Addis Ababa University
School of Graduate Studies

Protein Enrichment of Orange Wastes by Filamentous Fungi

By
Biniyam Yalemtesfa

A Thesis Submitted to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

July, 2007

Addis Ababa University
School of Graduate Studies

Protein Enrichment of Orange Wastes by Filamentous Fungi

By
Biniyam Yalemtesfa

A Thesis Submitted to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

Approved by Examiners Board:

Dr. Amare Gessese (Examiner) ______________________

Dr. Dawit Abate (Examiner) ______________________

Dr. Tesfaye Alemu (Advisor) ______________________

July, 2007

ACKNOWLEDGEMENT

I would like to express my deepest gratitude and appreciation to my advisor, Dr. Tesfaye Alemu, Department of Biology, Addis Ababa University, for his continued guidance, academic support and encouragement throughout the research work. I am also very much grateful to Dr. R. Amutha for her valuable ideas and help in my laboratory work.

Special thanks goes to Lulayn Awgichew, my fiancée, for her unreserved support and encouragement during the study period. I would like to appreciate my parents, Yalemtesfa Mengistu and Zewdi Gebru, Hanna Elias and whole family for being concerned and helping.

I am also indebted to my friends: Adi, Dagi, Check, Fiki, Bire, Fasil, Kas, Mike, Ante, Yobi, Ser, Teshe, Miscov, Endro, Josi and Nunu. Finally, I would like to thank all students and staff of Addis Ababa University, Deparment of Biology.

Table of contents

List of Figures

List of Tables

List of Appendices

Abbreviations

ANOVA= Analysis of variance
CP= crude protein
SCP= Single cell protein
SmF= submerged fermentation
SSF= solid state fermentation
TN= Total nitrogen
SSL= Spent sulfite liquor
OTR= oxygen transfer rate
MT= metric tone
W/W= weight per weight

Protein Enrichment of Orange Wastes by Filamentous Fungi

ABSTRACT

Orange peel and pulp are rich in carbohydrates but poor in protein and account for approximately 55 to 60% of the weight of the raw/whole fruit. Their high cellulose and low protein content prevents them from being used as non-ruminant feedstuff while their high moisture content leads to difficulty in storage. However, the orange waste is highly susceptible to hydrolysis by mixtures of cellulolytic and pectinolytic enzymes which makes it attractive potential feedstock for biological conversion to value added products. To this effect, two fungal species namely, *Aspergillus niger* (KA-06) and *Chaetomium spp* (KC-06) were used to enrich the orange wastes with protein in solid state fermentation. The mycelial biomass of KC-06 was found to have 37.64% protein and KA-06 had 34.201% protein when grown in malt extract broth. The effect of various process parameters were studied in solid media of orange pulp. The optimum duration of fermentation was found to be 5 days and 4 days for KC-06 and KA-06 respectively. The optimum conditions for the enrichment process were found to be moisture content 40%; temperature 25^0C and substrate load 10gm for both KC-06 and KA-6 . pH 7 gave highest protein yield by KA-06 whereas pH 5.5 was optimum for KC-06. Inoculum concentration 10^8 spores/ ml was the best dose for KC-06 as 10^6 spores/ ml was for KA-06. Among the nitrogen supplements studied, $(NH_4)_2SO_4$ gave the highest protein yield by both fungal species. The growth of KC-06 on orange waste has improved the protein content up to 39.64% and KA-06 was able to enrich the feed with 34% protein.

Keywords/Phrases: Orange wastes, Solid state fermentation, protein enrichment, *Aspergillus niger, Chaetomium spp*.

1. Introduction

Single Cell Protein (SCP) is a term coined in the 1960´s to embrace microbial biomass products, which were produced by fermentation. SCP production technologies arose as a promising way to solve the problem of worldwide protein shortage. They evolved as bioconversion processes which turned low value by-products, often wastes, into products with added nutritional and market value. Intensive research into fermentation science and technology for biomass production, as well as feeding, has resulted in a profound body of knowledge, the benefits of which now span far beyond the field of SCP production (Ugalde and Castrillo, 2005).

Many microorganisms are able to utilise cheap sources of nitrogen and abundant carbon sources such as molases, sulphite waste liquor, cheese whey, starch, fruit processing residues, animal waste, petroleum hydrocarbons and agricultural wastes so as to be used as feed for animals (Anupama and Ravinda, 2001). Besides, their rapid growth makes them very attractive as high protein crops; while only one or two grain crops can be grown per year, a crop of yeasts or moulds may be harvested weekly and bacteria may be harvested daily (Hecht, 1985).

As a source of SCP, bacteria, yeast and filamentous fungi can be used. In recent years attention has been directed to utilization of microbiological biosynthesis of protein by microscopic filamentous fungi exhibiting - similarly as yeasts – a fairly high growth rate, big protein content in biomass, high protein digestibility and an interesting amino acid composition (Oshoha and Ikenebomeh, 2005). Authors taking up utilization of microscopic fungi for protein biosynthesis stress moreover - other favorable properties of these organisms, namely their ability to form an enzymatic complex permitting transformation into protein of various raw materials as well as of different agricultural and industrial by-products; the low content of nucleic acids in fungal biomass is also emphasized (Rudravaram *et al*., 2006).

World citrus production has increased significantly since the 1980s. In 2010 the orange production is estimated to reach 66.4 million metric tones (MT), which represents a 14% increase compared to that of 1997–1999. About 30.1 MT of the orange production will be processed to yield juice, essential oils and other by-products (FAO, 2003). Therefore, a large and increasing quantity of citrus processing wastes will be disposed of every year mainly in the form of citrus pulp, which is the semi solid by-product obtained after juice extraction (Plessas, 2007). Citrus (mainly orange) pulp and peel are cheap and suitable substrates for cell growth. Therefore, various microorganisms in submerged or solid-state fermentation processes have been proposed for SCP production from orange wastes, with or without chemical and enzymatic pre-treatment, such as *Aspergillus niger* and *Trichoderma virdie* (De Gregorio *et al.*, 2002), *Chaetomium spp* (Karla et al., 1989), *Neurospora sitophila* (Shojaosadati et al., 1999), etc.

Orange waste is palatable to cattle and mature cows, when they are accustomed to the feed; consume about 10 kg per day. It has been used as the main energy source for beef cattle and heifers, and up to 45 percent has been used in calf rations. However, because of the high water content and the perishable nature of the waste, economically it can only be used close to the processing plant. The feed is rather difficult to handle, will ferment and sour quickly, and can be a fly breeding nuisance if allowed to spoil (Devendra, 1970).

Under-nutrition and malnutrition are major factors constraining animal production in Ethiopia despite the huge animal resource available. Nutritional stress causes low growth rates, poor fertility and high mortality which are compounded by animal diseases. Since utilization of the resources is highly inefficient, the return gained is low (Lemma and smith, 2005). A food source that is nutritionally complete and requires a minimum of land, time and cost to produce is highly desirable. To this effect, utilization of cheap and suitable substrates for the production of SCP can be considered as a solution for protein shortage in animal feeds.

Therefore, upgrading orange wastes in to protein rich feed would be economically favourable. In the present investigation, an attempt has been made to upgrade the protein content of orange pulp by two filamentous fungi namely *Aspergillus niger* and *Chaetomium spp.*

2. Objective of the study

1. To study the potential of orange waste for single cell protein in solid state fermentation
2. To evaluate fungal growth and protein production on orange waste
3. To optimize the growth conditions of two fungal species on orange waste in order to get maximum protein yield in solid state fermentation

3 Literature Review

3.1 Historical Development of Single cell protein

The pioneering research conducted almost a century ago by Max Delbrück and his colleagues at the Institut für Gärungsgewerbe in Berlin first highlighted the value of surplus brewer's yeast as a feeding supplement for animals (Ugalde and Castrillo, 2005). This experience proved more than useful in the ensuing First World War, when Germany managed to replace as much as half of its imported sources by yeast. The sustained interest in fodder yeast initiated in Germany in the inter-war years echoed elsewhere in the World. As part of a larger programme for utilizing natural sources, the Forest Products Laboratory of the United States Department of Agriculture undertook mass cultivation of yeast on sulfite waste liquor, the species used being *Candida utilis* (Ugalde and Castrillo, 2005).

In the 1950's some oil industries became interested in the growth of micro-organisms on alkanes. The micro-organisms which were used developed into a potential diet for feed and food. In the USSR 12 out of 86 plants making SCP were said to rely on hydrocarbons as the source of carbons and energy for the micro-organisms. However, the relatively low market selling price set for this non-conventional steered design towards low product cost and thus, large scale production. Abundant substrates with low prices were sought. By-products as wide ranging as cheese whey, molasses, starch, baggase and spent sulfite liquor were chosen to sustain commercial processes (Humphrey, 1975; Ugalde and Castrillo, 2005).

By the mid 60´s, some quarter of a million tons of food yeast were being produced in different parts of the world and the Soviet Union alone planned an annual production of 900,000 tons by 1970 of food and fodder yeast, to compensate agricultural production deficits. By 1980, SCP production processes were operating on a large scale in developed countries, and plans to extend SCP production to underdeveloped countries were being made. But a number of technical and political developments that occurred in the 80´s conditioned the expansion of the promising SCP industry. Marked improvements in plant breeding and crop production on a global basis allowed for a continued increase in agricultural output, beyond the expected ceilings (Department of Agriculture, Forest Service, 2000).

In view of these developments, many industrial SCP processes were discontinued, leaving behind them a wealth of skill and knowledge, which have been successfully bench marked in other fermentation processes. Specific research in the field also declined in consonance with the market trend (Ugalde and Castrillo, 2005).

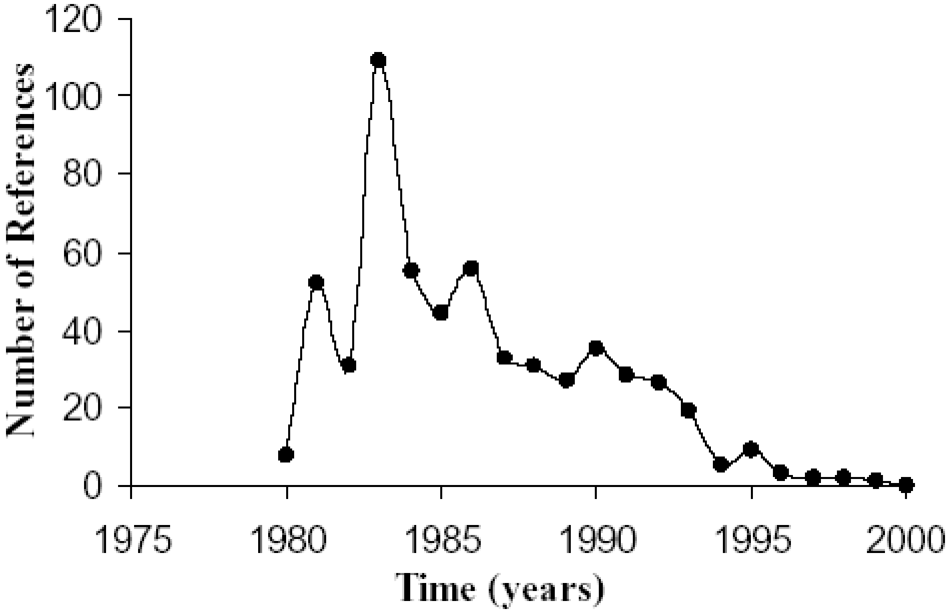

Fig. 1. Number of scientific papers cited including 'SCP' or 'Single Cell Protein' in their title or key words (Ugalde and Castrillo, 2005).

There is very little work in progress on a low-technology, labour-intensive, low-capital SCP system. It is an area with great potential for the developing countries, but of lesser interest to the industrialized countries or to the private sector of industrial countries because it does not appear to be a large potential market for sophisticated equipment and processes. It is in this area of controlled fermentation under non-sterile conditions with a minimum of equipment that methods may be developed for using agricultural wastes at the small farm level. Instead of being grown under sterile conditions in expensive stirred fermentors, the moulds are grown in a plastic container under conditions where the desired organisms predominate (Imrie, 1975).

3.2 Substrates for SCP

A wide range of substrates can be used to grow microbial proteins (whey, orange peel, sweet orange residue, sugarcane bagasse, paper mill waste, rice husks, wheat straw, cassava waste, sugar beet pulp, coconut waste, yam waste, banana pulp, mango waste, grape waste, sweet potato). The most widespread and commonly used substrates for SCP production have been those where the carbon and energy source is derived from carbohydrates. This is due to the fact that their building blocks (mono and disaccharides) are natural microbial substrates, and that carbohydrates are a renewable resource which is widely distributed (Israelidis, 1994; Ugalde and Castrillo, 2005).

3.2.1 Renewable Biological Feedstocks

Cellulose and lignocelluloses

Cellulose is the most widely occurring organic material in nature, and the principal source of biomass and therefore of a renewable resource; it is a complex of three classes of polymer, which consists of repeating glucose units largely in crystalline fibbers. Cellulose is usually mixed with substances such as lignin and hemicellulose. Therefore, the cellulose source must be pretreated physically and chemically in order to break down the cellulose into fermentable sugars (Moo-Young *et al.*, 1992).

The production of SCP from cellulose by hydrolysis with *Trichoderma reesei* and *Pellicularia filamentosa* has been reported (Clemeni *et al.*, 1985). The hydrolyzed cellulose supported the growth on both *Candida utiliz* and *Saccharomyces cerevisiae* (Hanrrison and Wren, 1976). *Neurospora sitophila* was cultivated in a SCP production batch process from wastes containing cellulose at pH 5.5 and temperature 35 – 37^{0}C with a yield of 31 % SCP (Moo-Young *et al.*, 1992). In the Waterloo SCP Process in Canada, the fungus *Chaetomium cellulolytium* was selected because of its ability to rapidly convert cellulose substrates directly to without the usual hydrolysis - type pre-treatment (Moo-Young *et al.*, 1981). Moo-Young *et al.* (1992) developed an SCP production process using cellulosic substrates, based on the filamentous fungus *Neurospora sitophila* which is used in oriental food preparation. Many microorganisms belonging to Basidiomycetes are able to convert lignocellulosic substrates directly into a fungal biomass which is suitable for both human and animal consumption (Waslein, 1975).

Starch and starch wastes

After cellulose, starch is the most abundant compound synthesized by plant cells. Starch can be extracted commercially from many raw materials including corn, cassava, potatoes, wheat barley, sorghum and sagopalm. Among the substrates that could be utilized as raw materials for SCP production, starch is especially attractive since it is easily metabolized by a large number of microorganisms. Important processes have been developed using starch materials for SCP production, such are the Symba process for potato wastes using *S. fibuligera* and *C. utilis*; the cassava starch process by *C. utilis* using fed-batch culture and the solid state fermentation process (Ghoul and Engasser 1983; Senez, 1980).

Raimbault (1981) developed a solid-state fermentation where the filamentous fungus, *Aspergillus hennebergii*, was grown in a starch biomass. Since the submerged culture processes have been developed successfully, filamentous fungi involved in SCP production and starch waste treatment have become more and more attractive. The fermentation of cassava meal was carried out in submerged cultures with *Rhizopus oligosporus* under non-aseptic conditions. The specific growth rate and the true contents of the product are 0.22/hour and 26 %, respectively. Balagopal and Maini (1977) grew fungi in a suspension culture containing 25 g/ l cassava starch wastes and found *Aspergillus niger* (NRRL 300) and *Rhizopus sp*. to be superior in terms of mycelium biomass and production. Muondi and Manssen (1981) grew *Trichoderma harzianum* in suspensions of cassava meal (4%) and obtained an enriched biomass product with a 38% content form original cassava which contained 2.4%.

3.2.2 Wastes from Renewable Feedstock Processes

The definition of “waste material” is changing continuously since more and more re-use is replacing simple disposal. By-products that have generally been considered as waste are now in routine use.

Whey

Whey is a by-product of cheese-making and contains lactose. The waste from large scale cheese plants can become a serious environmental problem if it is discharged without treatment. Utilization in the production of SCP was the subject of extensive study (Moulin & Galzy, 1984). One original solution concerned yeast production from crude sweet whey using *Candida* LY 496 in batch or continuous culture processes (Hiotizman, 1986). Many cheese companies in Europe have successfully used *Kluyveromyces fragilis* as the organism to produce SCP. This process, however, has to compete with fractionation of the whey as its component lactose and protein. The process also depends on large scale plants giving steady effluent as a source of raw material. The effluent is generally dilute and high transportation costs may be involved in the collection of the whey. This situation has been one of the major reasons why whey is still regarded as an unattractive substrate for SCP production (Moulin and Galzy, 1984).

Spent Sulfite Liquor

Spent sulfite liquor (SSL) is an effluent from pulp and paper mills, a sugar-containing waste product. These effluents have been identified as a possible source for the production of SCP. Romantschuk and Lehtomäki (1978) cultivated *Candida utiliz* for SCP production using SSL as the substrate. A reduction in sulfite processing and its replacement by the sulfate process has tended to reduce the available effluent and closed some USA facilities for SCP production. Pretorius *et al.* (1993) investigated a continuous system of selective cultivation of thermotolerant *Aspergillus sp.* on SSL for SCP production.

Molasses and Sugar Beet Pulp

Molasses and sugar beet pulp are two main by-products of the sugar industry and their production creates huge disposal problems. These fermentable sugars from pulp and molasses can be utilized by various microorganisms to produce SCP. An enzyme system for *Aspergillus ustus* and *Trichoderma viride*, which contains all three important cellulase constituents: hemi-cellulose, pectines, amylases and protease, has been developed. A multisystem with one and two stage processes was also tested to treat the effluent from a sugar plant and produced SCP using *Candida tropicalis*, 40% protein content and 95% chemical oxygen deamand (COD) removal were achieved (Senez, 1980).

3.2.3 Chemical feed stocks

Materials with a high commercial value such as energy sources or derivatives of these chemicals, such as gas oil, methanol, ethanol, methane and n-alkane, have attracted wide interest in the early stages. Many scientists have questioned the use of such compounds with a high energy potential for food production, including SCP, as health aspects (Rehm and Reiff, 1981).

SCP production from hydrocarbons presents practical complications due to the low water solubility of the substrate, as well as the high degree of aeration required for its metabolization (the substrate is essentially oxygen free and therefore presents a high oxidation potential). In consequence the cost of aerating the culture is relatively elevated. In addition, the oxidation processes are highly exothermic and cooling costs are correspondingly higher (Rehm and Reiff, 1981). Besides, the toxicity of the substrates has raised suspicions on the safety of continuous feeding with SCP containing trace amounts of substrate. A few decade ago, many of the large-scale processes in operation were forced to close down because of health regulations and the high costs of materials used (Ugalde and Castrillo, 2005).

N-Alkanes

Alkanes, in particular those of intermediate size (C10 - C20) are the most rapidly metabolised hydrocarbons and were chosen as the substrate for the SCP projects of the 1960s. Though alkanes can be catabolsed by many yeasts, some fungi and bacteria, the following yeasts have been studied intensively for SCP*: Candida tropicalis, C. oleophia, and Saccharomycopsis lipolytica* (Fukui and Tanaka, 1981).

British Petroleum Company has developed an airlift bioreactor for SCP production using C15-C30 alkanes from crude oil and C10-C17 alkanes from gas oil. *Candida tropicalis* was tested in the gas oil process in a nonsterile continuous system (16000 tons/year) in Cap Lavera, France from 1973 to 1975. In Grangemouth (U.K.), *Saccharomycopsis lipolytica* was grown aseptically with n-alkanes in a system which produced 4000 tons SCP/year over a period of several years. The disadvantage of alkanes is that they are not easily soluble, which results in poor oxygen transfer and low yields (Rehm and Reiff, 1981).

Methanol

Methanol was, for some time, the most important substrate for SCP production and extensive research on methanol utilizing organisms was carried out. Many bacteria (e.g. *Methylobacter, Bacillus and Klebsiella*), yeasts (*Candida boidinii, Hansenula henricii and Torulopsis molischiana*) and fungi (*Paecilomyes varioti, Gliocladium delinquescene and Trichoderma lignorum*) may all be considered for SCP production from methanol (Sahm, 1979).

Methanol, as a carbon source for SCP, has many inherent advantages over n-paraffins, methane gas and even carbohydrates; composition is independent of seasonal fluctuations. There are no possible sources of toxicity in methanol, it dissolves easily in the aqueous phase in all concentrations and no residue of carbon source remains in the harvested biomass (Sahm, 1979).

3.3 Microorganisms for SCP

Most SCP processes are designed to take advantage of an available, readily degradable substrate using microorganism. The choice of the SCP microorganism is usually limited to that particular process, and a change in substrate often necessitates a change in the type of microorganisms used. Apart from substrate, other important factors in SCP production are the content and the quality of the biomass product. Although the content can be varied by growth conditions, genetic manipulation can also be employed to alter the amino acid spectrum (Oura, 1983).

Four types of microorganisms are used to produce biomass: bacteria, yeasts, fungi and algae. Their biomass doubling time is given by Israeldis, 1994 (Table 1).

Table 1. Mass doubling time (S)

Organism	Mass Doubling
Bacteria and Yeasts	10-120 min
Mold and algae	2-6 hr
Grass	1-2 wk
Chickens	2-4 wk
Pigs	2-6 wk
Cattle	1-2 month
People	0.2-0.5 year

Source: Israelidis, (1994).

Ideal microorganism should possess the following technological characteristics (Hélène *et al.*, 19): high specific growth rate and biomass yield, high affinity for the substrate, low nutritional requirements, that is few indispensable growth factors, ability to use complex substrates, stability during multiplication, capacity for genetic modification, good tolerance to extreme temperature and pH.

In addition to the above mentioned characterstics, it should have low nucleic acid content, good digestibility and be non-toxic.

3.3.1 Fungi

On account of the steadily increasing food deficiency on a world scale, microscopic filamentous fungi exhibiting reasonably high growth rate and content in biomass, high digestibility and an interesting amino acid composition, has got due attention for single cell protein. Besides their properties of easy harvesting, low nucleic acid content and acceptability as traditional food, filamentous fungi have become more and more attractive in SCP production and biotechnological waste treatment processes. A considerable amount of research is being devoted to the growth of fungi such as *Trichoderma sp*. on cheap cellulosic materials or waste products. Two fungi, *Trichoderma viride* and *Geotrichum candidum*, are grown in a submerged culture for 60 hours, giving rise to a product containing 20% crude protein and 23% fibre with an *in vitro* digestibility of about 65% (Ikenebomeh, 1981; Chen and Wayman, 1992). Attempts have also been made to grow filamentous fungi on plant cell biomass. Fungi, including *Botritis cinerea* and *Trichoderma viride*, grow well on waste plant cell biomass as the sole nutrient source. *Botritis cinerea*, a plant pathogen, which has a recognised ability to degrade plant cells rapidly, is a particularly suitable fungus for SCP production when grown on waste plant cells. The starch, using fungi such as *Aspergillus niger* or *Rhizopus arrhizus*, is hydrolyzed to glucose and the content increases considerably as the fungus grows (Tan *et al.*, 1984; Byrne and Ward, 1989).

In the Pekilo process (Romantschuk and Lehtomäki, 1978), mycelia of the filamentous fungi *Paecilomyces variotii* are continuously cultivated in a medium which contains dissolved carbohydrates. The yield of biomass approached 55% of the reducing substrate consumed a value exceeding that originally anticipated. The dried Pekilo protein is sold to feed compounding mills and has a crude protein content of 52 - 57%. Several fungal processes in a submerged culture for treatment of starch wastes have been described. Balagopal and Maini (1977) grew fungi in a suspension culture containing 25 g/l of cassava starch wastes and found *Aspergillus sp.* NRRL 330 and *Rhizopus* sp. to be superior in terms of mycelial weight and protein production. Muindi and Manssen (1981) grew *Trichoderma harzianum* in suspensions of cassava meal (4%) and obtained an enriched product with 38% protein from the original cassava containing 2.4% protein. *Aspergillus niger* mutants have been applied for increasing the protein content of cassava starch wastes up to 20%.

Many strains such as *Aspergillus*, *Fusarium*, *Chaetomium*, *Rhizopus*, *and Trichoderma* are recommended on commercial scale for production of SCP (Solomonas, 1985). Among filamentous fungi suitable for industrial use, *A. oryzae* presents an unusually broad range of applications as a producer of highly active proteins. The other important ability of this fungus is to provide form and texture and hence can be harvested with ease; also, the cost of production may be reduced. Furthermore, fungi can prosper on a variety of carbohydrates and they are best suited for agro residues, although growth rates vary considerably with different substrates (Humphrey, 1975).

3.3.2 Bacteria

Various species of bacteria can utilize a wide range of carbon and energy sources, including sugars, starch, cellulose (either in pure form or as agricultural or forest product waste) hydrocarbons and petrochemicals. Rapid growth rates and high contents make bacteria prospective candidates in the SCP production. The generation time of bacteria is only 20 to 30 minutes compared with 2 to 3 hours for yeast and 16 hours or more for algae, and fungi (Litchfield, 1979). Semi - batch and continuous SCP processes using bacteria are suitable for industrial waste recovery. The conversion efficiency of substrates into SCP by bacteria is very high (0.8 - 1.2 g /g substrate) (Litchfield, 1979).

In most SCP processes the cell concentrations of bacteria are in the range 10 - 20 g/l requiring large volumes of water. The production of bacteria is only about 1.003 g/l, and the cost of centrifugation may be four times as great as for yeast centrifugation. On the other hand, high proportions of nucleic acid and poor digestibility make bacteria a less than acceptable food source (Ugalde and Castrillo, 2005).

3.3.3 Yeasts

Yeasts were the best studied and generally best accepted microorganisms by consumers. Yeasts are rarely toxic or pathogenic and can be used in human diets. Although their content rarely exceeds 60%, their concentration in essential amino acids such as lysine (6 to 9%), tryptophan and threonine is satisfactory. In contrast, they contain small amounts of the sulfur-containing amino acids methionine and cysteine. They are also rich in vitamins (B group), and their nucleic acid content ranges from 6 to 12%. They are larger than bacteria, facilitating separation. They can also be used in a raw state (Ugalde and Castrillo, 2005; Litchfield, 1979).

3.3.4 Algae

The potential merits of algae are related to their ability to multiply with CO_2 as the only carbon source. Some genera (Cyanophyta) can also use atmospheric nitrogen. Algae production takes place in natural water bodies (ponds, lakes and lagoons). Algae are traditionally a food complement for some populations in Mexico (*Spirulina platensis*) and Chad (*Spirulina maxima*). However, algae have a low sulfur-containing amino acid content. Their nucleic acid content is about 3 to 8%. They are easy to recover, but multiplication is very slow, and investment costs involved in artificial shallow ponds result in low process profitability (John, 1980).

3.4 Cultivation Processes for SCP Production

The great interest in large-scale SCP production has stimulated research and development programs in order to improve the efficiency of the process (Rudravaram *et al.*, 2006). Large-scale SCP production is mainly a huge bioengineering venture, to which novel design criteria of the cultivation system have been applied. For more than 30 years, the great interest has been focused on SCP production on reused raw materials including the wastes and renewable materials. Most production processes developed are characterized by large volume, continuous operation, aerobic systems and high capital investment. The objective of an aerobic SCP production system is to obtain a maximum oxygen transfer rate (OTR) efficiency and biomass yield. The bubble column bioreactor has been found to be the preferable cultivation system for improving the OTR at low cost (Schügerl *et al.*, 1977).

SCP cultivation, in most cases, was carried out in a stirred tank reactor. However, this technology is too expensive in such a way that it can't compete with other conventional sources (Pandey, 2002). Therefore, a simple device should be developed for this purpose. Recently, the potential advantage of conducting the cultivation in a solid state system has triggered research interest. SSF comes from its simplicity and closeness to the natural way of life for many microorganisms (Laufenberg *et al.*, 2003) as large water quantity is not required to the substrates, fermenter volumes remain small, necessary manipulations become less expensive and cost of water removal in post fermentative process is minimized. A further advantage is the possibility to cultivate the fungus under nonsterile conditions (Pandey, 2002).

A comparison of the cultivations with steam pretreated straw in a strirred tank reactor and a fixed bed (solid state), where packed bed of solid substrate rests on a perforated plate and continuous forced aeration is applied with intermittent mixing, was reported by Hetch *et al* (1985). It was found that in both reactors the same cell mass fraction (30%) is attained, however, in a stirred tank much earlier so (t_F=60hrs) than in a fixed bed (t_F -130 hrs); about double the cultivation time is necessary for a solid state cultivation as compared to a submerged cultivation. Fairly similar result, 0.08%/hrs (in tumble reactor) and 0.098%/h (in tray reactor) produced mass from substrate used in % per hour was also reported by Pamment *et al*. (1978).

3.5 Nutritional Value and Safety of SCP

Chemical analysis of the microorganisms evaluated for SCP reveal that they are comparable in amino acid content and type of plant and animal sources with the possible exception of sulphur amino acids cysteine and methionine, which is lower in some SCP sources, while they exhibit better levels of lysine (Israelidis, 1994).

Table 2. Essential amino acid content of the cell protein in comparison with several reference proteins (grams of amino acid per 100 grams of protein)

Amino acid	*Cellulomonas*	*S. cerviciae*	*Spirulina maxima*	*Penicillium notatum*	Wheat	Egg	Milk	Cow
Lysine	7.6	7.7	4.6	3.9	7.0	2.8	6.3	7.8
Threnine	5.4	4.8	4.6	-	4.9	2.9	5.0	4.6
Methionine	2.0	1.7	1.4	-	1.8	1.5	3.2	2.4
Cysteine	-	-	0.4	-	-	2.5	2.4	-
Tryptophane	-	1.0	1.4	1.25	-	1.1	1.6	-
Isoleucine	5.3	4.6	6.0	3.2	4.5	3.3	6.8	6.4
Leucine	7.3	7.0	8.0	5.5	7.0	6.7	9.0	9.9
Valine	7.1	5.3	6.5	3.9	5.4	4.4	7.4	6.9
Phenylalanine	4.6	4.1	5.0	2.8	4.4	4.5	6.3	4.9
Histidine	7.8	2.7	-	-	2.0	-	-	-
Arginine	6.4	2.4	-	-	4.8	-	-	-

Source: Israelidis, (1994)

In addition to this, microorganisms contain adequate levels of carbohydrates, lipids, and minerals and are excellent sources of vitamin B. The fat content varies among these sources, with algal cells containing the highest levels and bacteria the lowest. On a dry weight basis, nucleic acids average 3 to 8 for algae, 6 to 12 % for yeasts, and 8 to 16 % for bacteria. B vitamins are high in all SCP sources (Ugalde and Castrillo, 2005).

Table 3. Average composition of the main groups of microorganisms (% dry weight)

	Fungi	Algae	Yeasts	Bacteria
Protein	30-45	40-60	45-55	50-65
Fat	2-8	7-20	2-6	1.5-3
Ash	9-14	8-10	5-9.5	3-7
Nucleic acids	7-10	3-8	6-12	8-16

Source: Ugalde and Castrillo, (2005)

Safety

Microbial products must be free of pathogenic microorganisms, viruses, microbial toxins, toxic heavy metals and they must have safe levels of nucleic acids. Bacterial cells such as *Alcaligenes eutropha and Klebsiella pneumoniae (Aerobacter aerogenes)* gave adverse reactions such as vertigo, nausea, vomiting, and diarrhea in human subjects, even though rats fed with these organisms showed no ill effects. These reactions have been attributed to toxins bound to or within the cells (Calloway 1974). Consumption of dried cells of the yeasts *S. cerevisiae* and *C. utilis* as a major portion of the diet has led to gastroenteric disturbances, and *C. utilis g*rown on sulfite waste liquor has led to dermatologic effects (Waslien 1975). Many molds, including species of *Aspergillus* and *Fusarium,* produce mycotoxins. Care must be taken to screen any mold culture to be used for microbial production for its potential for mycotoxin production. Consideration must also be given to residues of heavy metals from equipment and toxic components from substrates such as crude hydrocarbons. The gas oil process for producing *Candida spp*. was abandoned because of the costly purification required to give an acceptable product (Calloway, 1974; Waslien 1975).

Safety valuations of microbial products should include: feeding studies in rats, including gross and microscopic pathological examinations, hematology, blood biochemistry, urinalysis, and evaluation for carcinogenic effects. Mutagenicity should be evaluated by multigenerational studies in the target species. Allergenicity should be evaluated with rabbits and guinea pigs (Taylor *et al*., 1974). Success has been achieved in rat feeding studies with a variety of SCP products, but human feeding studies have been less successful, except in case of certain yeast cell products. Gastrointestinal disturbances are common complaints following the consumption of algal and bacterial SCP (Ugalde and Castrillo, 2005).

About 70-80% of the total cell nitrogen is represented by amino acids while the rest occurs in nucleic acids. This concentration of nucleic acids is higher than other conventional sources and is characteristic of all fast growing organisms. The problem which occurs from the consumption of SCP with high concentration of nucleic acids (78-25 g/100 gram of dry weight) is the high level of uric acid in the blood, sometimes resulting in the disease gout. Upon the degradation of nucleic acid, purine and pyrimidine basese are released. Adenine and guanine are metabolized to uric acid. Lower animals can degrade uric acid to the soluble compound allantion, and consequently the consumption of high levels of nucleic acids does not present metabolic problems to these animals as it does to humans (Israelidis, 1994).

Because of concern for digestibility, nucleic acid contents, and presence of toxins, there has been considerable interest in processing microbial cells to remove cell walls, and toxic constituents and to reduce nucleic acids to acceptable levels. Microbial cells have been disrupted by ball milling, homogenization, autolysis, and/or acid, alkaline, or enzyme hydrolysis. Nucleic acids can be reduced by endogenous nucleosis, by acid precipitation, or by acid and alkaline hydrolysis (Litchfield, 1979). Microbial cell extracts or concentrates prepared after removing the cell wall and nucleic acids can be spun into fibers, extruded, or succinylated to prepare functional products (Gierhart and Potter, 1978).

3.6 Economic and Market Considerations

Important economic considerations in microbial production include capital costs of facilities; location of facilities and markets; costs and availability of substrates and nutrients; water- and waste-treatment requirements; energy, labour, and maintenance costs; and desired depreciation schedules, profit margins, and return on investment (Moo-Young, 1977). Capital costs of facilities for microbial cell production vary widely depending upon whether or not sterile conditions are required. In 1976, ICI estimated that a 50,000 to 75,000 metric ton capacity plant for producing *Methylophilus methylotrophus* from methanol under aseptic conditions would cost $70 million (Anonymous, 1977).

Market considerations are also critical for the success of microbial products. For feed stuff uses soybean meal and fish meal have established markets. The price and nutritional quality of microbial products must compete with these products. As functional ingredients for food applications, the microbial products must compete with soy concentrates and species in price and functional effectiveness. Yeast products have been used for foods and feeds for many years. Whether or not similar markets can be developed for dried cells of other microorganisms produced on waste substrates has yet to be determined. Regulatory agency requirements in different countries may be as important as economic and market considerations in determining the ultimate success or failure of these products (Litchfield, 1979 and Moo-Young, 1977).

4. Materials and Methods

The study was undertaken in the laboratory of Mycology, Department of Biology, Addis Ababa University, Ethiopia from Oct 2006 to June 2007.

4.1 Sample collection and preparation of substrates

Fresh orange fruit waste was collected from different shops found in Addis Ababa. The orange pulp and peel was sun dried for few days. The dried orange waste was ground using mortar and stored in plastic bag for subsequent laboratory experiment.

4.2 Isolation and identification of microorganism

Two different fungal species, *Aspergillus niger* (KA-06) and *Chaetomium* (KC-06), were isolated from decaying wastes of municipal solid waste disposal site of Addis Ababa (Koshae). Isolation of the fungal species was done by serial dilution agar plating method (Aneja, 1993). Ten grams of the collected soil samples were aseptically homogenized with 90 ml sterile distilled water under aseptic conditions. One ml was taken from this solution and mixed in to 9 ml sterile distilled water to make 10^2 dilutions. Same amount was taken from 10^2 dilution solution and used to make 10^3. This was done until 10^5 dilution factor. One ml of from each dilution factor (10^2 to 10^5) was taken and plated on triplicate plates of Malt Extract Agar (MEA) by pour plate technique. Then, plates were incubated at 30°C for 4 – 5 days and checked daily for colony development. After incubation, the colonies on the plates were counted. Colony types and numbers were also noted. Representative colonies were picked at random from the plates and streaked out to obtain pure cultures.

The pure cultures were also kept on slants of PDA for fungi. They were identified according to their morphological and cultural characterstics. Young, actively growing moulds were picked with a sterile needle unto clean glass slides and prepared for microscopic observation using lactophenol as mountant and cotton blue as stain (Barnett and Hunters, 1992; Davis, 1969). The slides were carefully covered with slips to exclude air bubbles. Microscopic examination of the prepared slides was carried out first using the low power objective followed by the middle power magnification objective lens for a closer examination of a selected field. Microscopic identification was on the basis of the structures bearing the spores and on the spores themselves.

Aspergillus niger (KA-06) and *Chaetomium* (KC-06) were identified and pure cultures were preserved in PDA slants for further studies.

4.3 Inoculum Preparation

Spore suspension of *Aspergillus niger* (KA-06) and C*haetomium* (KC-06) was obtained by growing the fungal species on malt extract agar at 37^0C: 5 days for KC-06 and 4 days for KA-06. A single disc was taken by 5 mm cork borer aseptically from each plate into 20 ml sterile distilled water mixed well by adding few drops of 4 drops of tween 80. The number of spores was counted by microscope using haemocytometer (Neubauer counting chamber). The spore count of the suspension was found to be 10^6 spores/ml. The suspension was made fresh for every experiment.

4.4 Mycelium Production, Protein Synthesis and Secretion

The fungal species were grown in 100 ml Malt Extract broth: Malt extract 1.5g, Dipotassium hygrogen phosphate 0.1g, Ammonium chloride 0.1g and citric acid (1N) 1.5ml and 100ml distilled water, in 250 ml Erlenmeyer flask. The cultures were incubated at 30^0c for 7 days. The amount of mycelial growth was measured by filtering the broth out using Whatman No. 42 filter paper. The mycelium biomass was dried in the oven for 24 hour at 105^0c and was measured by sensitive balance. The dried mycelium and culture filtrate were kept for further analysis.

4.4.1 Analysis of Extracellular Protein

The amount of extracellular protein (soluble protein) secreted in to the medium (broth) was measured by Folin method of Lowry *et al.* (1951). KA-06 and KC-06 were grown in malt extract broth for six days. Extracellular protein was measured from the culture filterate. Sample from the filterate was taken by eppendoff and centrifuged at 6000 rpm for 45 minutes. The supernatant obtained was used for estimating protein content.

4.4.2 Analysis of Intracellular Protein

Dried samples of the mycelium from section 4.4.1 were first grounded by mortar and mixed with 5 ml of 1N sodium hydroxide (NaOH). Then, it was heated at 90^{o}c for 10 minutes in water bath. Samples were taken from treated solution in Eppendorf and were centrifuged at 6000 rpm for 45 minutes. The supernatant was used for estimation of soluble protein by Folin method of Lowry *et al*, (1951) which is stated in section 4.8.1.

4.5 Standardization of Protein Extraction

Three extraction methods were tested in order to select better method of protein extraction from the fermented product. These are: Phosphate buffer (pH 7.0), NaOH (1N) and sonication with lysis buffer.

- 25 ml of phosphate buffer was added to the fermented product and was homogenized at 6000 rpm and centrifuged subsequently at 6000 rpm for 45 minutes in order to get the supernatant.
- Two grams of the product was mixed with 10 ml NaOH (1N) and was placed in water bath at 90^{o}c for 10 minutes. The sample was centrifuged at 6000 rpm for 45 minutes.
- 10 ml of lysis buffer was added to two grams of the product. The cells were disrupted by sonication (30 second cooling on ice between the sonication cycle,

10 seconds. Insoluble materials were removed by centrifugation at 6000rpm for 45 minutes.

The amount of protein in the extracts was measured by Folin method of Lowry *et al* (1951) and the methods were evaluated for high protein extraction.

4.6 Solid State Fermentation (SSF)

Ten grams of orange waste adjusted to 70% moisture content with 0.05 g of $(NH_4)_2SO_4$, autoclaved at 120 ^oC for 15 min, and taken in 31 cm x 21 cm size plastic bags in triplicate. The substrate was inoculated with 2 ml of spore suspension (10^6 spores/ml) of *Aspergillus niger* and *Chaetomium spp*. The culture was maintained in stationary conditions at 37^0c for six days. Samples were taken every day from each organism and the soluble protein content and total nitrogen were measured by Folin method, modified Kjeldhal method described by Sahlemedhin Sertsu and Taye Bekele (2000) respectively.

4.7 Optimization of Protein Enrichment in SSF

4.7.1 Effect of Moisture Content on protein enrichment

In SSF, the moisture level of the substrate has been considered as an important factor for the growth and activity of microbial cultures. So, various moisture levels of orange waste were maintained to study the effect on protein enrichment. Orange waste in plastic bags was used to setup the experiment. Each bag containing 10 grams of the substrate was taken and moistened with distilled water to obtain four levels of moisture contents (40%, 50% 60% and 70%). The fermentation was carried out under the same conditions as described in section 4.6.

4.7.2 Effect of Temperature on protein enrichment

*Chaetomium spp (*KC-06) and *Aspergillus niger* (KA-06) were grown at different temperatures in order to study the effect of temperature on protein enrichment of orange waste. The cultures were incubaterd at 25, 30, 37 and 45 ^{0}c; four days for *Aspergillus niger* (KA-06) and five days for *Chaetomium spp (*KC-06). The protein content was quantified in order to identify the best temperature level at which highest protein content is obtained.

4.7. 3. Effect of Initial pH on protein enrichment

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 3-7. The pH was adjusted either with 1N NaOH or 1N HCl to pH value of 3, 5.5, 7 and control (pH 4.6).

4.7.4. Effect of Inoculum Dose on protein enrichment

The effect of different inoculum size on protein enrichment was studied by adding spores suspension in the range of 10^4 to 10^8 spores/ml. The other parameters like moisture, pH and incubation temperature were kept constant.

4.7.5. Effect of Substrate concentration on protein enrichment

This experiment was designed in order to determine the best load of substrate that can be utilized by a certain amount of inoculum in a given area. Different amount of substrate with the same level of innoculum were used to investigate if there is any substrate inhibition. Ten (10), 20 and 30 grams of substrate were put in plastic bags of the same size (31x21cm) and were given equal amount of inoculum. The final protein amount was measured as described above.

4.7.6. Effect of Nitrogen Source

$(NH4)_2S04$, NH_4NO_3, $NaNO_3$, and a mixture of $(NH_4)_2SO_4$ and urea were used as nitrogen (**N)** sources in the medium. The media were supplemented with 1% (w/w) of the above mentioned nitrogen sources. The effect of these sources on protein enrichment was studied while other parameters are kept at the same.

4.8. Analytical Methods

4.8.1. Soluble protein Determination

The Soluble protein was determined by Folin method of Lowry *et al* (1951). The following reagents were prepared for the asssay:

- Reagent A: 2% Na_2CO_3 in 0.1 N of NaOH
- Reagent B: 2% $CuSO_4.5H_2O$
- Reagent C: 2% Sodium potassium tartarate or 2% trisodium citrate
- Reagent D: 0.5 ml of A + 0.5 ml of B in 99 ml of A (this should be prepared fresh every day)
- Folin: Dilute with distilled water to 1N

100 µl of sample and 900 µl distilled water was added in a test tube in triplicate. One (1) ml of freshly prepared reagent D was added and kept for 10 minutes at room temperature. Then, 0.5 ml of Folin phenol reagent was added and left for 30 minutes. Immediate vortex after each addition of the reagents is recommended in order to get best result. The optical density (OD) was measured by spectrophotometer at 740 nm.

The standard curve for the conversion of the result was constructed using bovine serum albumin (BSA).

4.8.2 Total Nitrogen Determination

Total nitrogen was determined by modified modified Kjeldhal method (Sahlemedhin and Taye, 2000). The dried samples were first finely ground using mortar and pistil followed by sieving with 0.5 mm sieve size of which 0.6 g of the ground samples were subjected to Kjeldahl procedure for determination of total nitrogen in the sample. Macrokjeldahl procedure for determination of total nitrogen involves three processes: digestion, distillation and titration. Two and half ml of the digesion mixture compsed of 1lt sulfuric acid, 3.5g selenium powder and 7.2 g of salicyclic acid/100ml H_2SO_4 – selenium mixture were added to each macrokjeldahl tube and swirled carefully so as to moisten the plant material and let them stand for 2hrs. The digestion tubes were placed on a heating block and heated at 100^{o}c for 2hrs, the tubes from the block were removed and allowed to cool; then three ml hydrogen peroxide were added and mixed thoroughly. The digestion tube was again heated at 300^{o}c on a preheated block until the digest turns to colorless or light yellow. After removing the tube from the block and cooling to room temperature, 48.3ml of distilled water was added, mixed and left overnignt. In the next day the mixtures were again mixed, filtered on 100 ml volumetric falsk, bring to volume with distilled water and made ready for further analysis.

The acid digest was transferred quantitatively to the macro-kjeldahl flasks. 20ml of boric acid solution was measured into a receiver Erlenmeyer flask corresponding to the number of samples. Two drops of mixed indicator solution composed of 0.5g bromocreson green, methyl red dissolved in 100ml of 95% ethanol were added and placed under the condenser. After adding 75ml of 40% NaOH solution to the digestion flasks containing the digests, it was fitted to the corresponding holder to start distillation. When the distillation was compeleted, Titration was then performed using 0.1N HCl until the color of the distillate turns from green to pink at the end and the amount of HCl utilized for titration was recorded volumetrically. Finally, the total nitrogen contents of the samples were determined by using the following formula after correcting for the blank.
$\%N= 0.014\ (a-b).N.\ 100/s$

Where
a= ml of HCl required for titration of sample
b= ml of HCl required for titration of blank
s= weight of dried sample
N= Normality of HCl (0.1)
0.014= meq weight of nitrogen in g

Note: The total nitrogen was converted to crude protein by multiplying the percentage of total nitrogen found by 6.25.

4.9 Data Analysis

One-way analysis of variance of the data was done at 0.05 level of significance using a computer statistical software (SPSS version 12).

5. Results

5.1. Mycelium Production

The study on mycelium production has revealed that *Aspergillus niger* (KA-06) and *Chaetomium spp* (KC-06) gave their maximum biomass on the sixth day. Besides, KA-06 was found to sporulate earlier than KC-06. KA-06 has produced 0.75 g of dry biomass while KC-06 produced 0.85gm however the statistical analysis has shown that there is no significant difference in biomass production between the two fungal species (fig.2). The protein content of the biomass quantified and has shown that the biomass of KC-06 is composed of 37.64 % protein and KA-06 has 34.201 % of protein as determined by Folin method. Crude protein content of biomass dry matter was also found to be 46.41% and 42.29% for KC-06 and KA-06 respectively. The result indicated that the mycelial biomass of KC-06 has more crude protein than KA-06 which makes it suitable for single cell protein production (Fig. 3).

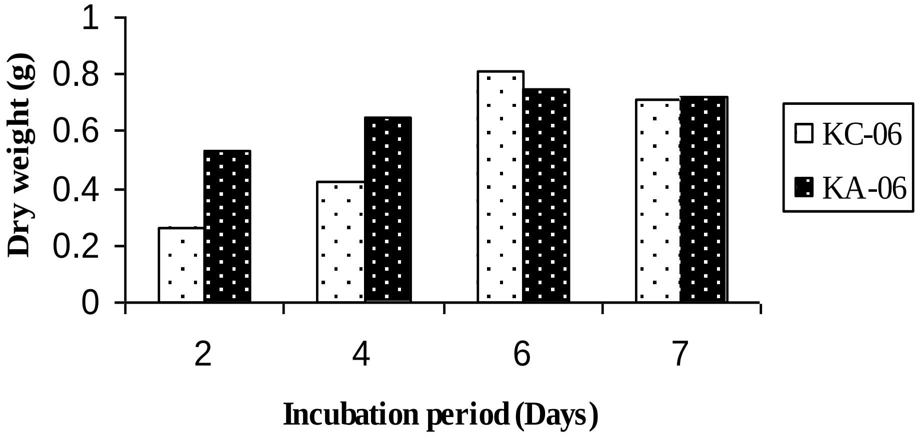

Fig. 2 The growth of *Chaetomium spp* (KC-06) and *Aspergillus niger* (KA-06) on malt extract broth

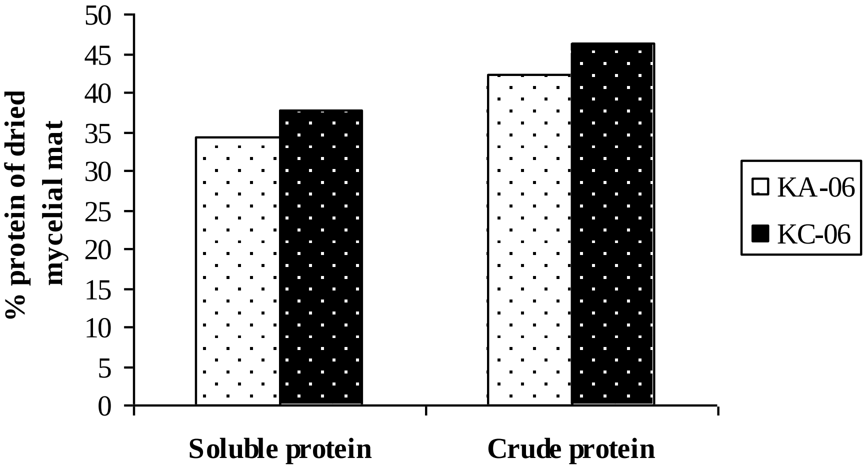

Fig 3. Protein content of biomass dry matter of KA-06 and KC-06

5.2. Protein production and secretion

Aspergillus niger (KA-06) has shown relatively higher amount of secretion of protein to the broth which comprises 34.97% of its Soluble protein. Whereas *Chaetomium spp* (KC-06) had more intracellular protein, 73.83%, in comparison with KA-06 which was found to have 65.03% of its Soluble protein as intracellular protein (Fig. 4). The secretion rate/synthesis rate ratio of KC-06, 0.26, was lower than that of KA-06 which had ratio of 0.35.

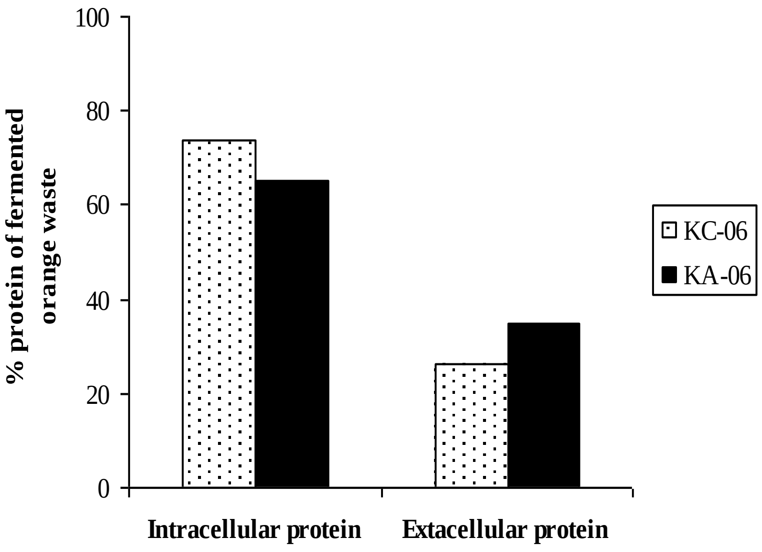

Fig. 4 The amount of intra and extra cellular protein of KA-06 and KC-06

5.3. Standardization of Protein Extraction

The study has found out that NaOH (1N) has extracted the highest protein, 13.65 and 11 g/100 gram of starting medium dry matter, from KC-06 and KA-06 respectively (Fig. 5). On the other hand extraction using phosphate buffer (pH 7) extracted the lowest amount of protein; 9.1 for KC-06 and 7.35 for KA-06 g/100 gram of starting medium dry matter, as determined by Lowry method (1951).

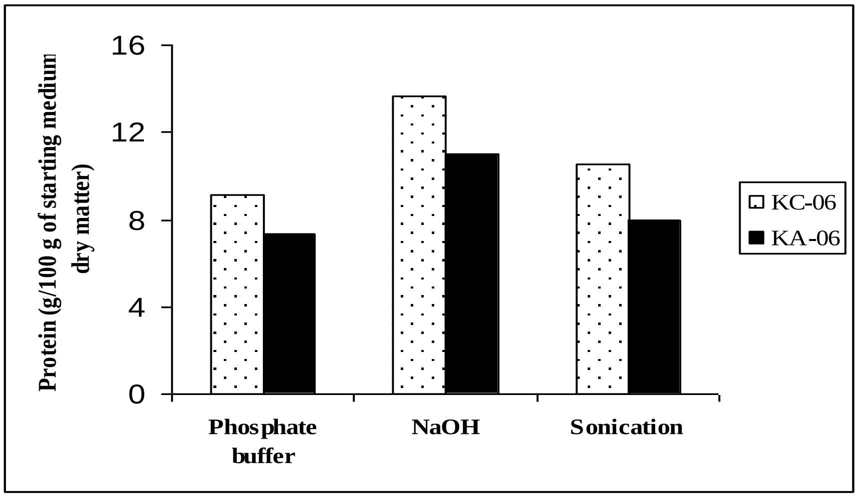

Fig. 5. Evaluation of different protein extraction methods from KA-06 and KC-06

5.4. Time course

Maximum protein enrichment was observed on the fifth day of fermentation by KC-06. This was confirmed by crude protein, 30.01 % and soluble protein, 21.31 % (Fig. 6). KA-06 has enriched the medium best on the fourth day with 30.28 % of crude protein and 21.51 % soluble protein (Fig. 7). After the maximum protein enrichment was attained, the time course has shown that the protein content started to decrease. So it was understood that four days for *Aspergilus niger* (KA-06) and five days for *Chaetomium spp* (KC-06) would be the optimum time for best protein production.

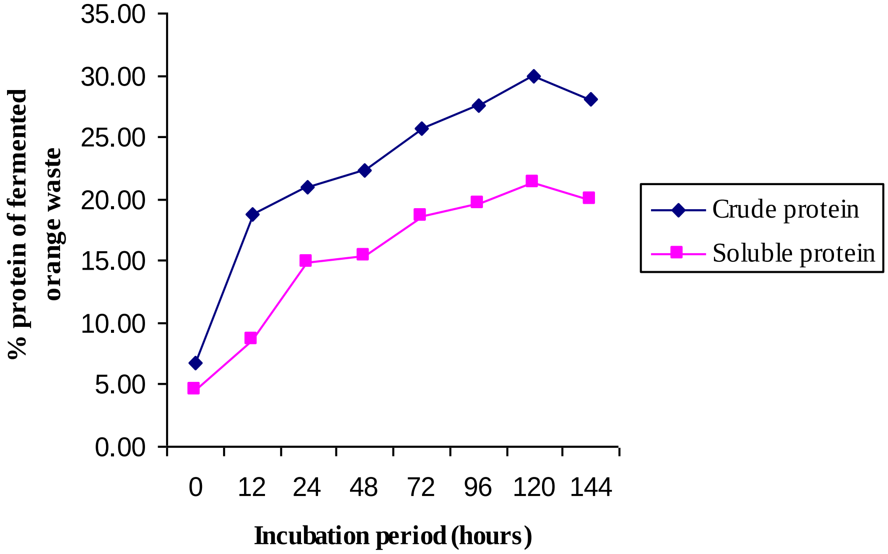

Fig. 6. Protein enrichment of orange waste by KC-06 over time

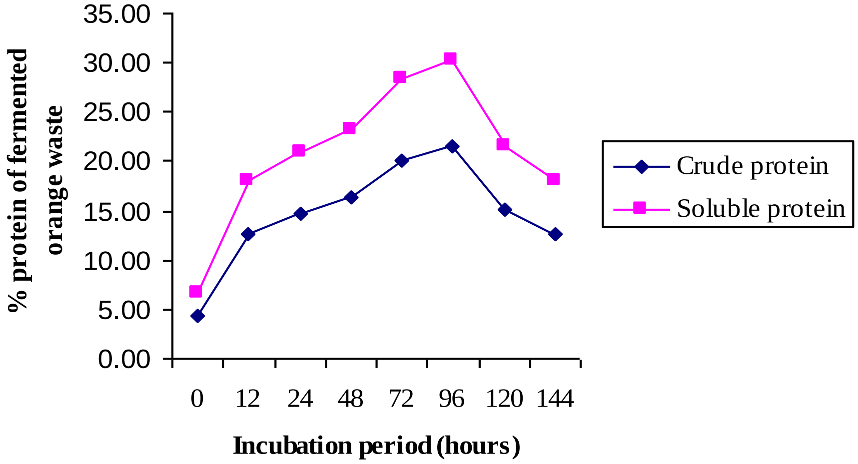

Fig. 7. Protein enrichment of orange waste by KA-06 over time

5.5. Optimization of Protein Enrichment in SSF

5.5.1. Effect of Temperature on protein enrichment by KA-06 and KC-06

Different incubation temperature has resulted highly significant change (at 95% confidence interval) in the final protein content of the product. In this study both organisms, KC-06 (25.28%) and KA-06 (27.38%) gave their highest protein yield at 25^{o}c. As shown in Fig. 8, the protein content of the fermented product decreases as the incubation temperature increases. Finally, the lowest protein content, KA-06 (18.04%) and KC-06 (16.1%) was found at 45^{0}c.

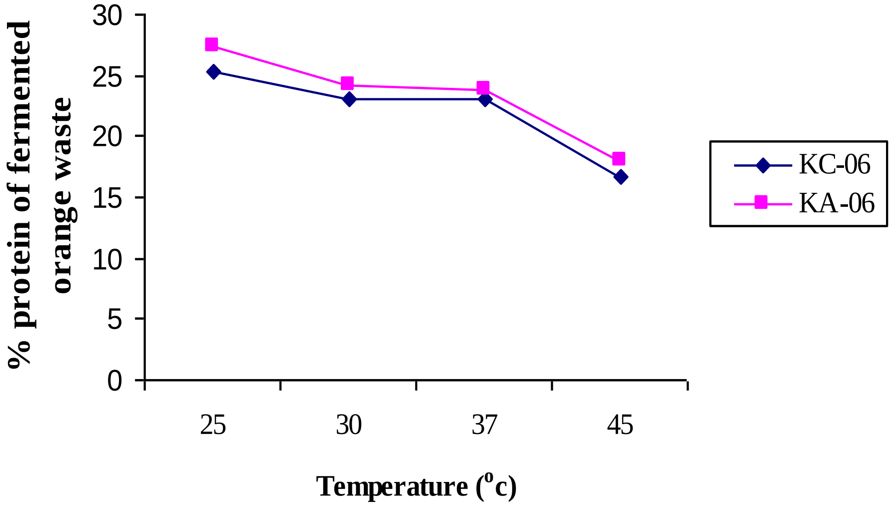

Fig. 8. Effect of temperature on protein enrichment of orange waste by KA-06 and KA-06

5.5.2. Effect of Moisture Content on protein enrichment by KA-06 and KC-06

Figure 9 shows the effect of different moisture levels for maximum protein enrichment in orange waste after 4 days for KA-06 and 5 days for KC-06 of incubation. The substrate with 40% moisture yielded maximum protein enrichment by both fugal species. KC-06 and KA-06 attained their highest figures of 29.59 % (w/w) and 31.7275 % (w/w) Soluble protein respectively which are significantly higher than the rest at 5% level of significance. Gradual decrease in the protein content was observed as the moisture content of the medium got increased. Mycelium at 40% moisture was very vigorous on the other hand at 70% the fermentation was less (Fig. 9).

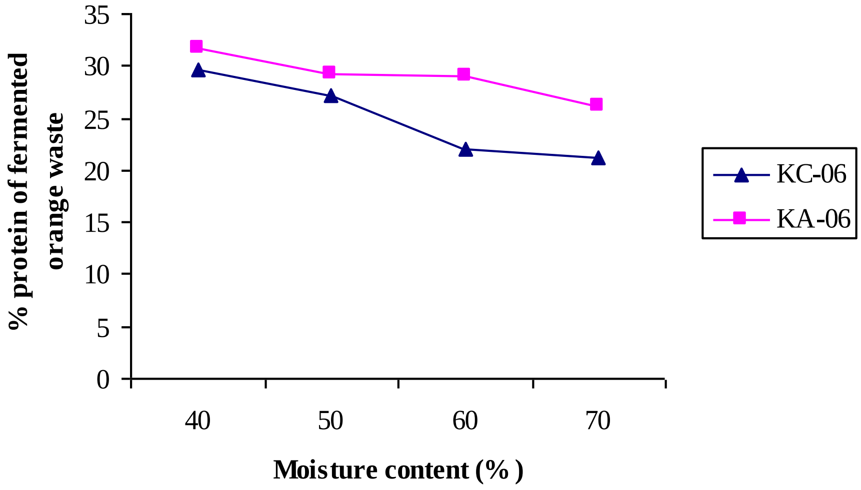

Fig. 9 Effect of Moisture content on protein enrichment of orange waste by kA-06 and KC-06

5.5.3. Effect of Initial pH on protein enrichment by KA-06 and KC-06

The maximum protein enrichment, 29.39% (w/w), was obtained with initial pH 5.5 for KC-06 whereas KA-06 attained its maximum protein content, 30.54 % (w/w), with initial pH of 7 (Fig. 10). It has been also observed that lower (acidic) initial pH (pH 3) has adversely affected the final protein content. Especially, neutral pH has enabled maximum enrichment in case of KA-06. After the fermentation the final pH was found to be 3.3 and 3.7 for KC-06 and KA-06 respectively. The decrease in pH is due to the release of certain organic acids. Generally, the effect of pH on the single cell protein production was found statistically significant in the present study.

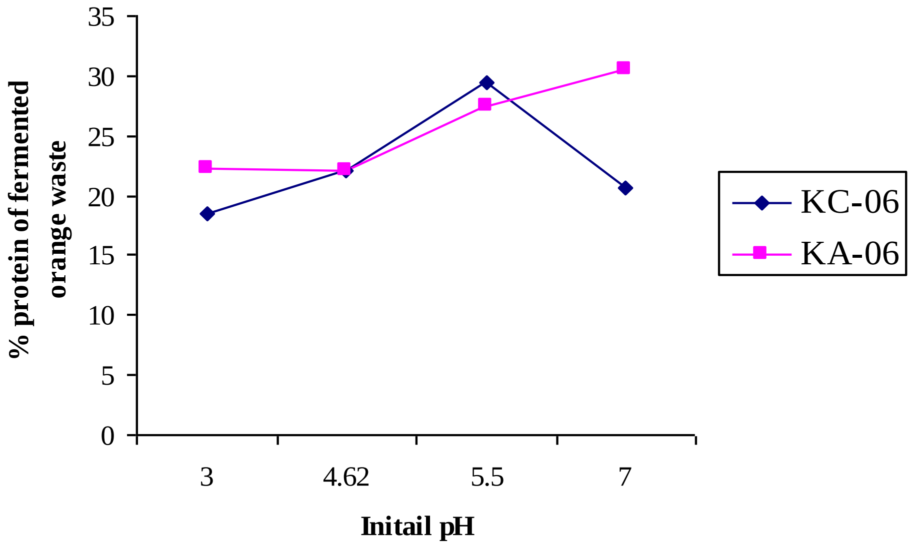

Fig. 10 Effect of initial pH on protein enrichment of orange waste by kA-06 and KC-06

5.5.4. Effect of Inoculum Dose on protein enrichment by KA-06 and KC-06

The size of inoculum, among others, was also found to be important factor influencing the growth of *A. niger* (KA-06) and *Chaetomium spp* (KC-06). As the dose of inoculum increases from 10^4 to 10^8 spores/ml, highly significant increase in protein yield was recorded on KC-06 in the same period. The highest protein content obtained from KC-06 culture was 39.65% (w/w). On the other hand, KA-06 had its optimum dose at 10^6

spores/ml which scored protein yield of 30.47 % (w/w); further increase beyond this level didn't assure increment in protein content rather very slight reduction was observed (Fig 11).

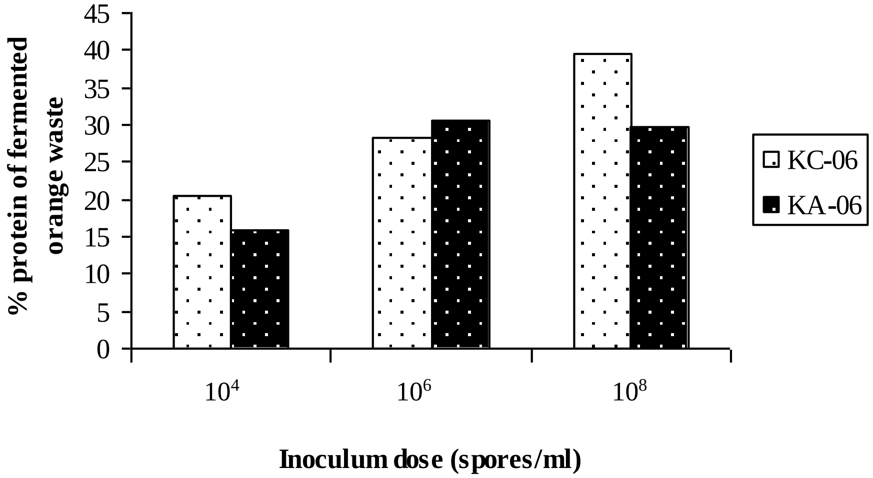

Fig. 11 Effect of Inoculum dose on protein enrichment of orange waste by kA-06 and KC-06

5.5.5. Effect of Substrate Dose on protein enrichment by KA-06 and KC-06

The level of substrate concentration was found to affect the final protein content of the product significantly (at 5% level of significance.) The result has shown that 10g of substrate was the optimum level that can be utilized by KA-06 and KC-06 inoculum to give 26.17 % (w/w) and 23.05% (w/w) protein respectively. The minimum protein content was recorded with 30g of substrate in case of both fungal species used (Fig. 12). This shows that there may be substrate inhibition.

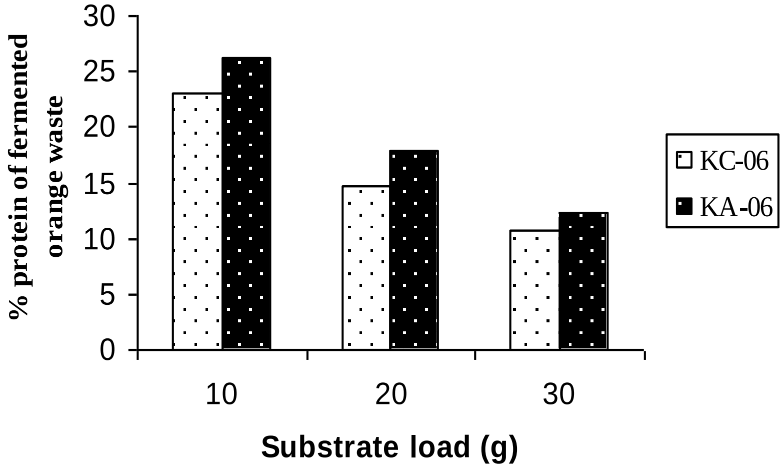

Fig. 12 Effect of amount of orange waste on protein enrichment by kA-06 and KC-06

5.5.6 Effect of different nitrogen sources on protein enrichment by KA-06 and KC-06

Nitrogen source has also shown highly significant difference on the protein yield of the fermented product in this experiment. The result has revealed that $(NH_4)_2SO_4$ gave the highest protein in both fungal species' culture: KC-06 (30.3%) and KA-06 (34.03%). So that, $(NH_4)_2SO_4$ can be considered as the best inorganic nitrogen source for protein enrichment of orange waste by *Aspergillus niger* (KA-06) and *Chaetomium spp* (KC-06) in this study. Very low yield of protein was observed with, among others, utilization of $NaN0_3$ and mixture of $(NH_4)_2SO_4$ and urea by KA-06 (20.97%) and KC-06 (20.59) respectively (Figure13).

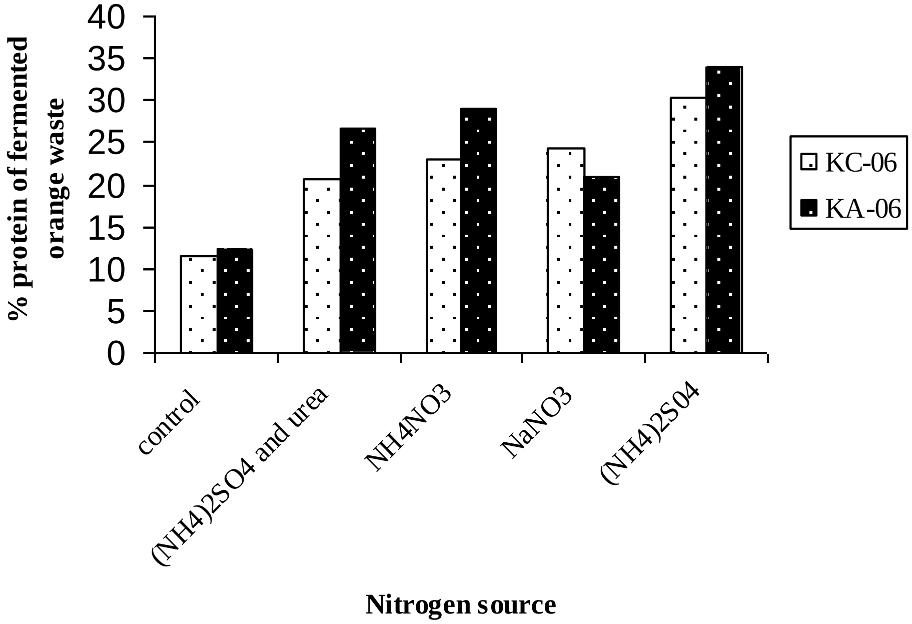

Fig. 13 The effect of different nitrogen sources on protein enrichment of orange waste by kA-06 and KC-06

6. Discussion

The global shortage of food and feed protein has prompted researchers to seek protein production improvements in both conventional and unconventional sources. One promising unconventional source is the mass cultivation of microbial biomass/SCP using renewable substrates which occur abundantly in nature. Fermentation processes have advantages over other unconventional routes which rely on agricultural by products, in that they are not subject to the variability of weather conditions, but can be controlled for product quantity and quality in virtually any geographic location by man-made environments (Czajkowska and Ilnicka-Olenjniczak, 1988; Rudravaram *et al*. 2006)

Orange waste, pulp and peel, were used as substrate for single cell protein in this study as it support growth of microorganisms well. For this purpose, two species, KA-06 and KC-06, were employed. One of the major factors which govern the success of SCP is the protein content of the fungi which in return depends on the species and type of medium. The biomass crude protein content of KC-06 and KA-06 was 46.41% and 42.29% respectively, falls in the range reported by Solomonas (1985), who cultured 130 fungal species and found crude protein contents of 36.7-49.5% of biomass dry matter. John (1980) has grown *Aspergillus 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 34.8 % of protein content for *Aspergillus oryze*. Moo-Young *et al* (1977) 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 percentage of proteins secreted to the medium out of the Soluble protein synthesized by KC-06 and KA-06 was 26.17% and 34.97% respectively. Similarly, the investigation by Pakula *et al* (2005) showed that 29 % secreted protein at low specific growth rate and only 8 % at high growth rate by *Trichoderma reesi*. Compared with *Chaetomium cellulolyticum,* more of the substrate had been utilized by *Trichoderma viride* energy and possibly, cellulase production rather than for synthesis of intracellular biomass protein as reported by Moo-Young *et al* (1977).

Identification of the optimum duration of fermentation helps to produce the higher amount of any fermentation product. The time course of *Chaetomium spp* (KC-06), has shown optimum fermentation time of 120 hrs (5 days) for maximum protein yield. Similar, finding was reported by Karla *et al.* (1989) which is five days (120 hrs) for *Chaetomium globosum* grown on Kinnow-mandarin waste. Czajkowska and Ilnicka-Olenjniczak (1988) reported 20-22 hrs optimum fermentation time for *Aspergillus oryze* which is too short. Rudravaram *et al*. (2006) recommended 3 days of fermentation for *Aspergillus oryze* which approaches the result of this study on KA-06 (4 days). On the other hand, Oshoma and Ikenebomeh (2006) got maximum protein from *Aspergillus niger* on the six day grown on rice bran. In most cases, a slight decrease in protein content was observed which might be due autolysis of fungal mycelium. Czajkowska and Ilnicka-Olenjniczak (1988) reported the shortest optimal fermentation time which is 20-22 hrs for *Aspergillus oryze*. Generally, the optimum duration of fermentation is governed by the type of substrate and organism used, the environmental conditions (moisture, temperature, pH) and method of fermentation (SSF or SmF).

The optimum temperature for growth of orange waste by KA-06 (27.3 %) and KC-06 (25.2 %) was 25^{O}c. This result is close to the report by Rudravaram *et al*. (2006), 28 ^{O}c for *Aspergillus oryze* on deoiled rice bran and attained 18.9% of Soluble protein. At low temperature, the decline in protein content was due to inactivation of cellular activities and at higher temperatures the enzymatic reactions in the cell are destroyed (Shojaosadati *et al.*, 1999). The overall rate or 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 lead to thermal gradients because of the limited heat transfer capacity of solid substrates. So, single cell protein production in SSF needs regulation of temperature.

In SSF, the moisture level of the substrate has been considered as an important factor for the growth and activity of microbial culture. Czajkowska and Ilnicka-Olenjniczak (1989) and Rudravaram *et al*. (2006) reported 60% of moisture content for optimal protein enrichment of starchy raw materials and deoiled rice bran, respectively Aspergillus oryze. In contrast to this, this KA-06 (31.7) and KC-06 (29.5%) gave maximum protein at 40% of moisture level. The investigation by Fan & Ding (1990) and Yang *et al.* (1979) has also found 60 % of moisture was most suitable for mushroom production in solid substrate fermentation. It has been reported that higher moisture levels cause particle agglomeration, which interfere with heat and mass transfer, in turn resulting in decreases microbial activity (Pandey, 1992a, b). A high moisture level leads to decrease 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 *Aspergillus nige*r on starchy substrates, such as cassava (Raimbault and Alazard, 1980) was optimal at moisture levels considerably lower than on coffee pulp (Penaloza *et al*., 1991) or sugarcane bagasse (Roussos *et al.*, 1991). This is probably because of the greater water holding capacity of the latter substrate (Oriol *et al*., 1988). The lower optimum moisture content found in this study can also be due to low water holding capacity of orange peel.

The highest microbial biomass was attained at pH value of 5.5 for KC-06 and pH 7 for KA-06. Besides, Czajkowska and Ilnicka-Olenjniczak (1989) have reported that acid pH resulting from the presence and gradual utilization of $(NH_4)_2SO_4$, by the fungus was shown to limit fungal growth and stressed the buffering action of urea added instead of $(NH_4)_2SO_4$. In contrast to this, $(NH_4)_2SO_4$ was found to give maximum protein enrichment for both fungal species used in this study. Similarly, Oshoma and Ikenebomeh (2005) found that $(NH_4)_2SO_4$ is the best among other inorganic nitrogen supplements. Several results showed that $NaNO_3$ was found high yielder in several studies (Anupama and Ravinda, 1989; Karla *et al.*, 1989).

Substrate load was also found to be important factor influencing the growth of both fungal species (KA-06 and KC-06). As the amount of orange waste increased, the protein yield has shown dramatic decline. This can be explained by the lower heat removal surface as the substrate load increases (Frank-Jan, 2002). Besides, it could be due to substrate inhibition by toxic substances present in waste. It has been reported that the seed of citrus fruits contain limonin which is a toxic factor for pigs and poultry (Driggers, 1951). This should also be checked for its anti microbial effect.

The highest protein yield by KC-06 was obtained with spore load of 10^8 spores/ml which is similar to the report by Czajkowska and Ilnicka-Olenjniczak (1989) and Rudravaram *et al*. (2006), 10^8 to 10^9 spores/ml. KA-06 reached maximum protein enrichment with spore load of 10^6 spores/ml and further increase in the dose didn't show increment the protein content. Ravinder (2004) stated that at low inoculum size the substrate is utilized and prolongs incubation time. On the other hand, high inoculum size will lead to competition of growth between fungal hyphae over the limited substrate.

Generally, the study has shown that orange waste can be efficiently utilized by fungi and be enriched with protein for animal feed. The protein content of orange pulp was upgraded by KC-06 from 4.61% to 39.64% whereas to 34% by KA-06. The feed was enriched by 8.6 and 7.4 folds than the unfermented orange pulp by KC-06 and KA-06 respectively.

7. Conclusion

From the present study the following conclusions can be drawn:

- Orange pulp is a potential renewable substrate that can be used for single cell protein production for animal feed without chemical pretreatment.
- *Aspergillus niger* (KA-06) and *Chaetomium Spp* (KC-06) are able to effectively transform orange pulp in to microbial biomass in solid state fermentation.
- The maximum protein enrichment of orange pulp was obtained at the fourth day by KA-06 and fifth day by KC-06
- Orange pulp can be biotransformed in to SCP feed by KA-06 and KC-07 applying the optimal cultural conditions. These conditions are:

  Temperature- 25^{o}c
  Moisture content- 40%
  pH- 7 for KA-06 and 5.5 for KC-06
  Innoculum dose- 10^{6} for KA-06 and 10^{8} for KC-06
  Nitrogen source- $(NH_4)_2SO_4$

- The maximum protein content in the final protein attained by KC-06 is 39.64% and by KA-06 is 34%.

8. Reference

Aneja, K.R. (1993). *Experiments in microbiology, plant pathology and tissue culture.* WISHWA Prakashan, New Delhi, pp. 117-147

Anonymous (1977). Full scale single cell protein plant. *Process Biochem.* **30**: 1-12.

Anupama and Ravinda, P. (2001). Studies on production of single cell protein by *Aspergillus niger* in solid state fermentation of rice bran. *Brazilian Archives of Bio. and Technol.* **44(1):**79-88.

Balagopal, C. and Maini, S.B. (1977). Fungal protein from starch. *J. Root crops* **3**:33–45

Barnett, H.L. and Hunter, B.B. (1998). *Illustrated General of the Imperfect Fungi, 4th edition*. APS press, St. Paul, Minnesota.

Belammy, W.D. (1974). Single-cell proteins from cellulosic wastes. *Biotech. and Bioeng.*, **16**: 869–880.

Byrne, G.S. and Ward, O.P. (1989) Growth of *Rhizopus* in fermentation media. *J. Ind. Microbiol.* **4:** 155 - 161.

Calloway, D.H. (1974). The place of SCP in man's diet. **In:** *Single-Cell Protein,* pp29-146, (Davis, ed.). Academic Press, New York.

Chen, S. and Wayman, M. (1992). Novel inducers derived for starch from cellulose production by *Trchodema reesei*. *Process Biochem.* **27**: 327 - 334.

Clementi, F., Moresi, M. and Ross, J. (1985). Effect of medium composition on microbial utilization of citrus waste by mixed fungal culture. *Appl. Microbiol. Biotechnol.* **22:** 26-32.

Czajkowska, D, Ilnicka-Olenjniczak, O. (1989). Biosyntheisis of protein by microscipic fungi in solid state fermentation. II. Optimization of A*spergillus oryzae* a. or 11 cultivation for protein enrichment of starchy raw materials. *Acta Biothecnol.* **(1)**:407-413.

Czajkowska, D., Ilnicka-Olenjniczak, O. (1988). Biosyntheisis of protein by microscipic fungi in solid state fermentation. I. Selection of *Aspergillus* strain for enrichment of starchy raw materials in protein. *Acta Biothecnol*. **8(5)**:407-413.

Davis J.D. (1969). A Seletive Technique for Determining *Chaetomium* in Soil. *Plant and Soil* **31**: 17181.

De Gregorio, A., Mandalari, G., Arena, N., Nucita, F., Tripodo, M.M. and Lo Curto, R.B. (2002). SCP and crude pectinase production by slurry-state fermentation of lemon pulps. *Biores. Technol*. **83:** 89–94.

Department of Agriculture Forest Service (2000). *The State of Food and Agriculture*. United States Department of Agriculture Forest Service, USA, pp110-119.

Driggers, J. Clyde, Davis, George K. and Mehrhof, N.R. (1951). *Toxic factor in citrus seed meal*. *Gainesville, Florida Agricultural Experiment Station Bulletin* **476**: 36.

Fan, L. F., and Ding, C. K. (1990). *Handbook of Mushroom Cultivation, Jiangxi Science and Technology Publishing House*, Jiangxi, China.

FAO Committee on commodity problems(2003). *Intergovernmental group on citrus fruit*. Havana, Cuba.

Fukui, S. and Tanaka, A. (1981). Metabolism of alkanes by yeasts. *Adv. Biochem. Eng.* **19**: 217-225.

Frank-Jan, N. (2002). *Process Control of Solid-State fermentation: Simultaneous control of temperature and moisture content.* PhD dissertation, Wageningen University, The Netherlands.

Ghoul, M. and Engasser, J.M. (1983). Microbiologie-Aliments-Nutrition, *Int. Microbiol.* **1**:277 - 297.

Gierhart, D. L. and Potter, N. N. (1978). Effects of ribonucleic acid removal methods on composition and functional properties of *Candida utilis.* J. *Food Sci.* **43**: 1705-1713.

Harrison, D.E.F. and Wren, S.I. (1976). Mixed microbial cultures as a basis for further fermentation process. *Process Biochem*. **11**: 30 - 43.

Hecht, V., Rosen, W., and Schugerl, K. (1985). Conversion of cellulose into fungal cell mass in solid state culture. *Appl Micriobiol Biotechnol,* 21: 189-191.

Hélène, B., Guy M. And Pierre G. (1984). *Production of Microbial Biomass*. Montpellier, Paris.

Hiotizman, D.O. (1986). Yeast production from crude sweet whey using *Candida* kefyr LY 496. **In**: *Microbial Biomass Protein,* pp. 27 – 45, (Moo-Young, M. and Gregory, K.F. eds). Elsevier Applied Science, London.

Humphrey, A.E. (1975). Production of food and feed by fermentation. *Proc. R. Aust. Chem. Inst,* **41 (11)**: 285 - 290.

Ikenebomeh, M.J. (1981). Upgrading “garri” with single cell protein of *Geotrichum candidum*: Preliminary investigation. *J. Canad. Inst. Food Sc. Technol.* **14 (3)**: 168-175.

Imrie, F. (1975). Single-cell protein from agricultural wastes. *New Scientist* 458–460.

Israelidis, C.J. (1994). Nutrition - Single Cell Protein, Twenty Years Later. *Procc. Balkan chem. days, chimica chronica* **9(3)**: 337-352.

John H.L. (1980). Microbial Protein Production. *BioSci., Food from Microbes* **30(6)**: 387-396.

Kalra, K.L., Grewal, H.S., and Kahlon, S.S. (1989). Bioconversion of Kinnow-mandarin waste into single-cell protein. *MIRCEN J.***5**:321-326.

Laufenberg, G., Kunz, B., and Nystroem, M. (2003). Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementation. *Bioresource technol*. **87**: 167-198.

Lemma Gizachew and Smith, G.N. (2005).Crude protein and mineral composition of major crop residues and supplemental feeds produced on Vertisols of the Ethiopian highland. *Anim. feed sci. technol.* **119(2)**: 143-153.

Litchfield, J.H. (1979) *Production of SCP for use in food or feed*, Academic Press, New York, pp. 93-112.

Lowry, O.H., Rosenbrough, N.J., Farr, A.I., & Randall, R.J. (1951). Protein measurement with the folin-phenol reagent. *J.Biol. Chem.* **193**: 265-227.

Moo-Young, M R., Chahal D. S., Swan J. E., And C. W. (1977). SCP Production by *Chaetomium cellulolyticum,* A new thermotolerant cellulolytic fungus. *Biotechnol and Bioeng* **(19)**:527-538.

Moo-Young, M. (1977). Economics of SCP production. *Process Biochem.,* **12(4)**: 6-10.

Moo-Young, M., Chahal, D.S. and Stickney B. (1981). Pollution control of swine manure and straw by conversion to *Chaetomium cellulolyticium* SCP feed. *Biotechnol. and Bioeng*. **3**: 2407-2415.

Moo-Young, M., Chisti, Y. and Vlach, D. (1992) Fermentation conversion of cellulosic substrates to microbial protein by *Neurospopa sitophila*. *Biotechnol. Letters*. **14(9)**: 863 - 868.

Moulin, G. and Galzy, A. (1984). Whey a potential substracte for biotechnology. *Biotechnol. Genetic Eng*. Rev. **2**: 344-349.

Muondi, P.J. and Manssen, J.F. (1981). SCP from suspensions of cassava meal by *Trichoderma harzianum J. Sci. Food. Agric*. **32**: 647 - 652.

Oriol, E., Raimbault, M., Roussos, S. and Viniegra Gonzales, G. (1988). Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger. Appl. Microbiol. and Biotechnol.* **27**:498-503.

Oshoma, C.E. and Ikenebomeh M.J. (2005). Production of *Aspergillus niger* Biomass from Rice Bran. *Pakistan J. of Nutrition* **4 (1):** 32-36.

Oura, E. (1983). Biomass from Carbohydrates. **In**: *Biotechnology,* pp, 3, (Rehm, H.J. and Reed, G. Eds). Verlag Chemie, Weinheim.

Pakula M.T., Salonene, K., Uusitalo, J. and Penttila, M. (2005). The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus *Trichoderma reesei*. *Microbiol.* **151**:135-143.

Pamment N., Robinson, C.W., Hilton J., Moo-Young, M. (1878). Solid state cultivation of *Chaetomium cellulolyticum* on alkali-pretreated sawdust. *Biotechnol Bioeng.* **20**: 1735- 1744.

Pandey, A. (1992a). Recent process developments in solid state fermentation. *Process Biochem.* **27**:109-116.

Pandey, A. (1992b). Production of strach saccharifying enzymes in solid cultures. *Starch* **39**: 75-77.

Pandey, A. (2002). Solid-state fermentation. *J. Biochem Eng.* **13**: 81–84.

Penaloza, W., Davey, C.L., Kell, D.B. and Hedger, J.N. (1991). Real time monitoring of the accretion of *Rhizopus Biotechnol. and Bioeng*. **35**:802-808.

Plessas, S., Bekatorou, A., Koutinas, A.A., Soupioni, M., Banat, I.M. and Marchant, R. (2007). Use of *Saccharomyces cerevisiae* cells immobilized on orange peel as biocatalyst for alcoholic fermentation. *Biores. Technol*. **98**: 860–865.

Pretorius, W.A. and Lempert, G.G. (1993). The selective cultivation of the thermotolerant a*spergillus sp*. on spent sulphite liquor. *Water SA*. **19(1)**: 67 - 72.

Raimbault, M. (1981). Fermentation on solid substrates. *ORSTOM.* 291 - 327.

Raimbault, M. and Alazard, D. (1980). Culture method to study fungal growth in solid fermentation. *Europ. J. appl. Microbiol. Biotechnol*. **9**: 199-209.

Rehm, H.J. and Reiff, I. (1981). Mechanisms and occurrence of microbial oxidation of long- chain alkanes. *Adv. Biochem. Eng.* **19:** 175 - 215.

Romantschuk, H. and Lehtomäki, M. (1978). Operation experience of first full scale Pekilo SCP-mill application. *Proc. Biochem*. **13**: 16 - 17, 29.

Roussos, S., Raimbault, M., Viniegra-Gonzalez, G., Saucedo-Castañeda, G. and Lonsane, B.K. (1991). Scaleup of cellulases production by *Trichoderma harzianum* on a mixture of sugar cane bagasse and wheat bran in solid state fermentation system. *Myco. Neotropical Aplicada* **4**:83-98.

Rudravaram, R., Chandel, A.K., Linga, V. R., and Pogaku, R. (2006). Optimization of protein enrichment of deoiled rice bran by solid state fermentation using *Aspergillus oryzae* MTCC 1846 *Int. J. of Food Eng.* **2(4):**

Saheleedhin Sertsu and Taye Bekele (2000). *Procedures for soil and Plant analysis.* National Soil Research Center, Ethipian Agricultural Research Organization, Addis Ababa, Ethiopia. pp. 70-76.

Sahm, H. (1979). Production of SCP by methanol utilizing microorganisms. *Int. Microbiol.and Food Ind.* **21**:135-142.

Schügerl, K., Lück, J. and Oels, U. (1977). Bubble column bioreactors, tower bioreactors without mechanical agitation. *Adv. Biochem. Eng.* **7**: 1 - 6.

Senez, J.C. (1980). Solid state fermentation of starchy substrates. **In*:*** *Food from Waste,* pp 169, (Birch, G.C., Parker, K.J. and Worgan, J.T., eds). Applied Science, London.

Shojaosadati, S.A, Faraidouni, R, Madadi-Nouei, A., & Mohamadpour. I. (1999). Protein enrichment of lignocellulosic substrates by solid state fermentation using *Neurospora sitophila. Resources, Conservation and Recycling* **27**: 73–87.

Solomonas, G. L. (1985). Production of biomass by filamentous fungi. *Comp. Biotechnol.* **3**: 483-505.

Tan, K.H., Ferguson, L.B. and Carlton, C. (1984) Conversion of cassava starch to biomass, carbohydrates, and acids by *Aspergillus niger*. *J. Appl. Biochem.* **6:** 80-90.

Taylor, J. C., Lucas, E. W., Gable, D. A., and Graber, G. (1974). *Evaluation of single cell protein for non-ruminants.* Academic Press, New York, pp179-186.

Ugalde, U. O. and Castrillo, J. I. (2005). Single Cell Protein from Fungi and Yeasts. *Biotech. and Bioeng.*, **27**: 269–280.

Waslein, C. I. (1975). Unusual sources of proteins for man. *CRC Crit. Rev. Food Sci. Technol.* **5:** 77-151.

Yang, H.H., Thayer, D.W., & Yang, S.P. (1979) Reduction of endogenous nucleic acid in single cell protein. *Appl. and Env. Microbiol*. **38**:143-147.

Yazicioqlv T. (1983). Production and Feeding of Single Cell Protein. Proceedings of a COST Workshop Appl. Sc. Publish., pp. 159.

APPENDICES

Appendix 1. ANOVA, effect of temperature on protein enrichment of orange waste by KA-06 and KC-06

		Sum of Squares	df	Mean Square	F	Sig.
protein * temp	Between Groups (Combined)	249.137	3	83.046	66.665	.000
	Within Groups	24.914	20	1.246		
	Total	274.052	23			

Appendix 2. ANOVA, effect of Moisture content on protein enrichment of protein enrichment of orange waste by kA-06 and KC-06

		Sum of Squares	df	Mean Square	F	Sig.
protein * moisture	Between Groups (Combined)	158.005	3	52.668	9.972	.000
	Within Groups	105.637	20	5.282		
	Total	263.642	23			

Appendix 3. ANOVA, effect of initial pH on protein enrichment of protein enrichment of orange waste by kA-06 and KC-06

		Sum of Squares	df	Mean Square	F	Sig.
protein * pH	Between Groups (Combined)	217.826	3	72.609	7.399	.002
	Within Groups	196.256	20	9.813		
	Total	414.081	23			

Appendix 4. ANOVA, effect of inoculum dose on protein enrichment of protein enrichment of orange waste by kA-06 and KC-06

		Sum of Squares	df	Mean Square	F	Sig.
protein * inoculum	Between Groups (Combined)	854.917	2	427.458	34.055	.000
	Within Groups	188.281	15	12.552		
	Total	1043.197	17			

Appendix 5. ANOVA, Effect of substrate load on protein enrichment on protein enrichment of orange waste by kA-06 and KC-06

		Sum of Squares	df	Mean Square	F	Sig.
protein * substrate load	Between Groups (Combined)	351.155	2	175.578	5.827	.013
	Within Groups	451.980	15	30.132		
	Total	803.135	17			

Appendix 6. ANOVA, the effect of different nitrogen sources on protein enrichment of orange waste by kA-06 and KC-06

		Sum of Squares	df	Mean Square	F	Sig.
protein * Nsource	Between Groups (Combined)	360.993	4	90.248	13.692	.000
	Within Groups	164.788	25	6.592		
	Total	525.781	29			

Declaration

I, the under signed, declare that this thesis is my original work. It has never been submitted in any institution and that all sources of materials used for the thesis have been dully acknowledged.

Name: Biniyam Yalemtesfa
Place: Addis Ababa University
Signiture ___________________
Date _______________________

This thesis has been submitted for examination with my approval as
Advisor: Tesfaye Alemu, Ph.D
Signiture _______________________
Date ___________________________

Table of contents

List of Figures

List of Tables