of addition of nitrogen sources

The addition of $(NH_4)_2SO_4$ as Nitrogen source produced the highest protein levels in both fungal isolates of *Chaetomium* spp (KC-06) and *A. niger* (KA-06) (Table 1). Hence $(NH_4)_2SO_4$ can be considered as the best inorganic Nitrogen source for protein enrichment of orange waste by *A. niger* (KA-06) and *Chaetomium* spp (KC-06) in this study. With Nitrogen supplementation, *A. niger* (KA-06) appeared to have a more promising potential for protein production compared to *Chaetomium* spp. (KC-06) (Table 1)

DISCUSSION

The citrus processing residues are rich in both soluble and insoluble carbohydrates (Kesterson and Braddok, 1974) which gives them potential as value added products. The traditional re-use of orange pulp and peel waste are not yet practiced in Ethiopia and thus these agricultural wastes have been accumulating in the soil and potentially causing serious environmental problems. Therefore, the aim of this work is not only to overcome the problems of waste accumulation but also to convert this orange waste using SSF process into protein enriched biomass with isolates of fungal spp. The use of plastic bags to carry out SSF instead of Erlenmeyer flasks showed that these bags, made of polyethylene can retain temperature and allow the passage of O_2 which stimulates the growth of fungal strains during fermentation. The production of fungal protein from orange wastes under SSF has been found to be a viable way of producing protein. Ravindra et al. (2009) showed that the major constituent of dried fungi *Aspergillus oryzae* is crude protein, at levels of 43% for strain MTCC 1846 and

Table 1. Yield of soluble proteins from fermentation of orange waste enriched with four different nitrogen sources compared with un-enriched control.

Nitrogen Sources	*Chaetomium* spp KC06. Soluble Protein (%)	*A. niger* KA 06. Soluble Protein (%)
$(NH_4)_2SO_4$	30	35
NH_4NO_3	22	30
Na No_3	25	20
$(NH_4)_2SO_4$ and Urea	22	28
Control: Un-enriched orange waste	12	15

39.2% for MTCC 1842 on dry basis. The protein obtained by *Chaetomium* spp (KC-06) with spore load of10^8 spores/ml is similar to what was reported by Czajkowska and Ilnicka-Olenjniczak (1989) and Rudravaram et al. (2006). On the other hand high inoculum may reduce protein yield because of nutrient competition among fungal hyphae on limited substrate. As was observed in this study, high substrate load may also limit protein yield. This could be explained by the lower heat removal surface as the substrate load increases (Frank-Jan et al., 2002). It could also be due to substrate inhibition by toxic substances present in the orange waste. It has been reported that the seed of citrus fruits contain limonin which is a toxic factor for pigs and poultry (Driggers et al., 1951).

In this study *A. nige*r (KA-06) and *Chaetomium* spp (KC-06) produced maximum protein at 40% of moisture level. The investigations by Fan and Ding (1990) and Yang et al. (1979) reported that 60% of moisture was most suitable for mushroom production in SSF. It has been observed that higher moisture levels cause particle agglomerations, which interfere with heat and mass transfer, in turn resulting in decreased microbial activity (Pandey 1992a, b). A high moisture level leads to decreased substrate porosity, preventing oxygen transfer and facilitates contamination by fast growing bacteria. Low moisture level leads to poor accessibility of nutrients to microbial cultures resulting in poor yield of microbial biomass. Additionally, the optimum moisture content varies with the type of substrate and organism used which ranges between 40 and 70%. Cultivation of *A. nige*r on starchy substrates, such as cassava (Raimbault, 1981) was optimal at moisture levels considerably lower than on coffee pulp (Penaloza et al., 1991) or sugarcane bagasse (Roussos et al., 1991). This was probably because of the maximum water holding capacity of the latter substrate (Oriol et al., 1988). The optimum moisture content of 40% found in this study could also be due to low water holding capacity of orange peel. At low temperatures, the decline in protein content may be due to inactivation of cellular activities and at higher temperatures the enzymatic reactions in the cell may be denatured (Shojaosadati et al., 1999). The overall rate of heat transfer may be limited by the rates of intra- and inter-particle heat transfer and by the rate at which heat is transferred from the particle surface to the gas phase. Heat generated by high levels of fungal activity within the solids may lead to thermal gradients because of the limited heat transfer capacity of solid substrates. The ambient (room) temperature at Addis Ababa at the time of experiment was 25°C and the process can take place at normal atmospheric condition. In this study both organisms, *Chaetomium* spp (KC-06) (25.28%) and *A. niger* (KA-06) (27.38%) produced protein yield at 25°C. This result was similar to Survase et al. (2008), who used *T. inflatum* MTCC557 to produce cyclosporin from cottonseed cake and observed the optimum temperature was 25°C. Previous study of biodegradation of orange waste showed that, after degradation it could be used as a biofertilizer to enhance seedling growth of wheat (Shahera and Ashour, 2002). Oshoma and Ikenebomesh (2005) found that the addition of $(NH_4)_2SO_4$ improved the protein production among the other inorganic supplements. In this study the addition of ammonium sulfate improved the protein content in both *Chaetomium* spp (KC-06) and *A. niger* (KA-06). The fact that orange waste could be produced in a small area and short time period made this product a promising biofertilizer source in addition to its use as waste recycling tool. The results of this study indicate that the protein content of the orange waste can be significantly increased to equivalent protein level of other conventional protein feed sources. It should also be noted that to make use of this product, the fermentation conditions must be optimized to enhance yields of biomass and protein.

In conclusion the production of protein from orange waste using fungal spp showed that *Chaetomium* spp (KC-06) and *A. niger* (KA-06) are able to degrade orange waste under SSF. This finding can be applied on large scale and research on scale up and low cost technology alternatives should be explored intensively. Furthermore, the biomass produced from orange waste can be evaluated for its suitability to be used either as a biofertilizer or as animal feed.

ACKNOWLEDGEMENT

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