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ORIGINAL ARTICLE

Production of microbial biomass protein from mixed substrates by sequential culture fermentation of *Candida utilis* and *Brevibacterium lactofermentum*

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Abstract The aim of the current study was to apply a mixed culture of *Candida utilis* and *Brevibacterium lactofermentum* instead of using a mono-culture of *B. lactofermentum* or yeast to produce higher amount of amino acids, crude protein and true protein by using the maximum amount of substrate mixture, which *B. lactofermentum* alone cannot utilize. This mixed culture offered a combination that yielded high crude protein content and converted the substrate mixture efficiently into microbial biomass protein. Best results were obtained with sequential fermentation carried out with *B. lactofermentum* added after 3 days to *C. utilis* culture grown on beet pulp hydrolysate supplemented with molasses and glucose. Mixed culture of *C. utilis* and *B. lactofermentum* maintained the properties of each individual fermentation, with high production of microbial biomass protein in optimized medium. Crude protein increased from 11.3% to 54.5% and dry cell mass to 48 g/L with mixed culture. The amino acid profile of the final microbial biomass protein obtained by mixed culture of *C. utilis* and *B. lactofermentum* in a 75-L fermentor remained unchanged, and was enriched with all essential and non-essential amino acids. Mixed culture in this study thus exhibits a synergistic effect with possible industrial application.

Keywords Beet pulp · *Candida utilis* · *Brevibacterium lactofermentum* · Single cell protein

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Introduction

The increasing world demand for food and feed protein has enhanced the search for non-conventional protein sources to supplement existing protein sources (Sheikh et al. 2003). Various alternatives are exercised to meet this increase in demand, including techniques involving elimination, purification and recycling (Saadia et al. 2008; Saleem et al. 2008; Ahmed et al. 2007). Biomass is an alternative natural source of chemical and feedstock with a replacement cycle short enough to meet the demands of the world fuel and feed protein markets (Ahmed et al. 2009a, b, 2005, 2003). Bioconversion of agricultural wastes through microbial fermentation is a 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) (Jin et al. 2001). Conversion of a carbohydrate by-product to a value-added product is of great importance in the current drive to produce products from renewable resources to attain a sustainable society (Taherzadeh et al. 2003; Jamil et al. 2005). Low-cost agro-industrial residues accumulate in amounts of up to 50 million tons in Pakistan, and can be fermented to produce single cell protein (SCP; Ghori et al. 2011). Cycling and recycling of these residues through microbial fermentation will not only reduce the burden of pollution but also serve as a potential source of energy for the production of low-cost high-quality SCP (Athar et al. 2009). Several agro-industrial wastes have been used to produce SCP for livestock and poultry feeds (Ghanem 1992).

Sugar beet pulp is a sugar industry waste, and contains sufficient amounts of cellulose and hemicellulose for efficient production of SCP but lacks both essential and non-essential amino acids. Lysine and other essential amino acids are nutritionally important for humans and animals. They cannot be synthesized internally and require supplementation (Sattar et al. 2008). The lysine market alone has increased to a current annual market volume of about 800,000 tonnes (Ali et al. 2009), with a corresponding increase in cost. Amino acids are produced by many bacteria, including *Brevibacterium lactofermentum*, *Bacillus subtilis* and *Corynebacterium glutamicum* (Ekwealor and Obeta 2005). *B. lactofermentum* synthesizes mainly lysine and glutamic acid and supports a higher yield of lysine (Shiratsuchi et al. 1995). *Candida utilis* has been used frequently in SCP production (Athar et al. 2009) in mono- and mixed culture.

Beet pulp is a "waste product" of the sugar beet industry, and is therefore one of the more inexpensive feeds available to supplement the diet of horses. It is soaked in molasses to increase its sugar content but is deficient in essential amino acids. Increasing these amino acids will enhance the nutritive value of beet pulp for rearing horses in northern areas of Pakistan.

The properties and growth of mixed microbial cultures are of potential interest in the field of biological processing, particularly for food production, because of the sophisticated compositional and structural requirements of food products. The important principles and properties of mixed culture have been largely overlooked in the past, due mainly to the fact that most high technology 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 for, or advantage of, using mixed cultures (Ghanem 1992; Ahmed et al. 2010). Mixed cultures of microorganisms, however, are also a very good method of converting carbohydrate wastes into high yields of SCP using short fermentation times (Paul et al. 2002).

The aim of the current study was to apply a mixed culture of *Candida utilis* and *Brevibacterium lactofermentum* instead of a mono-culture of *B. lactofermentum* or yeast to produce higher amount of amino acids, crude protein and true protein by using the maximum amount of substrate mixture, which *B. lactofermentum* alone cannot utilize. In this investigation, we studied the bioconversion of beet pulp supplemented with molasses and glucose into MBP by sequential culture fermentation of *C. utilis* and *B. lactofermentum*.

Materials and methods

Chemicals

All chemicals used were of analytical grade unless otherwise stated.

Beet pulp

Beet pulp was obtained from Charsadah Sugar Mills, Mardan, Pakistan. Growth medium containing 90 g beet pulp/L medium (found to be optimum) was autoclaved for 60 min at 121°C after pretreatment with concentrated H_2SO_4 (final concentration 1.5%) to hydrolyze the hemicellulose content. After cooling, the pH was adjusted to 6 or 7 with pre-sterilized 1 *N* NaOH (Athar et al. 2009). The medium was fortified with 30 g total reducing sugars/L medium using a calculated amount of molasses and 30 g glucose/L medium, autoclaved separately for 10 min at 121°C.

Culture cultivations

Inoculum medium used for *C. utilis* contained (g/L): KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 5.7; $CaCl_2$, 0.13; $MgSO_4$, 0.5; yeast extract, 0.5 (pH 6.0±0.1) (Athar et al. 2009) and grown at 35°C on an orbital shaker (150 rpm) for 24 h. For inoculum preparation of *B. lactofermentum*, glucose broth medium was used (yeast extract, 2 g ; peptone, 5 g ; NaCl, 5 g ; glucose , 10 g in 1 L distilled water). Inoculum was grown on an orbital shaker at 35°C (150 rpm) for 24 h.

Time course studies for MBP production on beet pulp fermentation medium

The composition of the beet pulp fermentation medium used for the production of MBP by sequential culture fermentation was as follows: 90 g beet pulp/L medium (contained 20 g soluble sugars), 30 g sugars/L from a known weight of cane molasses, 30 g glucose/L medium and 30 g corn steep liquor (CSL)/L (pH 6.0±0.1). All media were adjusted to pH 6.0, which was controlled automatically using 1M NH_4OH or 1M HCl.

Brevibacterium lactofermentum was added to beet pulp medium under optimized conditions for MBP production at 0, 12, 24, 48, 72 and 96 h grown *C. utilis* medium. *B. lactofermentum* produced maximal biomass protein after 48 h of fermentation, after its addition to a *C. utilis* culture grown in beet pulp fermentation medium for 72 h. The biomass product so produced was harvested and analyzed.

Effect of CSL on MBP production by sequential culture fermentation of *C. utilis* and *B. lactofermentum*

The CSL concentration was optimized by changing the nitrogen content (1.2 g nitrogen/L) used in the growth medium given above. CSL was added to the medium at 25, 30, 40, and 50 g/L to determine the optimum concentration for MBP production. For this purpose, batch fermentation by *C. utilis* for 72 h, followed by *B. lactofermentum* fermentation for an additional 48 h was used, using a 5-L fermentor fitted with automatic controls for temperature, pH, antifoam addition, stirring speed, and a dissolved oxygen (DO) measuring device. During batch cultivation, the pH was controlled with ammonium hydroxide, and foaming was controlled by adding polyoxyethylenesorbitan monopalmitate (Sigma, St. Louis, MO) diluted ten times and autoclaved. This technique—designated as sequential fermentation—resulted in higher MBP yield, and was used in subsequent experiments to optimize other parameters for production of MBP.

Large scale MBP production in a 75-L fermentor

The optimum conditions determined for the sequential culture fermentation of *C. utilis* and *B. lactofermentum* were extended to ferment mixed substrates (as above) in a 75-L (50 L working volume) fermentor for the production of biomass protein. Mixed or mono-cultures were grown at 35°C (unless mentioned otherwise) for 72 h. Samples were collected periodically. After 72 h growth of *C. utilis*, inoculum of *B. lactofermentum* was added and the mixed culture was allowed to grow for an additional 48 h at 35°C as described above. Samples from mixed culture were also collected aseptically for an additional 48 h. The biomass product obtained on this large scale was dried in an air oven at 60–65°C and analyzed.

Analytical methods

The MBP obtained by mixed culture fermentation of beet pulp by *C. utilis* and *B. lactofermentum* was analyzed following AOAC methods (1984). Aliquots of 200 mL were taken at different time intervals to determine cell biomass protein, crude protein and true protein, and RNA as described previously (Paul et al. 2002). Briefly, culture samples were centrifuged (7,000 *g* at 10°C for 10 min) to remove substrate, which was washed twice and dried to estimate unutilized solid material. The culture broth (100 mL portion) was also centrifuged (10,000 *g*, 10 min). The cell pellet was washed twice with saline, suspended in 10 mL distilled water and then dried at 70°C. The remaining 100 mL sample was also centrifuged (10,000 *g*, 10 min). The pellet containing the cell mass, soluble products and unutilized substrate were all dried. The latter was routinely analyzed for crude protein, true protein, and RNA as described above. The amino acid composition of the MBP produced by sequential culture fermentation of *C. utilis* and *B. lactofermentum* was determined using standard methods. Briefly, fermentation product containing unutilized beet pulp, cell mass and fermentation broth was dried at 70°C in an oven with hot air. A 1 g aliquot of this sample was hydrolyzed with 50 mL 1 *N* HCl in a boiling water bath. The hydrolyzate was recovered by centrifugation at 10,000 *g*. A 100 μL aliquot of this mixture was injected into an automatic amino acid analyzer (Evans Electro-Selenium, London, UK) and quantified according to the method described earlier (Rajoka et al. 2006). The chemical score of SCP was calculated following method of AOAC (1984).

Net metabolizable energy

Net metabolizable energy was determined by the Parr oxygen method using a Parr oxygen bomb calorimeter. The calorific value was calculated from the heat generated by the combustion of a known weight of sample in the presence of 20 atmospheric pressure of oxygen (Paul et al. 2002).

Determination of kinetic parameters

All kinetic parameters were determined as described earlier (Aiba et al. 1973). The differential equations that capture the dynamics of cell mass formation (X), mixed carbohydrates consumption (S), and crude protein (CP) synthesis are given below:

$$dX/dt = \mu X \quad (1)$$

$$dCP/dt = Q_{CP}x\ X \quad (2)$$

$$-dS/dt = -\mu X/Y_{X/S} - Q_{CP}X/Y_{CP}/S. \quad (3)$$

$$dP/dS = Y_{CP/S} \quad (4)$$

$$dP/dX = Y_{CP/X} \quad (5)$$

where Q_{CP}, q_P, $Y_{X/S}$, $Y_{CP/S}$, and $Y_{CP/X}$ are the volumetric rate of crude protein formation, specific rate of protein

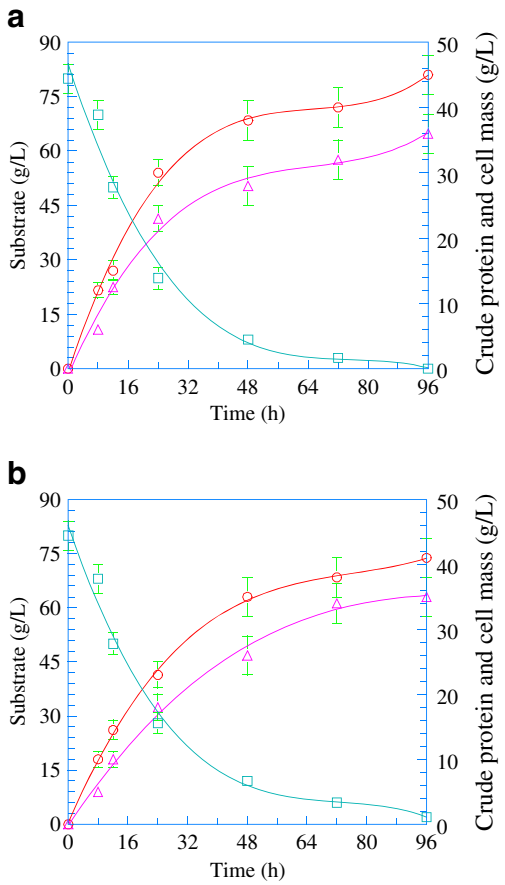

Fig. 1 Representative time course studies using mono-cultures of **a** *Candida utilis* or **b** *B. lactofermentum*. ○ Production of crude protein, Δ cell mass, □ soluble sugars present in a fermentor (5-L) containing 80 g reducing sugars/L. Data presented are mean values±SD of n=3 experiments

formation, substrate consumption yield coefficient with respect to growth, crude protein synthesis based on substrate uptake and cell mass formation, respectively.

Results and discussion

Production of MBP by pure culture of *C. utilis* or *B. lactofermentum*

Fermentations were performed in a 5 L fermentor with a working volume of 2.5 L. Fermentation medium contained reducing sugars from acid hydrolysis of 90 g beet pulp g/L medium, 30 g sugars/L medium using a known amount of sugarcane molasses and 30 g glucose/L medium (see Materials and methods). Time course studies (Fig. 1) using mono-cultures of *C. utilis* or *B. lactofermentum* showed that, using the basic medium given in Materials and methods, when *B. lactofermentum* was grown for 48 h, it supported maximum cell mass, and total crude protein while *C. utilis* supported maximum cell mass and crude protein formation in 72 h. The total sugars in the hydrolysate of beet pulp, along with glucose and molasses, were almost completely consumed by *C. utilis*, which produced more cell mass (40 g/L) and crude proteins (45 g/L) than the mono-culture of *B. lactofermentum* (cell mass 33 g/L and crude protein 42 g/L; Fig. 1).

The main purpose of this study was to revitalize the substrate mixture to obtain a protein-rich product. Crude protein and cell mass were the key products in this study. Results obtained with a mono-culture of *C. utlis* were almost the same as described earlier (Rajoka et al. 2006). Since *B. lactofermentum* does not consume some of the pentoses present in beet pulp hydrolysate, the mean values of all attributes were lower in the case of a mono-culture of *B. lactofermentum* (Fig. 1).

Influence of CSL on MBP production in sequential culture fermentation of *C. utilis* and *B. lactofermentum*

Nitrogen sources support formation of DNA, RNA and proteins of the metabolic network of the organism to

Table 1 Effect of some representative corn steep liquor (CSL) concentrations on the kinetics of crude protein production parameters in a continuously stirred tank reactor (CSTR)* by mixed culture on mixed substrate medium containing 80 g sugars/L (pH 6.0) fortified with different concentrations of CSL in 5-L fermentor. Samples were collected periodically and data collected as described in Materials and methods. Values are means ± standard deviation of n =3 experiments. Values with the same lower case letters are significantly different at $P < 0.05$. Q_{CP} Volumetric rate of crude protein formation, $Y_{CP/S}$ substrate consumption yield coefficient with respect to growth, $Y_{CP/X}$ substrate consumption yield coefficient with respect to crude protein synthesis, q_{CP} specific rate of protein formation

Kinetic parameter	CSL concentration (g/L)				*P*-value
	25	30	35	40	
Q_{CP} (g/L h)	1.25±0.04 c	1.55±0.05 a	1.49±0.04 b	1.42±0.04 b	0.0001
$Y_{CP/S}$ (g/g substrate)	0.49±0.02	0.56±0.03	0.5 2±0.03	0.50±0.03	0.0613
$Y_{CP/X}$ (g/g cells)	1.00±0.03 b	1.14±0.04 a	1.13±0.06 a	1.10±0.04 b	0.0134
q_{CP} (g/g h)	0.37±0.02	0.42±0.03	0.42±0.02	0.41±0.02	0.0817

*CSTR run under 1 vv^{-1} m^{-1} aeration rate, and 300 rpm stirrer speed

support good growth. Optimization studies of CSL concentration on crude protein and cell mass formation were conducted. The results (Table 1) showed that CSL concentration influenced the MBP production, with protein yields of varying levels. Responses were measured by determining four kinetic parameters (Table 1). CSL at 30 g/L supported significantly higher Q_{CP} ($P \geq 0.0001$) and $Y_{CP/X}$ ($P \geq .0.0134$) but non-significantly higher $Y_{CP/S}$ ($P \geq 0.0613$) and q_{CP} ($P \geq 0.0817$). Thus CSL at 30 g/L was adequate for recovering high crude protein kinetic parameters. Higher CSL levels were most probably inhibitory to both organisms. In addition to being rich in nutrients, CSL is a cheaper fermentation nutrient source (Rajoka et al. 2006; Athar et al. 2009) and is economically more suitable for a fermentation process.

Production of MBP in a 75-L fermentor

Results of MBP production extended to a 75-L fermentor are shown in Fig. 2. From time course studies of monocultures of *B. lactofermentum* (Fig. 2a), and *C. utilis* (Fig. 2b), and mixed culture (*C. utilis* for 72 h followed by addition of *B. lactofermentum* and growth of both organisms for an additional 48 h; Fig. 2c), kinetic parameters of crude protein production were calculated (Table 2). Studies on the effect of the organism on product formation kinetic parameters indicated that mixed culture supported significantly higher values of Q_{CP} ($P > 0.0008$), $Y_{CP/X}$ ($P > 0.0018$) and Q_X ($P > 0.0034$) but non-significant higher values of Y_{CP}/S ($_P > 0.0068$), Q_S ($P > 0.322$) and $Y_{X/S}$ ($P > 0.0945$). Mixed culture emerged as the best treatment to get the highest yields of crude protein. Hence sequential fermentation was the best combination for enhancing MBP production along with maximum enrichment of amino acids.

Previous studies have shown that, during mixed culture of yeast and bacteria, bacterial growth was stimulated by the presence of yeast. Ghanem (1992) found that a combination of *Trichoderma reesei* and *Kluyveromyces marxianus* gave high SCP yields and converted beet pulp efficiently into proteins (39.4%). Using mixed cultures of *T. reesei* and *K. marxianus*, when the beet pulp level increased from 2% to 4%, the highest efficiency of beet pulp conversion into protein was achieved (41.8%) and protein yields reached a maximum value of 54%.

Based on the data obtained in the present study, it was concluded that beet pulp substrate mixed with molasses and glucose can be fermented with *C. utilis* and *B. lactofermentum* to produce MBP. Mixed culture fermentation with *C. utilis* and *B. lactofermentum* improved the production of biomass and protein content, which may increase the suitability of the product in applications such as food ingredients or as protein supplements (Fonseca et al. 2008).

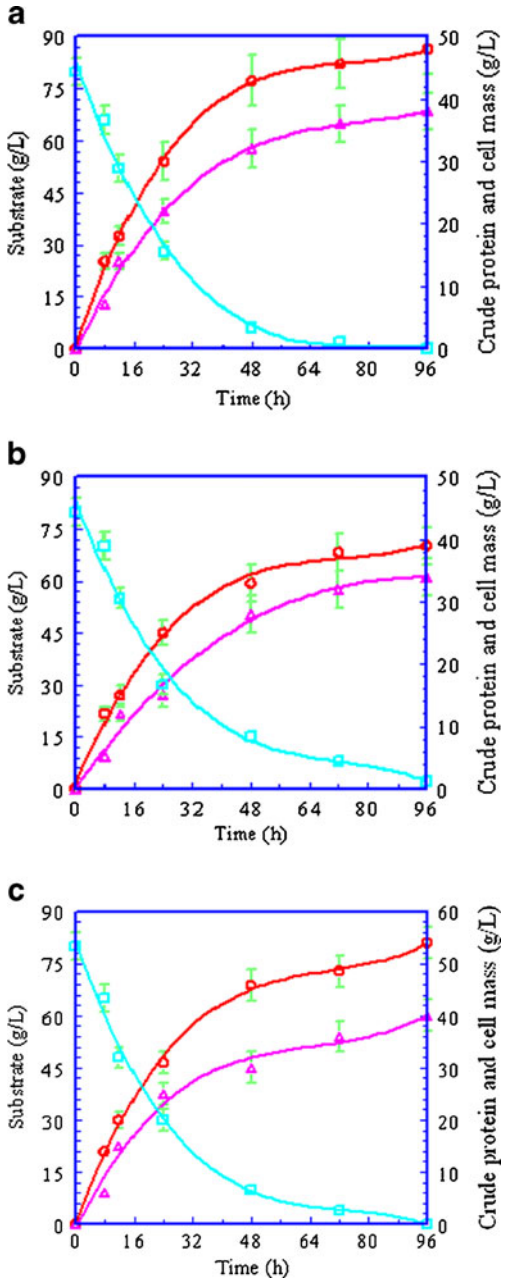

Fig. 2 Time course of crude protein (○), and cell mass (Δ), formation from soluble reducing sugars (□) by *C. utilis* (**a**), *B. lactofermentum* (**b**) and mixed culture (**c**) grown as described in Materials and methods. Data presented are mean values±SD of $n=3$ observations in 7a 5-L fermentor (working volume 50 L)

Chemical composition of final mixed MBP product

Candida utilis was grown for 72 h in a 75-L fermentor followed by *B. lactofermentum* fermentation for an additional 48 h. Aeration was kept constant at 1.0 vvm and agitation was varied. At 200 and 300 rpm agitation speed, crude protein yield was 45 and 54.5% respectively. This finding suggested that lower agitation (300 rpm) in a larger (20-fold) fermentor (75 L), i.e., the same agitation speed

Table 2 Kinetics of crude protein production by *B. lactofermentum* (BL), *C. utilis* (CU) and BL + CU following growth in CSTR* fed with CSL enriched mixed substrate medium (pH 6.0) at 35°C as in Table 1 but in 75-L fermentor. Values are means ± standard deviation of n =3 experiments. Values with the same lower case letters are significantly different at $P < 0.05$

Kinetic parameter	BL	CU	CU + BL	P
Q_{CP} (g/L h)	1.45.±0.04 b	1.56±0.02 b	1.76±0.03 a	<0.0008
$Y_{CP/S}$ (g/g)	0.45±0.035	0.51±0.02	0.57±0.03	>0.0613
$Y_{CP/X}$ (g/g cells)	1.1±0.04 b	1.27±0.04 a	1.37±0.05 a	> 0.0018
Q_S (g/L h)	2.52±0.15	2.72±0.25	2.82±0.26	>0.322
Q_X (g/L h)	1.10 ±0.05 b	1.180±0.05 b	1.32±0.04 a	>0.0034
$Y_{X/S}$ (g/g)	0.45±0.03	0.50±0.04	0.53±0.04	>0.0945

*CSTR run on 80 g reducing sugars/L medium fortified with 30 g/L CSL. Dissolved oxygen level was maintained as described in Materials and methods. Samples were collected periodically and data collected as described earlier

that supported maximum cell mass and crude protein formation, also improved crude protein synthesis rate as suggested earlier (Aiba et al. 1973). After observing the performance of the mixed culture in the 75 L fermentor, dry biomass product (SCP) was analyzed by chemical analysis as described in Materials and methods. The results are shown in Table 3. The total protein of MBP obtained by mixed culture fermentation of *C. utilis* and *B. lactofermentum* in 75 L fermentor was 33.21%. The MBP product had an apparent metabolic energy of 2,488 kcal and a true metabolizable energy of 2,563 kcal kg^{-1}.

The improvement in yield of crude protein from 11.26% to 54.5% indicated a great deal of CSL metabolism by the mixed culture. Of this nitrogen, most was utilized in the formation of true amino acids. The SCP 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 mixed microbial biomass product obtained by sequential culture fermentation of *Arachniotus* sp. and *C. utilis* contained 23.51% crude protein and 16.41% true protein content (Ahmed et al. 2010). This indicated that the mixed microbial biomass was superior to crude protein and true protein, and can serve as an energy source besides protein and amino acids, particularly when fed to poultry.

Table 3 Compositional analysis of beet pulp and mixed microbial biomass (% dry weight) by *C. utilis* and *B. lactofermentum* from beet pulp medium in a 75-L fermentor. Values are the mean of three observations. Standard deviation among replicates was 3–5% (not shown)

Component	Beet pulp	Microbial biomass protein
Moisture	5.50	0
Crude protein	11.3	54.5
True protein	0.00	32.00
Crude fat	0.91	0.10
Crude fiber	14.4	14.2
Ash	6.5	11.5
Nitrogen free extract	61.47	22.93
NDF	39.85	25.00
ADF	26.34	25.5
Hemicellulose	13.21	0.50
Carbon	47.50	44.5
Calcium	0.45	1.49
RNA	Not determined	1.32

Table 4 Amino acid (AA) profile of mixed microbial biomass protein of *C. utilis* and *B. lactofermentum* in beet pulp containing mixed substrate medium in a 75-L fermentor (working volume 50 L). Fermentation product containing unutilized beet pulp, cell mass and fermentation broth was dried with hot air. The dried test samples were hydrolyzed with HCl and analyzed using an automated amino acid analyzer

Amino acid	AA in beet pulp (%)	AA in biomass (%)
Aspartic acid	1.95	4.87
Threonine	1.21	3.93
Serine	0.99	1.24
Glutamic acid	7.67	12.00
Proline	1.47	2.74
Glycine	2.25	3.87
Alanine	2.16	2.82
Valine	0.94	2.81
Methionine	0.55	1.86
Isoleucine	1.05	3.45
Leucine	1.54	4.13
Tyrosine	0.96	2.49
Phenylalanine	0.95	1.65
Lysine	0.83	25.00
Histidine	1.29	3.12
Arginine	1.80	3.35

Nutritive value of mixed microbial biomass product obtained in a 75-L fermentor

The amino acid composition of a protein primarily determines its potential for nutritional value. As seen in Table 4, the MBP produced contained 16 amino acids. Biomass has sufficient lysine and threonine content, which suggests that this yeast protein could be utilized as a feed supplement, as it is better than a diet based on cereals. This indicates the possible exploitation of mixed culture of *C. utilis* and *B. lactofermentum* for the improvement of the nutritional value of feeds in Pakistan. The good nutritional quality of the biomass protein from the mixed culture of *C. utilis* and *B. lactofermentum* when fermented in beet pulp makes this combination potentially useful for the production of microbial protein from agricultural by-products. Thus, mixed culture of *C. utilis* and *B. lactofermentum* could be used profitably to produce MBP. In this context, the use of this biotechnological process could improve the quality of microbial biomass protein.

Conclusion

Compared to using mono-cultures of *B. lactofermentum* or yeast, mixed culture of *C. utilis* and *B. lactofermentum* produced higher amounts of amino acids, crude protein and true protein, notably using the maximum amount of substrate mixture, which *B. lactofermentum* alone cannot utilize. The results of this research indicate that sequential culture fermentation can produce high quality biomass protein effectively. The microbial protein product contains a fairly good quality protein rich in all essential amino acids that can be used for fortification of livestock and poultry feed.

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