INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 5, p. 62-69, 2017

RESEARCH PAPER OPEN ACCESS

Nutritional assessment of single cell protein produced with *Aspergillus flavus* and *Penicillium chrysogenum*

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Key words: Single cell protein (SCP), *Aspergillus flavus, Penicillium chrysogenum*.

http://dx.doi.org/10.12692/ijb/11.5.62-69 Article published on November 12, 2017

Abstract

The recent era has seen a great deal of interest in the bio-processing of fruit waste particularly prompted by their nutritional and economic exploitation through the application of emerging technologies like Single Cell Protein (SCP) Technology. The current study was conducted as a continuation of the global efforts to eradicate protein deficiency by ensuring the provision of protein- rich-food to the ever- increasing population of the world. Apple waste was used for the preparation of fermentation media as apple contains the greater bulk of carbohydrates required for the growth of the microorganism. The proximate analysis of apple waste was carried out and the data obtained showed that apple waste contains 17%,15.28%, 0.4%, 0.50%, 0.06% and 0.76% of Dry matter (DM %),Carbohydrates, Crude protein (CP %), Ash, Fats and Crude fiber respectively. Pure cultures of *Aspergillus flavus* and *Penicillium chrysogenum* were isolated and grown on fermentation media prepared with apple waste. The growth obtained was thus analyzed and it was found that *Penicillium chrysogenum* (91.19% Dry matter, 35% crude proteins and 5.38% ash) produces greater amount of Crude Proteins as compare to *Aspergillus flavus* (92.36% Dry Matter, 24.49% crude proteins and 6.49% ash) although *Penicillium chrysogenum* was a slow grower as compare to *aspergillus flavus* and it was assumed that *Aspergillus flavus* would possibly result in greater amount of proteins as compare to *Penicillium chrysogenum*.

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Introduction

Proteins are major macromolecules and essential component of all living organisms which carries out all biochemical reaction in form of enzyme. As compared to other macromolecules, our body requires plentiful quantity of proteins and its destruction is sometime lethal (Solomons, 1983). Though post-modern world is replaced from the modern but still poverty is flourishing at quicker face all around the globe. The data collected by the Food and agriculture organization, (FAO), suggest that twenty five percent of the world population has protein deficiency, which is an obvious indication of protein gap. To compensate this deficiency, microbial proteins can be utilized as potential source to provide an alternative of protein to the economically marginalized segments of the society (Najafpur, 2007). For this purpose SCP technology has been developed to produce microbial proteins.

Single Cell proteins refers to the dried cell mass of fungi, algae, moulds and bacteria grown on larger scale to enhance the net protein availability in animal and human foods consumption (Yalein *et al.*, 2008). SCP was produced in Germany during First World War with *Sacharomyces cerviceae* by using molasses as carbon source and ammonium salts as nitrogen sources. For the production of SCP a wide variety of microorganisms are used. SCP production with algae accounts for a high protein content as compared to other microorganisms and can be easily harvested, but apart from these merits such proteins are indigestible due to cellulosic wall and high concentration of metals (Gad *et al.*, 2010). A number of different types of agro-industrial wastes, fruits and wooden pulps are used for growing yeast. Such cells are larger in size but similar to algae these cells are also poorly digestible. Temperature required for growing yeast cells ranges 30-34°C and pH 3.5-4.5. Similar to yeast cells, bacteria can also be grown on wide range of different substrates (Harris,1949). However the rapid growth and high protein content of fungi and bacteria have made them the prime contenders for use as sources of SCP. Many fungi like *Aspergillus niger* AS101with corn cobs (Singh *et al.*, 1919). Pakistan is famous all around the globe for the production of a wide variety of fruits and vegetables. The country is one among major producers of good quality apple which greatly subsidize to the economic well-being of the country by exporting it to the global market. Apart from that, these fruits are locally utilized by various industries for production of different industrial products and discard the remnant as waste product. The discarded waste is a major cause of environmental pollution which can badly affect the environment and also results in various infectious diseases due to growth of pathogenic microorganisms. These fruit wastes are rich sources of carbohydrates that could be used as potential natural substrates for the growth of essential useful microorganisms. In this regard single cell protein technology is a newly introduced approach that uses these cheap out-of-use fruit waste for the production of valuable microbial products of human consumption.

The single cell protein technology promises great socio-economic impact as the technology provides an effective tool for the large scale production of valuable products of human consumption from cheap out-of-use organic agro-industrial waste. The Economic and Social Affairs of the United State (ESAUN) in 2011 recorded that the total number of humans in the world is well over 7 billion with a regular increase of approximately seventy seven million annually(ESAUN,2011). Conventional agriculture may be unable to satisfy such demands and to supply sufficient food particularly proteins to this ever increasing population (Smith, 2000).Hence substitute protein sources that are rather more value-added in quality is a must to deal with such problems (Glazer and Nikaido, 2007).This particular study was intended as possible approach towards utilizing apple waste as fermentation substrate for the large scale production of single cell protein. SCP technology is also an effective approach towards eradication of environmental pollutants as these residues (apple waste) are utilized for the preparation of fermentation media for the production of single cell proteins by the microorganisms.

Materials and methods

Selection and isolation of fungal species

The selection of fungal species for the production of single cell proteins was a crucial stepand so was the isolation of pure cultures. The extensive literature review prior to the current research work used different fungal species and techniques for its isolation. However for the current study two widely used fungal species i.e. *Aspergillus flavus*and *Penicillium chrysogenum* were chosen.

Isolation of Aspergillus flavus and Penicillium chrysogenum

In an effort to isolate pure cultures of either *Aspergillus flavus* bread, with amazing ability to support the growth of a number of fungal species was collected from a local Bakery in District Swabi. The bread showed clear signs of fungal growth that were carefully maintained under complete aseptic conditions in order to allow the fungal colonies growth, even bigger and allow easier isolation of the required fungal species. Lemon owing to their amazing ability to allow the growth of large number of fungal species i.e. *Penicillium chrysogenum* were collected, made porous with help of a needle and placed in open air to allow it develop fungal colonies. Either of the two samples was carefully maintained under the proper lab conditions in order to ensure that they develop pure fungal cultures and are free from any kind of contamination. The selected samples were investigated on regular basis for fungal growth. After a couple of days the bread was founded to have developed clear large greenish patches, whereas the lemon was also founded to have developed a large bulk of somewhat blackish mass all around.

Preparation and inoculation of Potato dextrose agar media

A known quantity i.e. 9.75gm of commercial PDA powder was dissolved into 250ml of sterile distilled water in a conical flask. The conical flask having mixture is placed on a hot plate stirrer, so that the media warmed up. The media is stirred at 400 rpm for 5 to 10 minutes until it is completely dissolved. Then it is removed from the stirrer, and placed for some time to let it cool. The flask is sealed with a cotton plug and wrapped around by an aluminium foil to avoid contamination. After being allowed to cool down, the media from the conical flask was poured into Petri dishes for further use. The media plates are kept at ambient temperature for about 24 hours to allow the media to solidify. The plates are tightly sealed as if kept in refrigerator for days; the agar releases water and becomes unsuitable for pure fungal growth. The plates are simply stacked and stored in sterile plastic bags and kept in refrigerator. The inoculation was preceded by preparing inoculums from two samples, with two media plates (S1=BI i.e. sample 1is taken from bread inoculums and S2=CI i.e. Sample 2 is taken from citrus fruit inoculums) being inoculated from sample inoculums while the remaining two plates were inoculated directly from the sample (S1=B i.e. sample is taken directly from bread and S2=C i.e. sample is taken directly from citrus fruit) in a sense that fungal biomass from the either sample were taken with the help of an inoculating loop and transferred onto media plates. The inoculated media plates were carefully sealed, labelled and stored is an incubator set sharply at around 35°c that is thought to be the most favourable temperature for fungal growth. The plates were then frequently investigated for fungal growth on regular basis and to ensure that all of them are free from any kind of contamination.

Fermentation media preparation from rotten apples

A series of experimental setups was conducted during this study in order to optimize the proper growth conditions for either of the two fungal species used for the production of maximum yield of single cell proteins from rotten apples. As a matter of fact, the healthy growth of almost all microorganisms is greatly affected by the presence of nutrients in the fermentation media and equally by the growth conditions provided during the course of their growth. During each proceeding setup all the necessary conditions were kept constant indefinitely while changing just a single one at a time.

Fermentation media was prepared by measuring 800gm of rotten apples through digital balance. Small quantity of water was added and then allowed to boil for an hour for hydrolyzing the polysaccharides. A total of 400ml of extract was obtained through filtration which was supplemented with small quantity of urea as a source of nitrogen and salt as source of minerals. The concentrated apple extract was diluted with water and were placed into 4 separate flasks. Then fungal species from each PDA plate were inoculated in separate flasks. These flasks were initially kept in shaking incubator for about 1 week keeping temperature 28°c, PH 4.8 and RPM 100; the flasks were then removed and placed in regular incubator and after about 1 week significant amount of growth was observed.

Nutritional analysis of rotten apples mass and single cell protein

Rotten Apples fruit samples were analyzed at dry weight basis for proximate crude protein, crude fat, crude fiber, ash, moisture and crude CHO content by following their respective procedures. The biomass of *Aspergillus flavus* and *Penicillium chrysogenum* on each batch was analyzed for its ash, crude fiber, crude fat and crude protein contents using the procedure mentioned in AOAC methods (AOAC, 2006).

Results

Identification and selection of fungal species

Morphological characteristics of the fungal colonies on potato dextrose agar media and high resolution microscopy assisted the identification and confirmation of two fungal species i.e. Aspergillus *flavus* and *Penicillium chrysogenum* (Fig. 1 and 2).

Optimization of fermentation media

A series of experiments were performed for optimization the proper growth conditions for two fungal species and to achieve maximum yield of single cell proteins. The optimum conditions provided at which fungal species showed maximum growth (Table 1).

Table 1. Experimental setup for optimization of fermentation media.

Experimental Setups	Substrate Quantity	Temp	RPM	PH	Incubation Period	Supplementary Nutrients	Growth
setup 1	800mg	28°c	150	5.3	1week	Galactose, Urea	Negative
setup 2	40mg	28°c	100	5.3	1week	Galactose, Urea	Negative
setup 3	200mg	28°	100	4.8	1week	Galactose. Urea	Negative
setup 4	800mg	28°c	100	4.8	1week	Galactose, Urea	Positive

Nutritional assessment of net protein content of apple waste

The net protein content of the apple waste was calculated at the Directorate of livestock research and development, veterinary research institute, Peshawar in order to know the proximate composition of the apple waste. The concentration of various components of the apple waste in terms of Dry Matter, Moisture Content, Carbohydrates, crude proteins, ash, fat and crude fiber calculated from 2gm of the finely grounded apple waste (fig.3). The data provides valuable insights regarding the use of apple waste as potential substrate for the preparation of fermentation media as apple waste provides the entire repertoire of all the essential components desperately required for microorganisms to grow profoundly and provide us with plentiful of single cell protein.

Proximate analysis of the fungal biomass

The obtained fungal biomass was investigated for the presence of various components, the most important being the net production of crude proteins by both *Aspergillus flavus* and *Penicillium chrysogenum*. The results turned out to be surprising in the first place as *Penicillium chrysogenum* showed a higher concentration of the crude proteins as compare to the extensively used *Aspergillus flavus* despite the fact that *Penicillium chrysogenum* was observed to be a slow grower on the fermentation media as compare to *Aspergillus flavus* (Fig. 4).

Discussion

Identification and selection of fungal species

There is wide variety of microorganisms used for the production of single cell protein including bacteria, fungi and algae being the most prominent agents. Algal single cell protein, however have limitations such as the need for warm temperatures and plenty of sun light in addition to carbon dioxide, and also that the algal cell wall is indigestible. Bacteria, although are capable of growing on a wide variety of substrates, have a short Generation time and have high protein content.

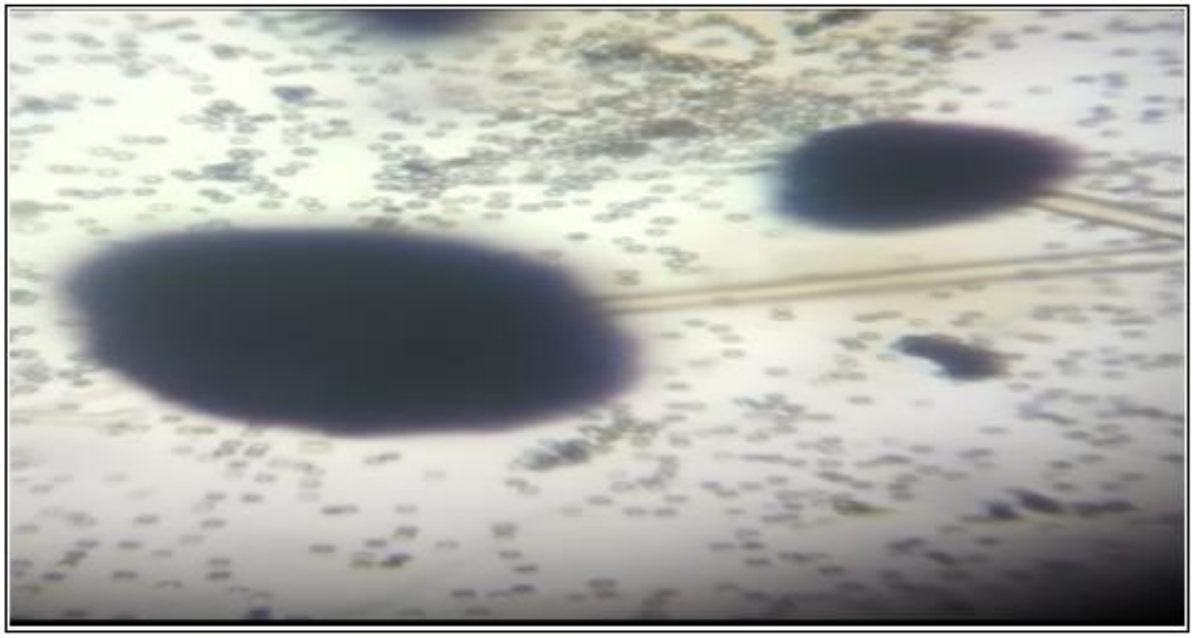

Fig. 1. Microscopic view of *Aspergillus flavus*.

Their use is somewhat limited by poor public acceptance of bacteria as food, small size and difficulty of harvesting and high content of nucleic acid on a dried weight basis. This makes fungi comparatively the best candidate for the production of single cell protein (SCP), once the only associated risk of producing toxin is avoided in the process by selecting the generally regarded as safe (GRAS) fungal species.

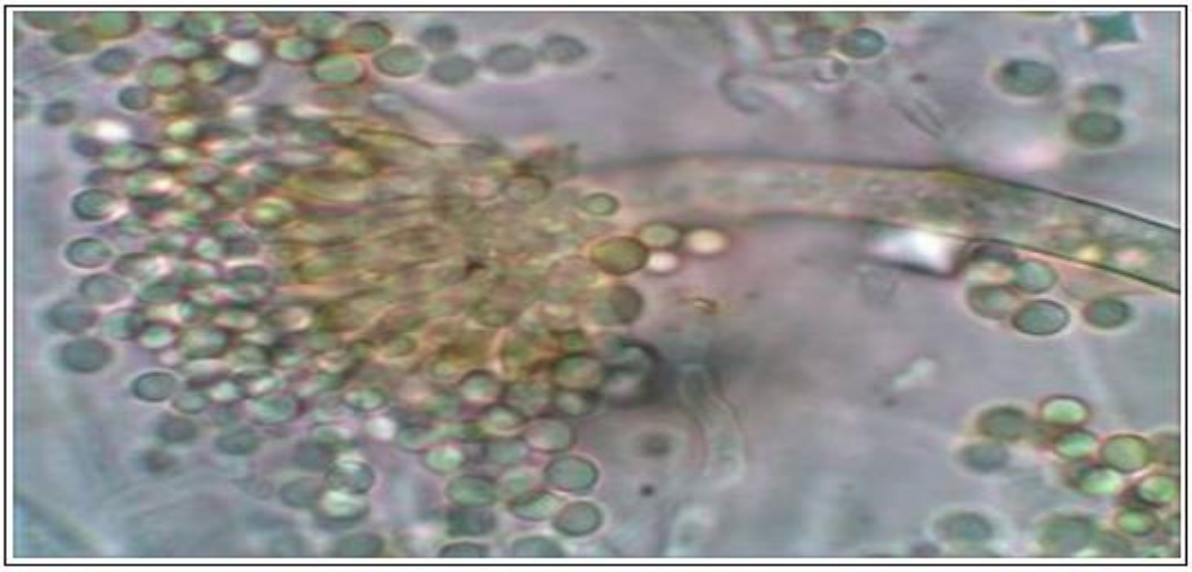

Fig. 2. Micrograph of *Penicillium Chrysogenum* under light microscope.

Two fungal species are well suited for the purpose as both are largely regarded as less pathogenic or even non-pathogenic once all the growth condition are properly optimized and sufficient amount of nutrients are available in the media. *Aspergillus flavus* is predominately a saprophyte and grows on dead plant and animal tissue in the soil. For this reason it is very important in nutrient recycling (Scheidegger *et al.*, 2003). *Penicillium chrysogenum* plays a significant role in the medical community as a powerhouse of antibiotics because it synthesis penicillin which inhibits the biosynthesis of bacterial cell walls affecting lysis of the cell (Fleming, 1929). *Penicillium chrysogenum* is rarely pathogenic except in extenuating circumstances such as people with severely suppressed immune systems, like those with human immunodeficiency virus (HIV).

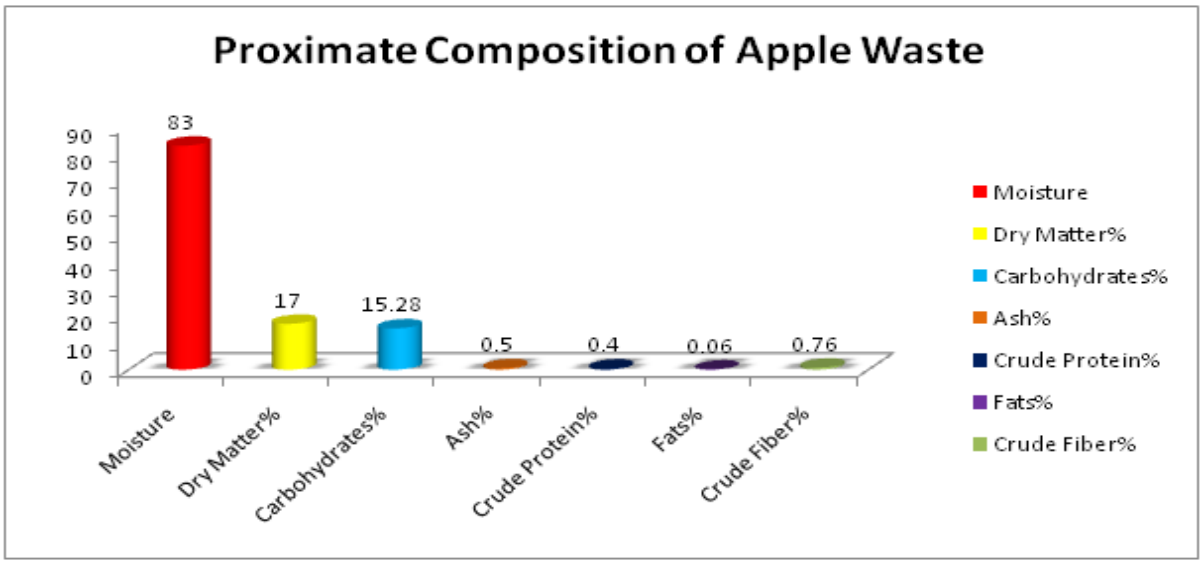

Fig. 3. Nutritional assessment of apple waste in term CHO, crude protein and Ash.

Optimization of growth condition of fermentation media

Once microorganisms are provided with all optimum conditions, they have high metabolism, utilizing maximum quantity of the nutrients in the media and grow profoundly providing large quantity of the required fermentation product. Optimization of the growth conditions and the availability of the required nutrients are particularly important for the smoother progression of the fermentation process. If surplus salts and sugar concentrations are present in the media it could possibly cause death of the cells. In an effort to properly optimize the growth conditions particularly required for the growth of *Aspergillus flavus* and *Penicillium chrysogenum* a series of experimental setups were carried out. In the process, the growth conditions were randomly manipulated in terms of PH, Temperature, amount of substrate (Apple waste) used for the preparation of fermentation media, RPM and the nature and quantity of supplements if any added to the media. It was found that both *Aspergillus flavus* and *Penicillium chrysogenum* grows profoundly under the following range of growth conditions i.e. PH 4.8, Temperature 28°c and a minute quantity of potato dextrose agar, galactose and urea added as media supplements. Apple waste is the residue left from juice extraction and constitutes about 25% of the weight of fresh fruit (Walter *et al.,* 1976). Apple waste is rich in carbohydrate (11.08gm/100gm of dry weight), but its protein content is low (0.3gm/100gm of dry weight) (Bomben *et al.,* 1971).Significant research effort has been made to use apple waste as an energy source, alleviating the waste disposal problem (Walter *et al.*, 1976). During the current research setup apple waste collected from various juice outlets of District Swabi were thoroughly washed with water and then a fermentation media was prepared using these apple residues.

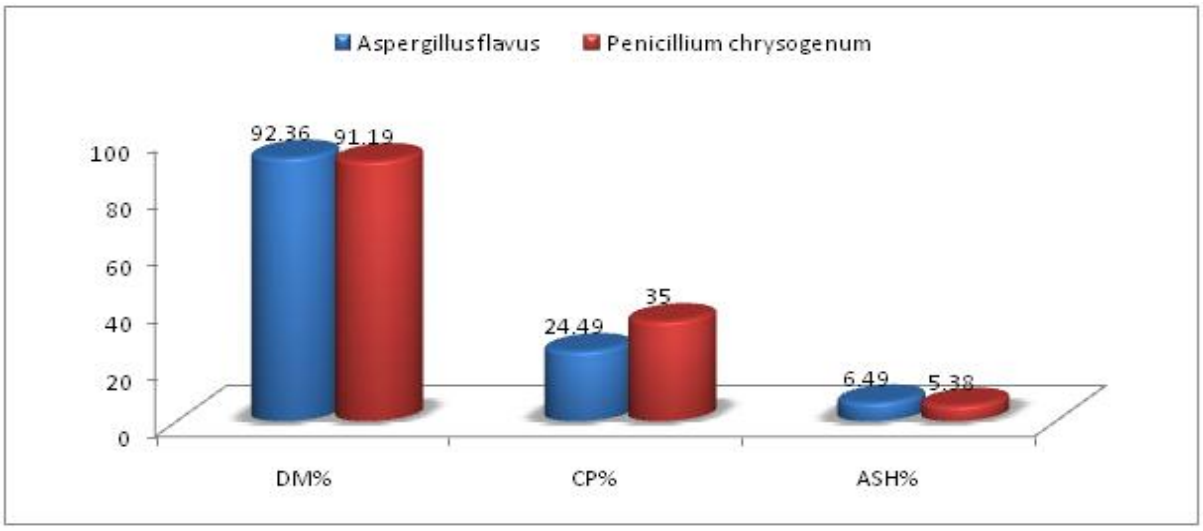

Fig. 4. Proximate analysis of SCP production *by A. flavis* and *P. chrysogenum*.

Nutritional assessment of net protein content of apple waste

The proximate analysis of apple waste revealed that apple waste contain considerable amount of carbohydrates i.e. 15.28% in addition to handsome quantity of all other nutritional components desperately required for the growth of microorganism. The proximate analysis, however revealed a much lower quantity of the net proteins (about 0.4%/17% of Dry Matter) contained in fixed amount of apple waste but even this very minute protein quantity fail to pose any problem to the growth of fungal cultures, as fungal cultures tends to grow profoundly on any source containing large quantity of carbohydrates. Thus a fermentation media using apple waste as potential substrate could possibly produce a large quantity of the required fungal biomass provided that proper growth conditions are also provided to the microorganisms used.

Proximate analysis of the fungal biomass

The net protein content of both *Aspergillus flavus* and *Penicillium chrysogenum* were determined by the standard kjeldahl method of protein analysis. The results obtained showed a much higher quantity of crude proteins (35.00%/91.19% of Dry Matter) produced by *Penicillium chrysogenum* as compare to the amount of crude proteins produced by *Aspergillus flavus* (24.49%/92.36% of Dry Matter).It was surprising to see that *Penicillium chrysogenum* produced relatively higher concentration of SCP as compare to that produced by *Aspergillus flavus* because *Penicillium chrysogenum* was initially observed to be a slow grower as compare to *Aspergillus flavus* and it was expected that *Penicillium chrysogenum* would produce a nominal amount of the required SCP as compare to *Aspergillus flavus*, a much rapid grower on fermentation media. In a similar kind of study conducted in India showed a higher concentration of SCP approximately 50.86%/100gm of substrate (apple waste) used with *Saccharomyces cerevisiae* (Mahnaz *et al.,* 2010). Similarly, in a study conducted with *Aspergillus oryzae* in India using a combination of apple waste and watermelon waste as potential substrate the total protein obtained were 24.2mg/100gm of substrate used (Mahmood khan yousufi, 2012).

The degree of fungal biomass growth depends on a number of factors with the most important being the type of substrate used for the preparation of fermentation media. This variation could possibly be attributed to ability of a particular class of microorganism to effectively utilize a particular substrate due to its unique ability to produce the necessary enzymes required for the degradation of the fruit waste and their subsequent conversion into valuable products of human welfare.

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