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Production of Single Cell Protein from Sugarcane Bagasse by *Saccharomyces cerevisiae* in Tray Bioreactor

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ABSTRACT

In this study, solid state fermentation (SSF) was carried out to produce single cell protein (SCP) from sugarcane bagasse using *Saccharomyces cerevisiae*. The SSF experiments were performed in a tray bioreactor. The influence of several parameters including extraction buffer, initial moisture content of substrate, fermentation time, relative humidity in bioreactor, the bioreactor temperature and pretreatment of substrate on SCP production yield was considered. Among the extraction buffers used in this work, carbonate-bicarbonate buffer was the most effective one for protein extraction. Results revealed that suitable fermentation conditions were initial substrate moisture content of 70%, fermentation time of 72 h, relative humidity of 85%, bioreactor temperature of 35 ^{o}C and pretreatment of substrate using 2% NaOH solution; at this optimum condition protein production yield of 13.41% was attained. The amino acid analysis of the produced protein showed that the product contained almost all of the essential amino acids as well as some non-essential ones.

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1. INTRODUCTION

Population growth is one of the major challenges that world is facing today; this has increased the demand for food resources. In the near future, providing food will become the major concern in undeveloped and poor countries. Single cell protein (SCP), which is the protein extracted from cultivated microbial biomass, can be used as supplement in human foods or animal feeds to address part of this issue.

A variety of microorganisms and substrates have been used to produce SCP. Algae, fungi and bacteria are the chief sources of microbial protein that can be utilized as SCP. As compared to other microorganisms, yeasts are more suitable to be used as source of food. They contain more nitrogen than fungi and algae and more ash as compared to bacteria [1]; yeasts also offer advantages such as larger size (which makes them easier to harvest), lower nucleic acid content, high lysine content and ability to grow at acidic pH [2]. They are suitable for solid state fermentation (SSF) because of their low water activity. In SSF, the water content is quite low and the microorganism is almost in contact with gaseous oxygen in the air, unlike the case of submerged fermentation (SmF) [3]. Since SSF products are not highly diluted, their recovery would be easier and less downstream processing is required as compared to products produced in submerged culture [4].

Lignocellulosic materials, including agricultural residues, have attracted much attention as abundant and low-cost carbon source all around the world for protein production. Use of such materials for SCP production is to meet two main objectives; one is the mitigation of environmental pollution and the other is production of protein with affordable price, good quality and very high nutritional value. In this regard, SSF technology provides many new opportunities as it allows the use of agricultural wastes as fermentation substrates, without the need for extensive pretreatment of the substrate.

Sugarcane bagasse is the residue, left over after extraction of sucrose from sugarcane which is burnt for steam generation or left to natural degradation. This lignocellulosic agricultural waste can serve as an ideal

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substrate for microbial processes for production of variety of value added products [5]. In this study, sugarcane bagasse was used as substrate for SCP production in SSF process in a tray bioreactor. To enhance the protein content of the fermented product, the influence of several parameters including extraction buffer, initial moisture content of substrate, fermentation time, relative humidity in bioreactor, the bioreactor temperature and pretreatment of substrate was investigated.

2. MATERIALS AND METHODS

2. 1. Substrate Sugarcane bagasse used in this study was obtained from a local sugar factory. It was washed with distilled water several times and dried in an oven at 80 ºC for 24 h and then milled.

2. 2. Microorganism and Inoculum Preparation A pure culture of *Saccharomyces cerevisiae* PTCC 5269 was obtained from the Iranian Research Organization for Science and Technology (IROST). The yeast was maintained on nutrient agar slant at 4 °C. The seed culture was prepared by transferring a loop-full of yeast from the agar slant into the Erlenmeyer flask containing 50 ml of medium containing (g/l): peptone, 2; yeast extract, 2; glucose, 4; KH_2PO_4, 1 and $MgSO_4$, 0.2; the culture was incubated in a rotary shaker incubator at 30 °C for 24 h.

2. 3. Laboratory Scale Tray Bioreactor and SSF In this study, SSF was carried out in a tray bioreactor for SCP production. A Plexiglas cabin with dimensions of 45×35×55 cm including three metal trays, equipped with temperature and humidity controllers was used as tray bioreactor. The trays were located at equal distances from one another. The bottom tray contained nutrient solution (yeast extract 1 g/l and KH_2PO_4 0.2 g/l) for growth of microorganism that was circulated in the cabin using a peristaltic pump and sprayed on the solid bed which was spread over the two other perforated trays. The liquid medium was circulated inside the bioreactor until the desired relative humidity, as monitored by digital humidity controller (FOX-1H, Korea), was achieved. Each three grams of bagasse were poured in filter papers which were formed as open boxes and fermentation medium was sprayed on them. This was for convenient sampling and also for exact calculation of yield of protein from substrate ($Y_{P/S}$). Thin and wet fabrics were placed below the sample boxes in order to provide moisture and avoid clogging of tray holes. The required cabin temperature was provided by an incandescent bulb; four small fans were used for uniform distribution of air inside the bioreactor. A digital temperature controller (Samwon Eng, SU-105IP, Korea) was used to control the temperature.

The sugarcane bagasse, nutrient solution and all the piping and attachments were sterilized before the start of fermentation. The cabin was also cleaned using bleaching and oxidizing agents, and finally disinfected by ethanol (70%) before each use. SSF was started by spraying pre-cultured cells of *S. cerevisiae* (24 h old, grown in previously described medium) on sterilized sugarcane bagasse spread over the first and second trays and the bioreactor was incubated at 30 °C for 4 days. In cases where sampling was required during fermentation, one pack of sugarcane bagasse from each tray was taken out of the bioreactor and corresponding tests were performed.

2. 4. Protein Extraction After completion of each batch of fermentation, the fermented biomass was poured in Erlenmeyer flask containing extraction buffer and shaken at 200 rpm. The resulting suspension after the extraction was filtered under vacuum to separate the solid residues. The filtrate was centrifuged at 6000 rpm for 20 min; the supernatant was collected for protein assay.

2. 5. Analytical Methods The lignocellulosic composition of sugarcane bagasse, either raw or pretreated, was determined according to standard methods.

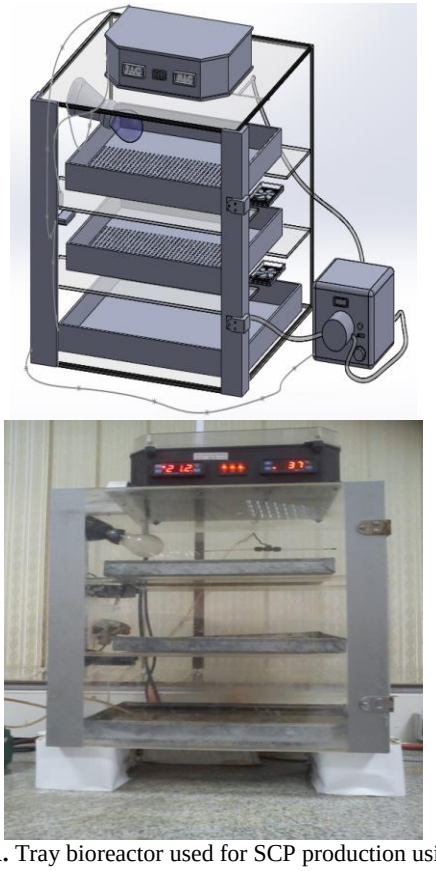

Figure 1. Tray bioreactor used for SCP production using SSF

The lignin content was determined by TAPPI test method T-222 om-22 [6], cellulose content was measured by TAPPI test method T-203 cm-99 [7] and extractive content was obtained by TAPPI test method T 204 cm-07 [8]; the hemicellulose content was determined by difference.

As an indication of biodegradability, the cellulolytic activity (CA) of lignocellulosic biomass was determined. For this purpose, the same method as described for protein extraction was followed; the biomass residues that remained on filter paper were collected, thoroughly washed with distilled water and dried at 105 °C for 5 h. The value of CA was determined as follows [9]:

$$CA\% = \frac{W_1 - W_2}{W_1} \times 100 \quad (1)$$

where W_1 is the initial weight of dry bagasse and W_2 represents the final weight of dry bagasse.

Sugar consumption during the course of fermentation was measured by 3, 5-dinitrosalicylic acid (DNS) method at 540 nm as described by Miller [10]. The ash content of sugarcane bagasse was determined by NREL method [11]. The substrate moisture was measured by oven drying at 105 °C until a constant weight was achieved. The acidic number of substrate was obtained by ASTM method based on color-indicator titration [12]. The sugarcane bagasse used in this study was characterized and its properties were determined as summarized in Table 1.

Protein content of the samples was measured by colorimetric Bradford assay at 595 nm [13]. The amino acids of produced SCP were analyzed using reverse phase (C18 column) high performance liquid chromatography (HPLC, Knauer, Germany) after derivatization by OPA (O-Phthalaldehyde); homoserine was used as internal standard.

TABLE 1. Properties of sugarcane bagasse

Properties	**Value (w/w %)**
Moisture	9.1
Ash	5
Sugar	1.23
Protein	2.65
Acidic number	0.5
Lignocellulosic composition	**Value (w/w %)**
Extractive	0.8
Cellulose	42.5
Hemicellulose	33.7
Lignin	23

2. 6. Process Optimization for SCP Production

2. 6. 1. Extraction Buffer Different buffers, namely; citrate (pH 5.0), phosphate (pH 7.0) and carbonate-bicarbonate (pH 10.0) as well as distilled water were used for the extraction of proteins from *S. cerevisiae*.

2. 6. 2. Initial Moisture of Substrate The initial moisture content of substrate was defined as the volume of sprayed culture to the mass of dry sugarcane bagasse. This parameter was varied in the range of 30 to 90% (v/w), with an increment of 10%. At this condition, the incubation temperature was 30°C and humidity of bioreactor chamber was fixed at 85%.

2. 6. 3. Fermentation Time In order to find the suitable fermentation time at which the highest protein concentration could be achieved, samples were taken from the bioreactor every 24 h during four days of fermentation and their protein content was measured.

2. 6. 4. Relative Humidity in Bioreactor To demonstrate the influence of relative humidity (RH) inside the tray bioreactor on SCP production, various RHs in the range of 70 to 90%, with an increment of 5% were tested and their effect on protein content and cellulolytic activity was investigated.

2. 6. 5. Incubation Temperature The temperature of bioreactor is one of the factors that have profound influence on the production of end product. To determine the optimum temperature, the SSF was carried out at different temperatures in the range of 20 to 40°C, with an increment of 5°C and the protein content, cellulolytic activity and product yield were determined.

2. 6. 6. Substrate Pretreatment To study the influence of alkaline pretreatment of sugarcane bagasse on destruction of its lignocellulosic structure and thus enhancement of protein production, the substrate was pretreated using NaOH solution at concentrations of 1, 2, 3 and 4% (w/v). A 50 g of bagasse powdered was soaked in 500 ml NaOH solution for 2 h at room temperature. Then, the bagasse was filtered and washed repeatedly with distilled water until a neutral pH in the filtrate was achieved; it was then dried in oven at 80°C for 24 h.

3. RESULTS AND DISCUSSION

For enhancement of protein production from sugarcane bagasse using *S. cerevisiae* in SSF process, the influence of some parameters including extraction buffer, initial moisture content of substrate, fermentation time, RH in bioreactor, bioreactor

temperature and pretreatment of substrate on SCP production was investigated; results and corresponding discussion are presented here.

3. 1. Effect of Protein Extraction Buffer For analysis of the protein content of fermented biomass, the first step was to extract protein from yeast cells. This could be conducted either by direct use of buffer solution, or disruption of cells and tissues followed by extraction of protein using buffer solution. Choice of suitable extraction method depends on the nature and structure of sources from which the protein is extracted. Solid samples are usually lysed in the presence of buffer solution.

In this study, we used three buffer solutions and distilled water for protein extraction and then protein concentration was determined using Bradford assay. The true protein content with various buffers was as follows (mg/g of sample): citrate buffer, 5.198; phosphate buffer, 4.84; carbonate-bicarbonate, 11.494 and distilled water, 3.452; the effectiveness of the extraction agents was in the order of carbonate-bicarbonate buffer > citrate buffer > phosphate buffer > distilled water. The superior performance of carbonate-bicarbonate buffer for protein extraction over other buffers was likely attributed to the extraction buffer pH (pH 10) where the extracted protein had a ready solubility in mildly alkaline solution. Separation and purification of protein for foodstuff is usually carried out under pH 10 [14]. Therefore, the carbonate-bicarbonate buffer with highest amount of protein extraction was used as the protein extraction agent in the next experiments.

3. 2. Effect of Substrate Initial Moisture SSF is defined as the fermentation involving solids in the absence (or near absence) of free water; however, substrate must have enough moisture to support the growth and metabolism of microorganism [15]. The initial moisture content of substrate in SSF critically affects cell growth and product formation. The optimum value for this parameter should be carefully determined in SSF, as low moisture level reduces the solubility of nutrients in the substrate and swelling degree of substrate; on the contrary, very high moisture content decrease the porosity of substrate and thus limits the oxygen transfer to the cells.

The influence of initial substrate moisture content on protein production was investigated in the range of 30 to 90%; the results are presented in Figure 2. Along with increase of the moisture content from 30 to 70%, the protein concentration increased; this was attributed to the increased water activity and promoted growth of the yeast which eventually resulted in higher SCP production. However, further increase of the moisture content to 90% adversely affected the protein concentration. At such high moisture content, the inter-particle spaces were probably decreased due to water filling and limited accessibility of the biomass to the air hindered the cell growth and thus SCP production. Based on the results, the initial moisture content of 70% at which 7.2% protein production was achieved was selected as the optimum condition for the SSF process.

3. 3. Effect of Fermentation Time In order to find the optimum fermentation time at which the highest amount of protein production would occur, cultivation of *S. cerevisiae* in SSF was monitored during a batch of 4 days (96 h). Figure 3 depicts the concentrations of protein and sugar versus fermentation time. The highest amount of protein (7.8%) was observed at 72 h. Prolonging the fermentation time to 96 h reduced the protein concentration. Monitoring sugar consumption during the course of fermentation revealed that the yeast cells consumed sugar for cell mass and protein production and the amount of sugar remained at 96 h was almost negligible. This limited availability of nutrients and depletion of carbon source during prolonged fermentation probably forced the yeast cells to consume the produced protein to preserve their viability and metabolic activity; as a result, a slight reduction in protein content was observed at 96 h.

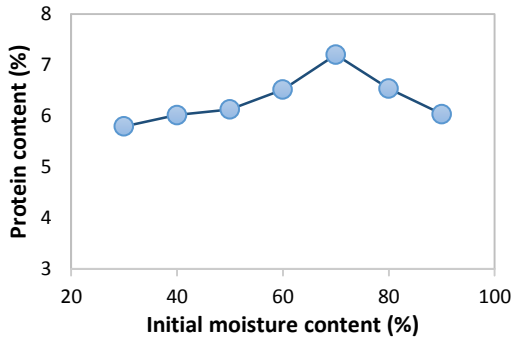

Figure 2. Effect of substrate initial moisture on protein production in SSF at 30 °C and 96 h

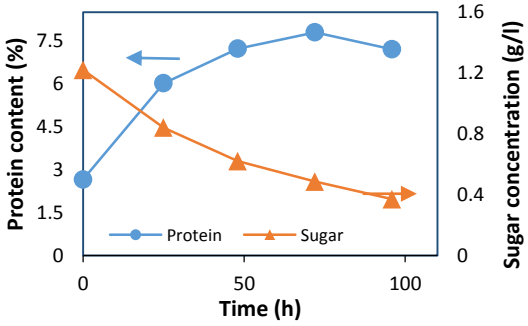

Figure 3. Effect of fermentation time on protein production and sugar consumption by *S. cerevisiae* at 30 °C for SCP production in SSF

3. 4. Effect of Relative Humidity in Bioreactor Figure 4 illustrates the influence of relative humidity on protein production and cellulolytic activity. The RH in the tray bioreactor was varied in the range of 70 to 90%; as results show the highest protein production was achieved at RH of 85%. As mentioned earlier, in the SSF process no free water is present in the environment; nevertheless, the substrate must be sufficiently moistened to support the growth of microorganism. At low humidity, due to reduced fluid flow within the bioreactor, the substrate is dried and there is not sufficient moisture for microbial growth and protein production. In contrast, at too high humidity, increase of fluid flow inside the reactor establishes a semi-solid state and reduces the cell mass production. It is generally accepted that protein production in SSF is more than submerged or semi-solid state fermentation.

Cellulolytic activity (CA) which shows the ability of microorganisms to degrade the cellulosic material was measured during these experiments and the results are shown in Figure 4 (b). As expected, there was a direct relation between CA and protein production. In fact, the higher the CA and substrate consumption, the more was the protein production. The highest CA of 35.5% was achieved at relative humidity of 85% wherein the protein yield was 7.81%.

3. 5. Effect of Bioreactor Temperature Temperature of fermentation medium is one of the key parameters which affect the cell growth and product formation. The influence of incubation temperature on SCP production and CA was investigated at temperatures in the range of 20 to 40°C with an increment of 5°C. The results of this investigation are exhibited in Figure 5. Low protein production (5.8%) was observed at 20°C. Protein production (8.43%) and CA (40.55%) were highest when the temperature was 35 °C. However, increasing temperature to 40°C hindered the activity of the microorganism.

The optimum growth temperature of *S. cerevisiae* has been reported in the range of 30 to 37°C [16]; so, it was expected that the optimum protein production could be achieved in this temperature range.

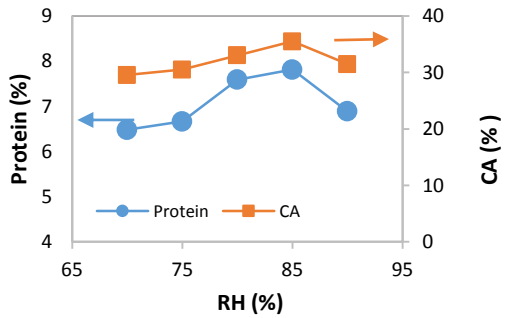

Figure 4. Effect of RH in bioreactor on protein production and CA

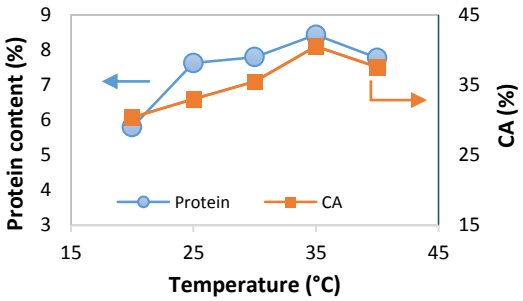

Figure 5. Effect of bioreactor temperature on protein production and CA

At low temperatures flow of nutrients across the cell membrane may be decelerated; also, the enzyme activities are low which results in slight cell growth and protein production. High temperatures also adversely affect the cell productivity due to the partial deactivation of enzymes involved in metabolic pathway and also reduced moisture content of the substrate.

3. 6. Effect of Substrate Pretreatment Physical or chemical pretreatments are usually required for lignocellulosic materials to destruct their polymeric structure and enhance the accessibility of microorganisms to the substrate. As another advantage, pretreatment can enhance the moisture holding capacity of the solid substrate. Typically, pretreatment of soft cellulosic materials is carried out using dilute acid or alkaline solutions. Use of strong solutions calls for complicated downstream processing. Alkaline pretreatment requires a lower temperature compared to other treatments and can be done at room temperature [17]. In this study, the effect of alkaline pretreatment of sugarcane bagasse using 1 to 4% (w/v) NaOH solution on SCP production was investigated; the results are presented in Figure 6. As could be inferred from the results, protein production considerably improved in pretreated samples where the bagasse sample pretreated using 2% NaOH solution yielded in protein production of 13.41% as compared to untreated sample with protein production of 8.41%. Low concentration alkaline solutions may not effectively contribute to the hydrolysis of carbohydrate polymers and high concentration solutions may cause solid loss or release of sugars to liquid phase.

In order to investigate the effect of pretreatment of bagasse on its lignocellulosic structure, the extractives, cellulose, hemicellulose and lignin contents of pretreated and untreated samples were compared (Table 2). According to the results presented in this table, the implemented pretreatment method was able to reduce 71.81% of hemicellulose, but not effective in destruction of cellulose and lignin; thus, the share of cellulose and lignin increased after pretreatment.

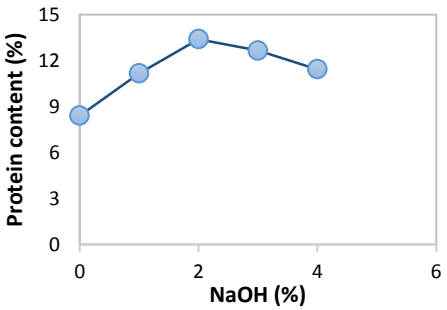

Figure 6. Effect of substrate pretreatment on protein production

The hydrogen bond between adjacent strands in cellulose creates crystalline structure which makes cellulose relatively resistant to breakage. Lignin also contains polyphenolic compounds and is hardly affected by simple acid or alkaline pretreatments; however, hemicellulose which has a highly branched and amorphous structure easily undergoes hydrolysis [18-20]. Considering that in this study pretreatment was carried out at low temperature (30^{o}C) for a short period of time (2 h), it was only effective for hydrolysis of hemicellulose, but not cellulose and lignin. The extractives content of bagasse, which is comprised of non-structural components including fats, waxes and phenolics, soluble either in polar or non-polar solvents, also reduced from 0.8 to 0.5% (w/w) along with alkali pretreatment. Based on the above discussion, alkali pretreatment under mild condition effectively hydrolyzed hemicellulose and provided sugar substrate for the microorganism; as a result, the protein production using alkali treated bagasse enhanced by around 60% as compared to that obtained from pristine bagasse.

In this study, after optimization of several operation parameters, a protein content of 13.41% was obtained from SSF of alkali pretreated sugarcane bagasse using *S. cerevisiae* (the protein content of pristine biomass was 2.65%). This result was interesting as compared to those reported in the literature. Anupama and Ravindra [21] reported a protein content of 4.17% for solid state fermented rice bran using *Aspergillus niger*.

TABLE 2. The lignocellulosic composition of sugarcane bagasse before and after pretreatment

	Extractive	Hemicellulose	Lignin	Cellulose
Untreated sugarcane bagasse	0.8	33.7	23	42.5
Pretreated Sugarcane bagasse	0.5	9.5	47	53

Units in w/w %

In another study undertaken by Hatakka and Pirhonen [22], wood-rotting white-rot fungus was grown on alkali treated straw (protein content of 3.5%) through which the protein content enriched to 9.1%.

3. 7. Determination of Amino Acids in Produced SCP Table 3 summarizes the amino acid composition of the produced SCP. The product contained 17 amino acids including almost all the essential amino acids; this means that the product is acceptable in terms of nutrition. The produced protein contains relatively high concentrations of glutamic acid, alanine and glycine. Among essential amino acids, methionine level is less than others and valine has the highest concentration. The yeast protein is almost identical to soya protein [23]. To show this, the amino acid composition of SCP obtained in this work was compared to that of soybean [24] as presented in Table 3.

TABLE 3. Amino acids composition (% dry matter) obtained from SCP produced from *S. cerevisiae* in SSF of sugarcane bagasse as compared to soybean protein

Amino acids	SCP, this work	Soybean [24]
Aspartic acid	5.08	4.44
Glutamic acid	3.79	8.14
Asparagine	0.59	-
Serine	1.12	2.46
Glutamine	2.98	-
Cysteine	-	0.8
Histidine	1.88	1.05
Glycine	0.96	1.92
Citrulline	5.00	-
Alanine	1.33	1.78
Tyrosine	5.37	1.35
Arginine	0.68	2.91
Methionine	0.58	0.59
Valine	5.39	1.94
Phenyl alanine	2.38	2.25
Isoleucine	2.22	1.97
Leucine	2.89	3.47
Lysine	3.04	2.37
Proline	-	2.23

4. CONCLUSION

The objective of this work was to use sugarcane bagasse as a low cost lignocellulosic substrate for SCP production. The SSF experiments for SCP production were successfully carried out in a tray bioreactor equipped with temperature and humidity controller. The fermentation condition was optimized in terms of initial moisture content of substrate, fermentation time and temperature and relative humidity of bioreactor. Moreover, the influence of pretreatment of substrate on SCP production was investigated. The profound effect of pretreatment of substrate on protein production could be realized by comparison of protein yield of untreated (8.41%) and 2% NaOH pretreated sample (13.41%). The produced SCP contained several amino acids including almost all of the essential ones. The protein content of sugarcane bagasse considerably enhanced during fermentation; this protein enriched lignocellulosic biomass can be a good feedstock for animal feeding.

5. ACKNOWLEDGEMENTS

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Amino Acids

چکیده

در این مطالعه، تخمیر حالت جامد (SSF) برای تولید پروتئین تک‌یاخته (SCP) از باگاس نیشکر با استفاده از مخمر *ساکارومایسیس سرویسیه* انجام شد. آزمایش SSF در یک بیوراکتور سینی‌دار انجام گرفت. تاثیر پارامترهای مختلف از جمله بافر استخراج، رطوبت اولیه بستر، زمان تخمیر، رطوبت نسبی راکتور، دمای راکتور و پیش‌تیمار سوبسترا بر بازده تولید SCP بررسی شد. از میان بافرهایی که برای استخراج استفاده گردید، بافر کربنات–بی‌کربنات بهترین عملکرد را برای استخراج پروتئین داشت. نتایج نشان داد که شرایط تخمیر مناسب شامل رطوبت اولیه‌ی بستر ۷۰٪، زمان تخمیر ۷۲ ساعت، رطوبت نسبی ۸۵٪، درجه حرارت راکتور ۳۵ سانتی‌گراد و پیش‌تیمار سوبسترا با استفاده از محلول سود ۲٪ وزنی بود. در این شرایط بهینه بازده تولید پروتئین ۱۳.٤۱٪ به دست آمد. آنالیز اسیدهای آمینه‌ی موجود در پروتئین تولید شده نشان داد که محصول تقریبا همه‌ی اسیدهای آمینه‌ی ضروری و همچنین برخی از اسیدهای آمینه‌ی غیرضروری را دارد.

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