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PRODUCTION OF MICROBIAL BIOMASS PROTEIN BY SEQUENTIAL CULTURE FERMENTATION OF *ARACHNIOTUS* SP., AND *CANDIDA UTILIS*

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Abstract

Sequential culture fermentation by *Arachniotus* sp. at 35°C for 72 h and followed by *Candida utilis* fermentation at 35°C for 72 h more resulted in higher production of microbial biomass protein. 6% (w/v) corn stover, 0.0075% $CaCl_2.2H_2O$, 0.005% $MgSO_4.7H_2O$, 0.01% KH_2PO_4, C: N ratio of 30:1 and 1% molasses gave higher microbial biomass protein production by the sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. The mixed microbial biomass protein produced in the 75-L fermentor contained 16.41%, 23.51%, 10.9%, 12.11% and 0.12% true protein, crude protein, crude fiber, ash and RNA content, respectively. The amino acid profile of final mixed microbial biomass protein showed that it was enriched with essential amino acids. Thus, the potential utilization of corn stover can minimize the cost for growth of these microorganisms and enhance microbial biomass protein production by sequential culture fermentation.

Introduction

The increased production of wastes in the world is of great concern. Various alternatives are exercised to diminish this increase by elimination, purification and recycling (Saadia *et al*., 2008; Saleem *et al*., 2008; Ahmed *et al*., 2009a). Biomass is an alternative natural source for chemical and feedstock with a replacement cycle short enough to meet the demands of the world fuel market (Ahmed *et al*., 2005; Ahmed *et al*., 2007; Ahmed *et al*., 2009b). Bioconversion of the agricultural wastes through microbial fermentation is the natural way to recover resources (Irshad *et al*., 2008). Biotechnological treatments of food processing wastes can produce a valuable end-product e.g., microbial biomass protein (MBP) (Moo-Young *et al*., 1992; Jin *et al*., 2001). Conversion of carbohydrate by-product to value added product is of great importance for the production from renewable resource in sustainable society (Taherzadeh *et al*., 2003; Jamil *et al*., 2005). Low-cost non-conventional agro-industrial residues, which accumulate up to 50 million ton in Pakistan, can be fermented to produce single cell protein. Cycling and recycling of these residues through microbial fermentation will not only reduce the pollution but also serve as potential source of energy for the production of low cost high quality MBP (Athar *et al*., 2009). Several agro-industrial wastes have been used to produce MBP for livestock and poultry feeds.

Arachniotus sp., is a white rot fungus and has been used for the economic utilization of many waste products (Shaukat *et al*., 2006). *Candida utilis* has been frequently used in single cell protein (SCP) production because of its ability to utilize a variety of carbon sources and to support high protein yield (Nigam, 2000). It has been used for production of several industrial products both for human and animal consumption (Zayed & Mostafa, 1992; Kondo *et al*., 1997).

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The properties and the growth of mixed microbial cultures are of potential interest to biological processing, particularly for food production because of the sophisticated compositional and structural expectations of food products. The important principles and properties of mixed cultures have largely been overlooked in the past, mainly due to the fact that most high technological bioprocess industries are developed for the manufacture of individual chemicals, such as antibiotics and primary and secondary metabolites, for which there may be no need or advantage in using mixed cultures (Fields *et al*., 1991, Ghanem, 1992). Mixed culturing of microorganisms, however, is also a very good method for converting carbohydrate wastes into high yields of microbial biomass protein using short fermentation times (Konlani *et al*., 1996; Paul *et al*., 2002). In the present study, we have investigated the possibility of bioconversion of corn stover augmented with molasses into microbial biomass protein by sequential culture fermentation of *Arachniotus* sp., and *Candida utilis*.

Materials and Methods

Substrate: Corn stover obtained from local market of Faisalabad, Pakistan, was air dried in an oven at 65°C to a constant weight. It was ground to 2 mm sieve and stored in air tight plastic jars till further use.

Microorganisms: *Arachniotus* sp., and *Candida utilis* were obtained from the stock cultures of National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan.

Media and Culture conditions: The inoculum medium for *Arachniotus* sp., consisted of (g L^{-1}) corn stover, 20; $CaCl_2$. $2H_2O$, 0.025; $MgSO_4.7H_20$, 0.025; KH_2PO_4, 2; poultry droppings (as urea's source), 18.9. The inoculum was grown at 35°C with pH 4 and shaking at 150 rpm for 24 h (Shaukat *et al*., 2006).

Seed culture medium was used as inoculum medium for *C. utilis* containing (g L^{-1}) KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 5.0; $CaCl_2$, 0.13; $MgSO_4$, 0.5; yeast extract, 0.5. The inoculum was grown at 35°C with pH 6 and shaking at 150 rpm for 24 h (Rajoka *et al*., 2006).

Sequential culture cultivation of *Arachniotus* sp., and *Candida utilis* for microbial biomass protein production: The ability of the microorganisms to produce microbial biomass protein from corn stover as a carbon source were examined in fermentation medium containing (g L^{-1}) corn stover, 60; $CaCl_2$. $2H_2O$, 0.05; $MgSO_4.7H_20$, 0.05; KH_2PO_4, 0.1; poultry droppings (as urea's source), 18.9 at 35°C. pH of the medium was adjusted to 4, and after 3 days of growth when *C. utilis* was added, the pH was adjusted to 6. After the addition of *C. utilis,* mixed culture was further grown for 3 days and autoclaved for 5 minutes. It was dried in an oven at 100°C for 1 hour, and to a constant weight at 60°C. The dried biomass was analyzed for crude protein content by Kjeldahl method (Anon., 1984).

Time course studies for microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis* were also conducted at 35°C using 5 % inoculum of both the cultures. To determine the optimum time for microbial biomass protein production by sequential culture fermentation, *C. utilis* was added at 0, 12, 24, 48, 72 and 96 h grown *Arachniotus* sp., culture. Since the optimum time for microbial biomass protein production by *C. utilis* was 72 h (Athar *et al*., 2009), therefore after

addition of *C. utilis* to the *Arachniotus* sp., grown culture at different time intervals, mixed culture was allowed to grow further for 72 h. The biomass product so produced was analyzed as described above.

Optimization of conditions for microbial biomass protein production by sequential culture cultivation of *Arachniotus* sp., and *Candida utilis*: As sequential culture fermentation by *Arachniotus* sp., at pH 4, 35°C for 72 h and then followed by *Candida utilis* fermentation at pH 6, 35°C for 72 h more, resulted in higher production of microbial biomass protein, therefore these conditions of sequential culture fermentation were used in all the subsequent experiments of optimization for the maximum production of microbial biomass protein. Different corn stover concentrations (0, 1, 2, 3, 4, 5, 6, 7 and 8 w/v %) were tested to get maximum microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. Different ionic concentrations of $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and KH_2PO_4 were tested to get optimal microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. Different carbon nitrogen ratios i.e., 10:1, 20:1, 30:1, 40:1, 50:1 were tested to find out the optimal C: N ratio to get the maximum microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*.

Different concentration of molasses (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 w/v %) were tested to get the maximum microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*.

Large scale biomass production in 75 L Fermentor: The optimum conditions determined for microbial biomass production by sequential culture fermentation of *Arachniotus* sp. and *C. utilis* were extended to ferment corn stover in a 75 L (50 L working volume) fermentor for the production of microbial biomass protein and amino acids. The biomass product obtained on large scale was analyzed (AOAC Methods, 1984).

Amino acid composition of microbial biomass protein thus produced in a 75 L fermentor was determined on an automatic amino acid analyzer according to the method described by Moore & Stein (1959).

Results and Discussion

Microbial biomass protein production by pure culture of *Arachniotus* sp., and *C. utilis*: Maximum microbial biomass protein from *Arachniotus* sp., was obtained at optimal pH, temperature and incubation time of 4, 35°C and 72 h respectively (Results not shown). Optimum pH, temperature and incubation time for maximum microbial biomass protein production from *C. utilis* was 6, 35°C and 72 h respectively as reported in our previous study (Athar *et al*., 2009).

Time course for the production of microbial biomass protein by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*: Sequential culture fermentation was found to be the best combination for enhancing the microbial biomass protein production. Best results were obtained with sequential fermentation carried out by *Arachniotus* sp. at 35°C for 72 h at pH 4 and then followed by *C. utilis* fermentation at 35°C for 72 h more at pH 6 (Fig. 1).

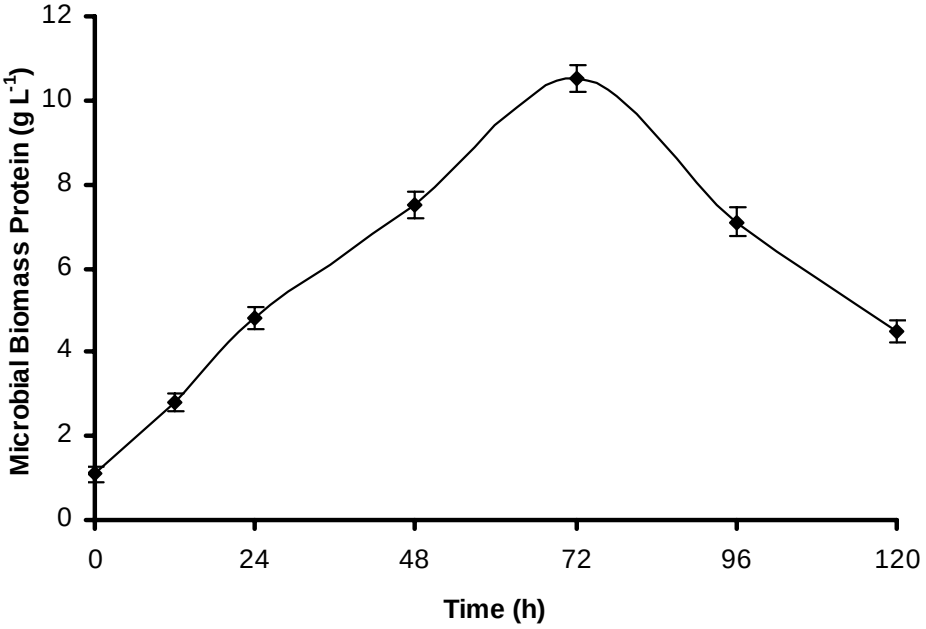

Fig. 1. Time course of microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. Error bars show standard deviation among three observations.

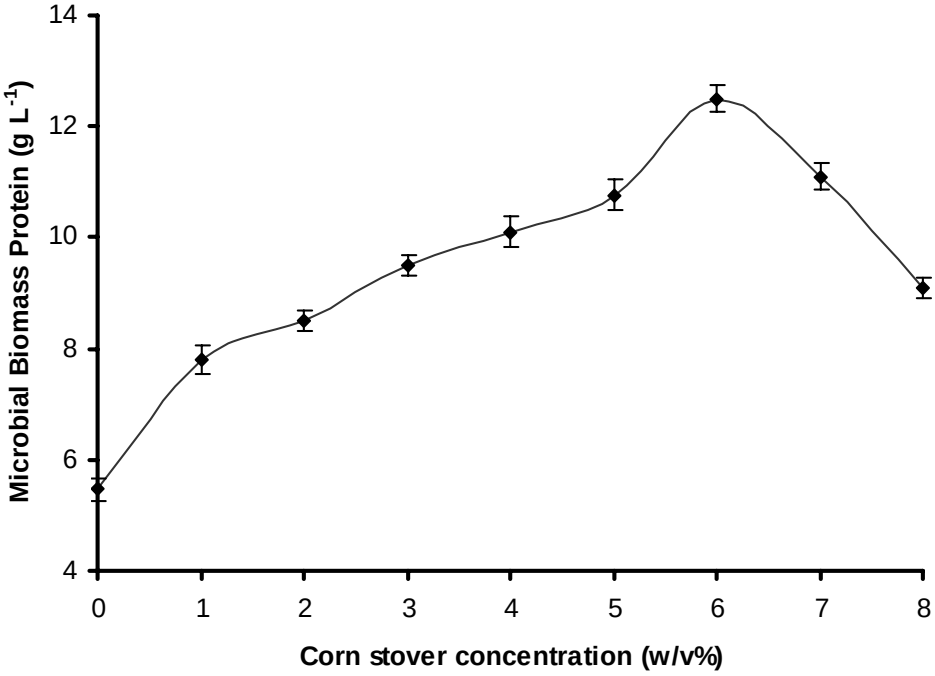

Fig. 2. Effect of different corn stover concentrations (w/v %) on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis.* Error bars show standard deviation among three observations.

Influence of optimum corn stover concentration on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*: Among different corn stover concentrations, 6% (w/v) corn stover produced higher microbial biomass protein production (Fig. 2). Microbial biomass protein did not increase with further increase in substrate concentration. The lignocellulosic biomass, especially agricultural wastes, is known to be an excellent carbon source for microbial biomass production. Corn stover which is very abundant, cheap and easily available can be used for economic production of microbial biomass protein (Yang *et al*., 2006).

Effect of ionic concentrations on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*: Economic production of microbial biomass protein is required on industrial scale. The optimum concentrations for the maximum microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis* were found to be 0.0075% $CaCl_2.2H_2O$, 0.005 % $MgSO_4.7H_2O$ and 0.01% KH_2PO_4 (Fig. 3).

The predetermined conditions optimized for the production of microbial biomass protein during sequential culture fermentation of corn stover with *Arachniotus* sp., and *C. utilis* such as 6 % (w/v) corn stover as a substrate, 0.0075% $CaCl_2.2H_2O$, 0.005 % $MgSO_4.7H_2O$ and 0.01% KH_2PO_4, at 35°C with shaking at 150 rpm were used in all subsequent experiments.

Effect of carbon: Nitrogen ratio on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*: Carbon nitrogen ratio in fermentation process influence fermentation of protein concentrates. C: N ratio of 30:1 gave maximum microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis* (Fig. 4). An appropriate amount of C: N ratio is the key to harvest maximum microbial biomass protein (Zheng *et al*., 2005). Generally the results confirmed that urea; a low cost fertilizer, supported maximum microbial biomass protein production and confirmed the previous findings (Hashmi, 1986; Ali *et al*., 2009).

Effect of supplementation with molasses on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*: Among different concentration of molasses, 1% molasses gave higher microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis* (Fig. 5).

Utilization of sucrose or glucose as carbon source is not economical in the production of microbial biomass protein and a less expensive carbohydrate source would be beneficial. Low cost substrates such as cane molasses can be used for the production of microbial biomass protein for animal feed supplements (Litchfield, 1983; Sattar *et al*., 2008). Molasses, a cheap by-product is widely available from the sugar industry and consist of water, sucrose (47–50%, w/w) which is the disaccharide most easily utilized by yeast cells, 0.5–1% of nitrogen source, proteins, vitamins, amino acids, organic acids and heavy metals (Roukas, 1998). Hence it is a very attractive carbon source for microbial biomass protein production by mixed culture from economic point of view. In this study, molasses were added to the fermentation medium to enhance the microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. The present results demonstrate the potential of molasses along with corn stover as a substrate for microbial biomass protein production by sequential culture fermentation.

One criterion that is crucial in the selection of microbial strains for microbial biomass protein production is its ability to grow on cheap substrates. This criterion is satisfied with the results obtained with the current strains of mixed culture used in this study, which were found to grow well and produce microbial biomass protein production on corn stover along with molasses.

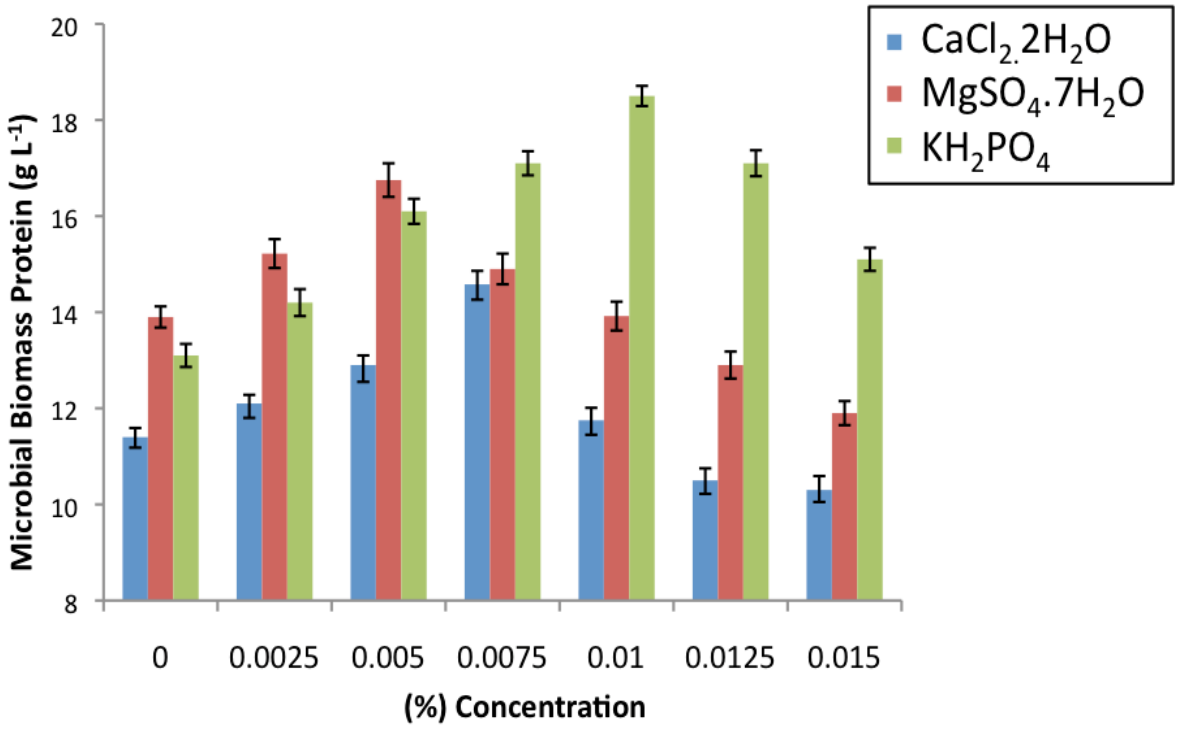

Fig. 3 Effect of various levels of $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and KH_2PO_4 on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. Error bars show standard deviation among three observations.

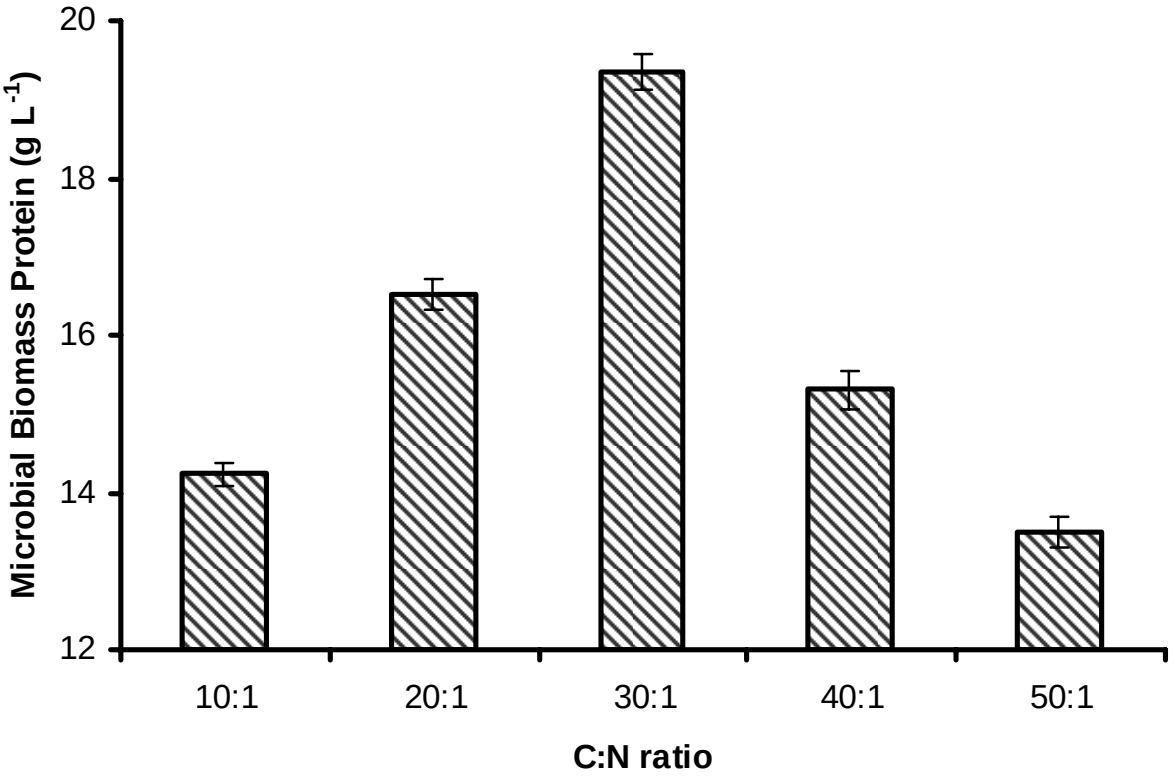

Fig. 4. Effect of different C: N ratio on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. Error bars show standard deviation among three observations.

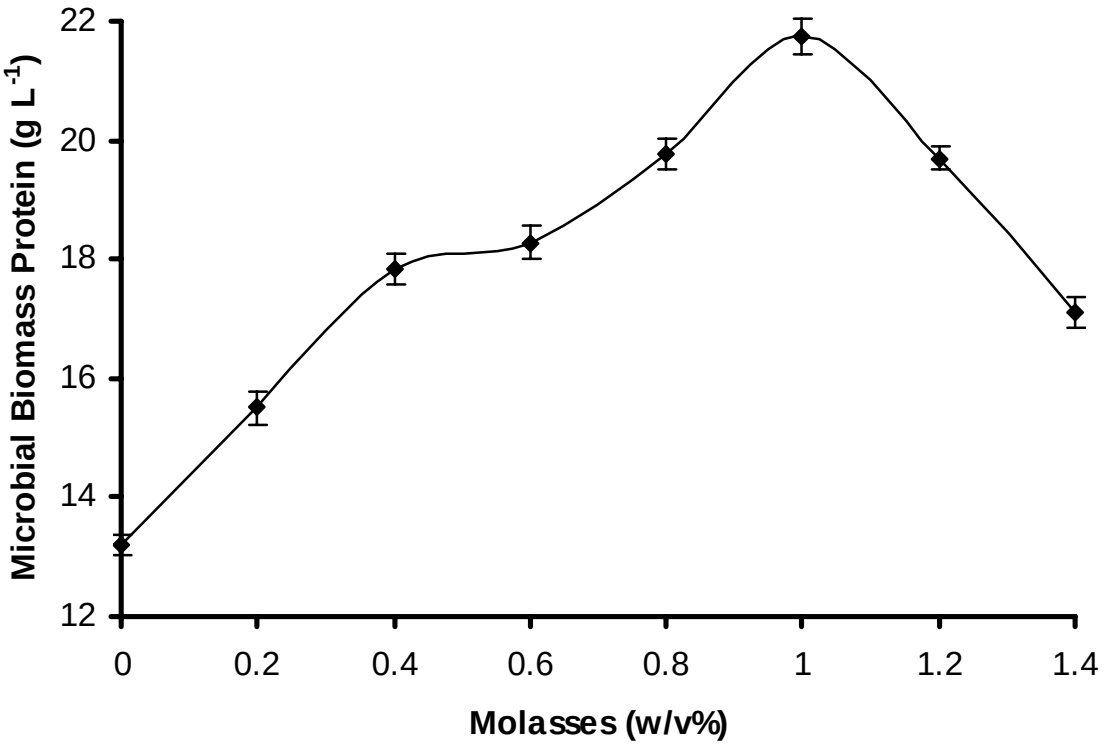

Fig. 5. Influence of molasses on microbial biomass protein production by sequential culture fermentation of *Arachniotus* sp., and *C. utilis*. Error bars show standard deviation among three observations.

Chemical composition of mixed microbial biomass product produced in 75 L Fermentor: *Arachniotus sp.*, was grown for 72 h in a 75 L fermentor followed by *C. utilis* fermentation of corn stover medium for an additional 72 h. The aeration was kept constant at 1.0 vvm and agitation was varied. The nutrient composition (%) of the mixed microbial biomass protein product is shown in Table 1. The crude protein was increased from 5.46% to 23.51%. The true protein content of 16.41% indicated that the final microbial biomass product can serve as an energy source beside protein and amino acids particularly when it may be fed to poultry.

Earlier 55.3% crude protein of *Candida utilis* SCP has been reported (Nigam, 1998). The single cell protein product reported by Singh *et al*., (1991) contained 30.4 % crude protein while *Kluyveromyces fragilis* biomass grown on deproteinized whey supplemented with 0.8% diammonium hydrogen phosphate contained 37% crude protein (Paul *et al*., 2002). The final microbial biomass product obtained in 75 L fermentor in this study by the sequential culture fermentation of *Arachniotus* sp., and *C. utilis* offers potential as an excellent protein supplement for animal feed.

Nutritive value of mixed microbial biomass product obtained in 75 L Fermentor: The amino acid composition of a protein primarily determines its potential of nutritional value. As is evident from Table 2, the microbial biomass protein produced in 75 L fermentor contained 16 amino acids. Microbial biomass has sufficient contents, which suggests that this protein should be utilized as feed supplement, as it is better than diet based on cereals. This indicates the possible exploitation of *Arachniotus* sp., and *C. utilis* by sequential culture fermentation for the production of microbial biomass protein.

Table 1. Nutrient composition (%) of corn stover and the mixed microbial biomass protein.

Components	Corn stover	Mixed microbial biomass protein product obtained in 75 L fermentor
Moisture	8.20 ± 0.95	9.63 ± 0.32
Crude protein	5.46 ± 0.84	23.51 ± 0.71
Crude fat	1.92 ± 0.04	5.30 ± 0.03
Crude fiber	22.41 ± 0.21	10.9 ± 0.11
Ash	33.22 ± 0.21	12.11 ± 0.15
Nitrogen free extract	56.79 ± 0.81	48.39 ± 0.62
RNA	Not determined	0.12 ± 0.02

Each value is a mean of three replicates ± stands for standard deviation among three independent analyses.

Table 2. Amino acid profile of corn stover and mixed microbial biomass product produced by sequential culture fermentation of *Arachniotus sp., and C. utilis* in a 75 L fermentor.

S. No.	Amino acid	Percent of corn stover	Percent of biomass product produced in a 75 L fermentor by sequential culture fermentation of *Arachniotus* sp. and *C. utilis*
1.	Aspartic acid	0.12	2.7
2.	Threonine	0.01	1.3
3.	Serine	0.05	1.8
4.	Gluatamic acid	0.32	4.4
5.	Proline	0.11	1.8
6.	Glycine	0.08	1.9
7.	Alanine	0.07	1.8
8.	Valine	0.04	1.4
9.	Methoinine	0.01	0.2
10.	Isoleucine	0.05	0.9
11.	Leucine	0.07	1.8
12	Tyrosine	0.14	0.4
13.	Phenylalanine	0.4	1.1
14.	Lysine	0.04	3.0
15.	Histidine	0.2	0.6
16.	Arginine	0.1	0.4

Test samples were hydrolyzed with HCl and analyzed on an automated amino acid analyzer

Conclusion

In this study, it was found that corn stover and molasses can be used to generate microbial biomass protein by sequential culture fermentation without costly pretreatment or nutrient supplementation. The present results contribute an increase in relevant information concerning microbial biomass protein production by sequential culture fermentation from waste products. The research indicated that sequential culture fermentation effectively produced high quality biomass protein. The microbial protein product contains fairly good quality protein rich in all essential amino acids. It can be used for fortification to livestock and poultry feed.

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