Bioconversion of Beet Pulp to Microbial Biomass Protein by *Candida utilis*

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(Received 11th June 2008, accepted in revised form 3rd September 2008)

Summary: The present article describes a study of the microbial biomass protein production from beet pulp to improve its nutritional value as a feed stuff. *Candida utilis* has important characteristics for the economic production of microbial biomass protein and for its supplementation in poultry and livestock feeds. The optimal conditions for microbial biomass protein production from *C. utilis* were 3 % (w/v) beet pulp, 0.0075 % $CaCl_2.2H_2O$, 0.125 % $MgSO_4.7H_2O$, 0.45 % KH_2PO_4, 0.03 % KCl at C: N ratio of 15:1 in the presence of 2 % cane molasses and 1 % corn steep liquor. The amino acid profile of final microbial biomass protein showed that it is enriched with all essential amino acids. The microbial biomass protein obtained could be employed in the protein enrichment of animal feed supplements.

Introduction

The increasing world demand for food and feed protein has led to the search for nonconventional protein sources to supplement the available protein sources. Much interest has been focused on the potential of converting agriculture, industrial, municipal or forestry wastes to microbial protein [1]. Production of single cell microbial biomass protein has been studied intensively as consumption of dried yeast and could be an important alternative to prevent malnutrition in developing countries [2]. Low-cost non-conventional agro-industrial residues, which accumulate up to 50 million ton in Pakistan [3], can be fermented to produce single cell protein. Cycling and recycling of these residues through fermentation will not only reduce the pollution but also will serve as a potential source of energy for production of low cost high quality microbial biomass protein. Microbial biomass protein is also a potential supplemental protein source for feeding poultry, livestock and humans [4-6]. Several agro-industrial wastes have been used to produce single cell protein for livestock and poultry feeds [7, 8].

Sugar beet pulp is the residue after sugar extraction. It is used as animal feed with relatively low price and it has been reported to contain large amounts of cellulose and hemicellulose [9]. Beet pulp is a very attractive carbon source for microbial biomass protein production. *Candida utilis* has been frequently used in biomass production because of its ability to utilize a variety of carbon sources and to support high protein yield [10]. It has been used for production of several industrial products both for human and animal consumption [11-13]. To be economically feasible, it is necessary to engineer optimum culture conditions for maximum biomass productivity. So In this study, beet pulp was used as a substrate to investigate optimum fermentation conditions for microbial biomass protein production by culturing *C. utilis* as fermenting agent.

Results and Discussion

Influence of Optimum Substrate Concentration

Among different beet pulp concentrations, 3 % (w/v) beet pulp supported significantly higher microbial biomass protein production (Fig. 1). Microbial biomass protein did not increase with further increase in substrate concentration.

Candida species are preferred for microbial biomass production since they have a better regulation between anabolic and catabolic pathways which prevent waste of substrate [14]. The predominantly aerobic metabolism of *C. utilis* and active participation of the pentose phosphate pathway for sugar metabolism predisposes this yeast to carbon balance in favors of biomass production as compared with other yeasts such as *Saccharomyees cerevisae*,

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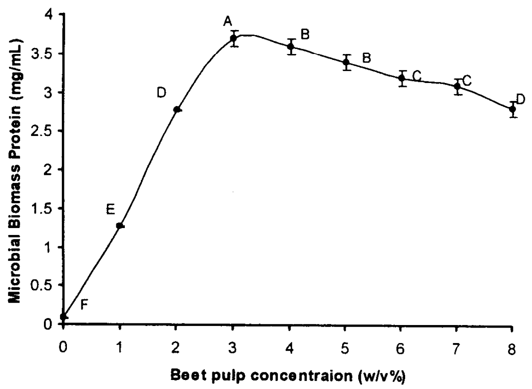

Fig. 1: Effect of different beet pulp concentration (% w/v) on microbial biomass protein production by *C. utilis* at 35 °C, pH 6 and 35 °C after 72 h. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

which are glucose sensitive and largely fermentative [15, 16].

Effect of Ionic Concentration on Microbial Biomass Protein Production

Economic production of microbial biomass protein from *C. utilis* is required for its production at industrial scale. We have formulated a medium with optimized ionic concentrations for the enhanced production of microbial biomass protein from beet pulp with *C. utils*. Optimum concentrations of the variables were found to be 0.0075 % $CaCl_2.2H_2O$, 0.125 % $MgSO_4.7H_2O$, 0.45 % KH_2PO_4 and 0.03 % KCl. The trend in Fig. 2 indicated that optimum concentration of $CaCl_2.2H_2O$ could be 0.0075 %. The trend in Fig. 3 indicated that optimum concentration of $MgSO_4.7H_2O$ could be 0.125 %. The trend in Fig. 4 indicated that optimum concentration of KH_2PO_4 could be 0.45 %. The trend in Fig. 5 indicated that optimum concentration of KCl could be 0.03 %. Effect of addition of $FeSO_4.7H_2O$ was found to be statistically non-significant (Fig. 6).

The predetermined conditions optimized for the production of microbial biomass protein during fermentation of beet pulp with *C. utilis* such as 3 % (w/v) beet pulp as a substrate, 0.0075 % $CaCl_2.2H_2O$, 0.125 % $MgSO_4.7H_2O$, 0.45 % KH_2PO_4, 0.03 % concentration of KCl, at 35 °C (pH 6.0) were used in all subsequent experiments.

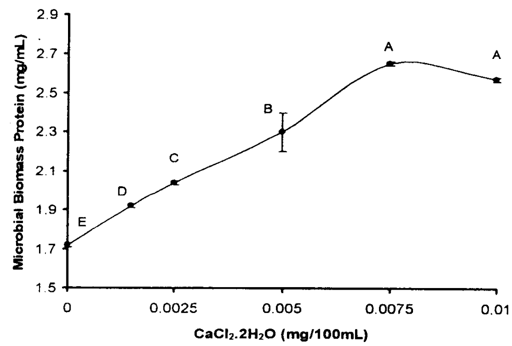

Fig. 2: Effect of various levels of $CaCl_2.2H_2O$ on microbial biomass protein production from beet pulp by *C. utilis*. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

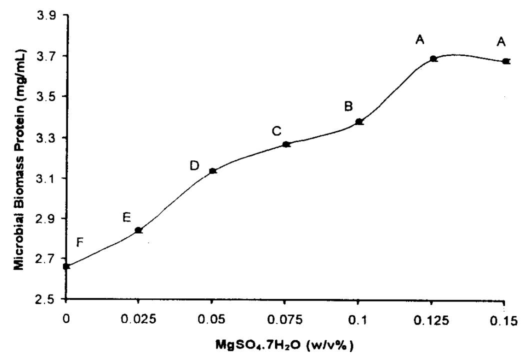

Fig. 3: Effect of different concentrations of $MgSO_4.7H_2O$ on microbial biomass protein production from beet pulp by *C. utilis*. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

Effect of Carbon: Nitrogen Ratio on Microbial Biomass Protein Production

Carbon nitrogen ratio in fermentation process influence fermentation of protein concentrates. To explore the influence of this variable on production of biomass protein, ratios were obtained by increasing the urea concentration in the medium. A ratio of 25:1, 20: 1, 15:1, 10:1, and 5:1 gave microbial biomass protein of 6.34, 7.92, 9.04, 8.62, 6.94 mg/mL protein respectively. C:N ratio of 15:1 gave significantly higher microbial biomass protein production (Fig. 7). An appropriate amount of C:N ratio is the key to harvest high quality microbial biomass protein [17]. Generally the results confirmed

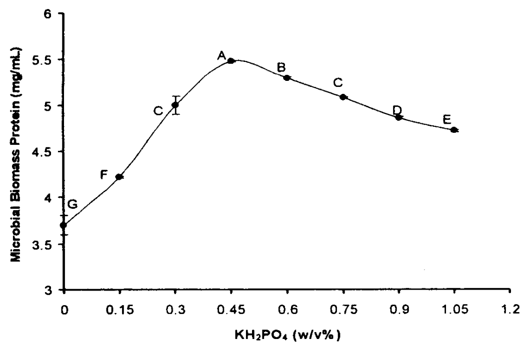

Fig. 4: Effect of different concentrations of KH_2PO_4 on microbial biomass protein production from beet pulp by *C. utilis*. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

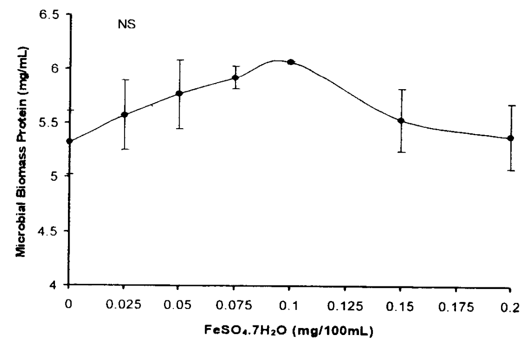

Fig. 6: Effect of different concentrations of $FeSO_4.7H_2O$ on microbial biomass protein production by *C. utilis* grown on beet pulp. Error bars show standard deviation among three observations. The results are non-significant statistically.

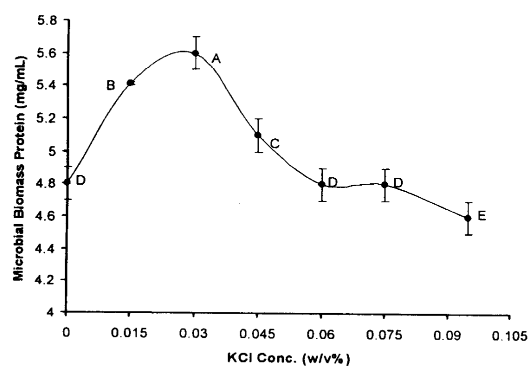

Fig. 5: Effect of different concentrations of KCl on microbial biomass protein production by *C. utilis* grown on beet pulp. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

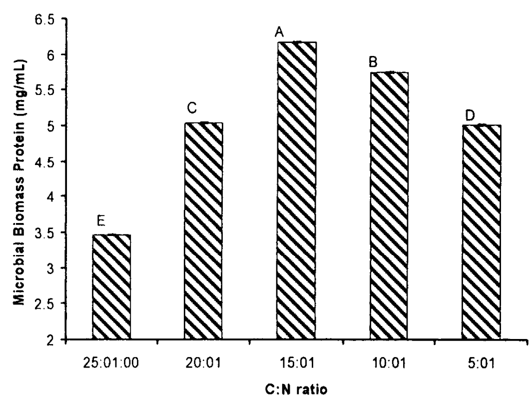

Fig. 7: Effect of different C:N ratio on microbial biomass protein production by *C. utilis* grown on beet pulp. Means sharing similar letters are statistically non-significant (P>0.05).

that urea; a low cost fertilizer, supported maximum microbial biomass protein production from *C. utilis* and confirmed the findings of Hashmi [18].

Effect of Supplementation with Cane Molasses and Corn Steep Liquor

Cultivation of *C. utilis* in beet pulp medium under previously optimized conditions with molasses (0.5-2 %) showed that 2 % molasses gave significantly higher microbial biomass protein production (Fig. 8). Corn steep liquor a cheap nitrogen source was added to the fermentation medium under previously optimized conditions to enhance the biomass protein production. The result shows that 1 % corn step liquor gave significantly higher microbial biomass protein production (Fig. 9). Generally, the results confirmed that corn steep liquor, a low-cost by-product of the starch industry, supported the maximum biomass production.

C. utilis can utilize various carbon sources to synthesize its own cell mass. Utilization of sucrose or glucose as carbon source is not economical in production of microbial biomass protein, and a less expensive carbohydrate source would be beneficial.

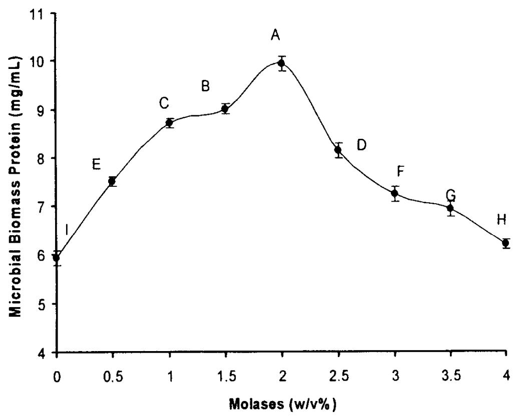

Fig. 8: Influence of molasses on microbial biomass protein production from beet pulp with *C. utilis* under optimum conditions. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

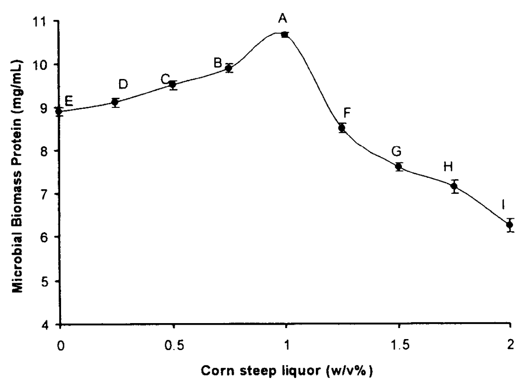

Fig. 9: Influence of Corn steep liquor on microbial biomass protein production from beet pulp with *C. utilis* under optimum conditions. Error bars show standard deviation among three observations. Means sharing similar letters are statistically non-significant (P>0.05).

Low cost substrates such as cane molasses and corn steep liquor can be used for the production of microbial biomass protein for animal feed supplements [19]. Molasses, cheap by-products, are 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 [20]. Hence it is a very attractive carbon source for biomass production from *C. utilis* from economic point of view. In this study, to improve the microbial biomass protein production of *C. utilis* produced from beet pulp, cane molasses and corn steep liquor were added to the fermentation medium. The present result demonstrates the potential of cane molasses and corn steep liquor along with beet pulp as a substrate for microbial biomass protein production. One criterion that is crucial in the selection of a yeast strain for microbial biomass protein production is its ability to grow on cheap substrates. This criterion is satisfied with the results obtained with the current strain of *C. utilis* used in this study, which was found to grow well and produce microbial biomass protein production on beet pulp along with cane molasses and corn steep liquor.

Chemical Analysis of the Beet Pulp

Chemical analysis of the beet pulp and its fermented product were carried out on dry weight basis following Association of Official Analytical Chemists Methods [21] to find out the relative improvement in the fermented product. Fermentation of beet pulp with *C. utilis* not only increased the lysine concentration but also increased the values of crude proteins. Compositional analysis of microbial biomass protein product obtained with *C. utilis* (Table-1) revealed that dried biomass was rich in crude protein. The improvement of crude protein from 11.26 to 44.29 % (Table-1) indicated a great deal of urea metabolism by the yeast. Of this nitrogen, most was utilized in the formation of true amino acids. The single cell protein product reported by Singh *et al.* [4], contained 30.4 % crude protein while *Kluyveromyces fragilis* biomass grown on deproteinized whey supplemented with 0.8 % diammonium hydrogen phosphate contained 37 % crude protein [8]. This indicated that the biomass can serve as an energy source besides protein, and amino acids particularly when it may be fed to poultry.

Table-1: Nutrient composition (%) of beet pulp and the *Candida utilis* microbial biomass protein.

Components	Beet pulp	Microbial biomass protein
Moisture	5.50	0
Crude protein	11.26	44.29
Crude fat	0.91	0.10
Crude fiber	14.35	14.17
Ash	6.51	8.51
Nitrogen Free extract	61.47	32.93

Amino Acid Analysis

The amino acid composition of a protein primarily determines its potential of nutritional value. The amino acid composition of our final microbial biomass protein product obtained under optimized fermentation conditions with *C. utilis* are given in Table-2. As evident from Table-2, the microbial biomass protein produced from *C. utilis* contained 16 amino acids. Yeast biomass has sufficient lysine and threonine contents which suggests that this yeast protein should be utilized as feed supplement, especially diet based on cereals [22]. This indicates the possible exploitation of *C. utilis* for the improvement of nutritional value of Pakistani beet pulp.

Table-2: Amino acid profile of microbial biomass protein produced from beet pulp fermentation with *Candida utilis* under optimized conditions.

S. No.	Amino acid	Beet pulp (%)	*C. utilis* yeast protein %
1.	Aspartic acid	1.95	2.70
2.	Threonine	1.21	2.33
3.	Serine	0.99	1.24
4.	Gluatamic acid	7.67	3.06
5.	Proline	1.47	1.29
6.	Glycine	2.25	2.78
7.	Alanine	2.16	2.87
8.	Valine	0.94	1.21
9.	Methoinine	0.55	0.60
10.	Isoleucine	1.05	1.14
11.	Leucine	1.54	1.56
12.	Tyrosine	0.96	1.27
13.	Phenylalanine	0.95	1.28
14.	Lysine	0.83	2.28
15.	Histidine	1.29	1.06
16.	Arginine	1.80	2.28

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

Hence in this study, it was found that beet pulp, cane molasses and corn steep liquor can be used to generate microbial biomass protein without costly pretreatment or nutrient supplementation. The final biomass protein may have the potential to be developed as animal feed. The demonstration of its successful production at laboratory scale is an incentive to develop an effective large scale method for the production of microbial protein using beet pulp, cane molasses and corn steep liquor. The present results contribute an increase in relevant information concerning microbial biomass protein production from waste products.

Experimental

Chemicals

All the chemicals used were of analytical grade unless otherwise stated.

Beet Pulp

Beet pulp a by product of sugar industry was obtained from Charsadah Sugar Mills Mardan, Pakistan. The growth medium containing 3 % beet pulp was autoclaved for 15 minutes at 121°C after adjusting its pH to 5 with 1 N H_2SO_4. After autoclaving it was cooled and its pH was adjusted to 6 with I N NaOH [18]. It was then inoculated with freshly prepared inoculum.

Microorganisms

The strain utilized in the present study was *Candida utilis* which was obtained from Japanese Federation of Culture Collection of Microorganisms, Institute of Applied Microbiology, University of Tokyo, Japan. Stock cultures were maintained on beet pulp agar slants.

Media and Culture Conditions

Seed culture medium was used as inoculum medium for *C. utilis* containing (g/L) KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 5.0; $CaCl_2$, 0.13; $MgSO_4$, 0.5; yeast extract, 0.5 (pH 6.0 ± 0.1) [23] and grown at 35 °C on an orbital shaker working at 150 rpm for 3 days [6]. Concentration of the microorganism was adjusted to an optical density (OD) of 0.6 at 610 nm by diluting the suspension with sterile distilled water to get the homogenous suspension containing 4 x 10^7 cells/mL [24]. This suspension was used as inoculum for the study of optimized conditions for microbial biomass protein containing beet pulp as a substrate. The ability of the *C. utilis* to produce microbial biomass protein from beet pulp was examined in fermentation medium containing (g/L) Beet pulp, 30; Urea, 0.30; KH_2PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.50; KCl, 0.50 (pH 6.0 ± 0.1). The microbial biomass protein was produced in fermentation medium under submerged culture for 3 days at 35 °C and was harvested by centrifugation at 10,000 rpm, for 20 min at 4 °C [25].

Optimal Conditions for Microbial Biomass Protein Production

With the view to get enhanced microbial protein production, different fermentation conditions were optimized. Initial studies were performed on micro shake flasks to optimize fermentation conditions for the production of microbial biomass protein through fermentation of beet pulp with *C. utilis*. The fermentation temperature was maintained at 35 °C and pH 6 throughout the fermentation period. The orbital shaker was operated at a speed of 150 rpm [6].

Effect of Substrate Concentration

Different beet pulp concentrations (0, 1, 2, 3, 4, 5, 6, 7 and 8 w/v %) were tested for microbial biomass protein through fermentation beet pulp with *C. utilis*.

Effect of Ionic Concentration

Different ionic concentrations of $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4, KCl and $FeSO_4$. $7H_2O$ were tested to get optimal microbial biomass protein through fermentation of beet pulp with *C. utilis*.

Effect of Carbon Nitrogen Ratio

The concentration of soluble carbohydrate of growth medium was determined in term of glucose that represents ''C''. While the total nitrogen of the growth medium was estimated thorough Kjeldahl's method. For constructing different C:N ratios, urea as nitrogen source and glucose as carbon source were adjusted accordingly. Different carbon nitrogen ratios *i.e* 25:1, 20:1, 15:1, 10:1, 5:1) were tested to find out the optimal C:N ratio to get the maximum microbial biomass protein production through fermentation of beet pulp with *C. utilis*.

Effect of Cane Molasses and Corn Steep Liquor

Different concentrations of cane molasses (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 w/v %) were tested to get the maximum microbial biomass protein production through fermentation of beet pulp with *C. utilis*. The dry weight contents of cane molasses volumes used; 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5 and 4 mL/100 mL of growth medium contains 0.37, 0.74, 1.1, 1.48, 1.85, 2.22, 2.59 and 2.96 g of cane molasses respectively.

Different concentrations of corn steep liquor (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 w/v %) were tested to get the maximum microbial biomass protein production through fermentation of beet pulp with *C. utilis*. The dry weight contents of corn steep liquor volumes used; 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 2 mL/100 mL of growth medium contains 0.125, 0.25, 0.375, 0.50, 0.625, 0.75 and 1.0 g of corn steep liquor respectively. Dry weight contents of cane molasses and corn steep liquor were estimated following AOAC Methods [21].

Microbial Protein Estimation

The protein content of the culture biomass was determined by Lowry method [26].

Analysis of Beet Pulp

Analysis of the beet pulp was performed before and after fermentation with *C. utilis* following AOAC Methods [21] to find out the nutritive value of the beet pulp.

Amino Acid Profile

Amino acid composition of microbial biomass protein thus produced was determined on amino acid analyzer according to the method described by Moore and Stein [27].

Statistical Analysis

The data obtained was subjected to Analysis of Variance Techniques [28] and, in case of significant differences; a Duncan's Multiple Range Test (DMR) was applied [29].

Conclusions

Beet pulp (3 %, w/v) has shown excellent potential as a substrate for microbial biomass protein production. The production of microbial biomass protein from *C. utilis* at C:N ratio of 15:1 was significantly higher as compared to other ratios. 2 % cane molasses and 1 % corn steep liquor gave significantly higher microbial biomass protein production from *C. utilis*. The research indicated that *C. utilis* effectively produced high quality yeast protein. The microbial protein product contains fairly good quality protein rich in all essential amino acids. It can be produced at large scale for fortification to livestock and poultry feed.

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