OPTIMIZING OF SCP PRODUCTION FROM SUGAR BEET STILLAGE USING ISOLATED YEAST

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ABSTRACT: *In this study fungi isolated from the effluent of ethanol factories were identified. Optimal conditions for single cell protein (SCP) production and COD reduction of sugar beet stillage are specified for a species of Hansenula in a continuous culture. Under these conditions 5.7 g dm^{-3} biomass was produced and 31% of COD was reduced without addition of further nutrients to the beet molasses stillage. Adding nitrogen and phosphorus sources, increased the biomass production and COD reduction to 8.5 g dm^{-3} and 35.7%, respectively. The crude protein content of SCP in the absence and presence of additives was 39.6% and to 50.6% respectively. The amounts of essential amino acids measured were greater than that of the FAO standards reference and are comparable with some other food proteins, such as soya bean and fish meal.*

KEY WORDS: *Single cell protein (SCP), Stillage, Yeast, Hansenula.*

INTRODUCTION

Because of enhanced industrial activities and increasing standard of living, our environment is now polluted by industrial wastes, especially liquid ones. Among the liquid industrial wastes, distillery wastes pose a serious problem to our environment.

Distilleries producing ethanol from raw materials such as cane, beet,grains, fruit, etc., shows considerable disposal or treatment problems. Molasses stillage is particularly more difficult than others to treat, because of its high ash content, low pH, high concentration of mineral salts and BOD_5 as high as 45-65 g dm^{-3} [1].

In a typical ethanol distillery, production of 1 L of alcohol from molasses gives rise to approximately 10-14 L of stillage [1,2]. Because of the high BOD value of this effluent it is a serious pollution source. This is

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because 90% or more of the organic compounds used as raw materials (non-fermentable) end up as a waste [3]. The sugar beet molasses stillage consist of different organic compounds such as acetic acid, lactic acid, glycerol and various reducing sugars [4].

Thus degradation of these various compounds requires the selection of microorganisms which are able to assimilate them. The microorganisms curently used for microbial protein production from stillage are mainly fungi [1,2,6-14].

The aim of this study was the optimization of growth conditions for single cell protein (SCP) production and COD reduction by of *Hansenula sp.* culture in sugar beet stillage. *Hansenula sp.* previously isolated from stillage has been shown to give the highest biomass yield and COD reduction on the nonsupplemented stillage than the other microorganisms [14].

In the present work a process which combines the disposal of stillage with the production of useful protein production is reported. This process could specially be a highly economical for the third world countries which need to import proteins for animal feed.

EXPERIMENTAL

Microorganism

Hansenula sp. was isolated from stillage effluent and identified using the methods and identification keys for fungi [15,16]. The culture was grown on Sabouraud-dextrose agar (SDA) slants for 48 hours at 30°C and then stored at 4°C, with subculturing every 2 months.

Stillage

The sugar beet stillage used for this study was obtained from the Estalak Alcohol Plants and was kept in refrigerator at 4°C. Stillage was autoclaved at 121°C for 15 minutes before utilization.

Inoculum preparation

Yeast cells of *Hansenula sp.* from a stock culture on SDA slant were transferred into 250 mL Erlenmayer flasks containing 50 mL of stillage medium previously sterilized at 121°C for 15 minutes. The flasks were incubated for 48 hours at 30°C on a rotary shaker at 200 rev min^{-1} .A 10% (v/v) inoculum of active cells in all experiments was used.

Batch culture

Conical flasks (250 mL) containing 50 mL sterile stillage were inoculated with 5 mL inoculum and incubated for 48 hours at 30°C on a rotary shaker at 200 rev min^{-1}.

Continuous culture

Continuous culture was performed in a 2 L fermenter (Model Multign TM New Brunswick Scientific Company) with a working volume of 1 L. The fermenter was charged with the sterile stillage after sterilization (at 121°C for 20 minutes) and inoculated by 10% (v/v) inoculum. After sufficient growth, continuous fermentation was started by feeding the sterile stillage at various dilution rate (D). Temperature, agitation and aeration rate were kept constant, and the foam controlled manually by addition of silicone.

Optimization procedures

Optimal conditions for temperature, agitation, aeration and dilution rate were determined in the continuous culture. Optimal amounts of nitrogen and phosphorus sources were determined under batch condition. Ammonium sulphate and KH_2PO_4 were used as nitrogen and phosphorus sources, respectively.

Analytical methods

Growth was estimated by optical density at 600 nm using a spectrophotometer (Shimadzu UV-120). The pH was measured using a digital pH meter (Metrohm 620). Cells were counted via a Neubar chamber. Chemical oxygen demand (COD) was estimated by the ampule method [17].

The microbial samples were centrifuged at 2500 g for 20 minutes at 5°C and washed twice with distilled water. The biomass was determined as dry weight at 105°C until constant weight. Crude protein content was expressed as total nitrogen (N) multiplied by 6.25. Total nitrogen was determined according to Kjeldahl method. The amino acid analysis was carried-out on an automatic Beckman amino acid analyzer after hydrolysis of the sample in 6 N HCl for 20 hours.

RESULTS AND DISCUSSION

Growth kinetic of Hansenula sp.

The growth curve of *Hansenula sp.* in the batch fermentation is shown in Fig. 1. The cell growth showed a diauxic form after 18 hours from the begining of fermentation, which could have been caused by the change of an enzyme system related to the degradation and absorption of macromolecular components of the sugar beet stillage reported. for *Candida rugosa* grown in the sugar beet stillage at 30°C [9]. Similar results has been reported by *Taghavi* et al. [14].

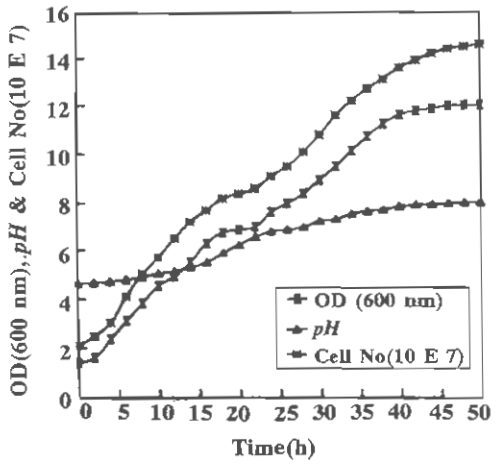


Fig. 1 : Batch culture of Hansenula sp. in the sugar beet stillage (35°C, 500 rpm,1 vvm, initial COD= 59900 mg dm^{-3}, initial pH = 4.5)

From these results, the specific growth rate during first and second phase were calculated 0.09 h^{-1} and 0.03 h^{-1}, respectively. The increasing in the pH during the first step is larger than second step, which could have been caused by the assimilation of organic acids such as lactic acid and acetic acid in the first step and those of the reducing sugars in the second step as reported by *Malnou* et al. [4].

Continuous culture

Continuous culture has many advantages over the batch system. One of these being to the growth conditions which are fixed and reproducible in the continuous culture. Thus it is possible to study the influence of a single parameter(dilution rate, temperature, dissolved oxygen, added elements, etc.) on growth.

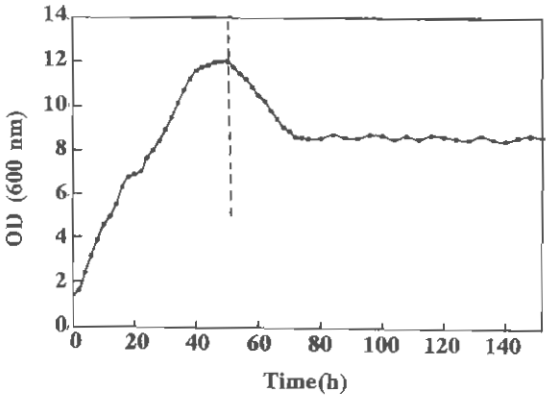


Fig. 2 : Continuous culture of Hansenuld sp. in the sugar beet stillage (D=0.04 h^{-1}, 35°C, 500 rpm, 1 vvm, initial COD= 59900 mg dm^{-3}, initial pH=4.5)

The establishment of continuous culture for *Hansenula sp.* is shown in Fig. 2. The results exhibit successful establishment of the continuous culture.

In order to determine the dilution rate suitable for optimization, it was increased stepwise until washout started and then it was lowerd and fixed at a point close to the critical value, where the sensitivity of the system to the changes is high under enviromental conditions [11]. Once the steady state was established, biomass dry weight, COD reduction and the crude protein content of biomass were measured.

Fig. 3 shows the effect of the increase in the dilution rate on biomass production, COD reduction

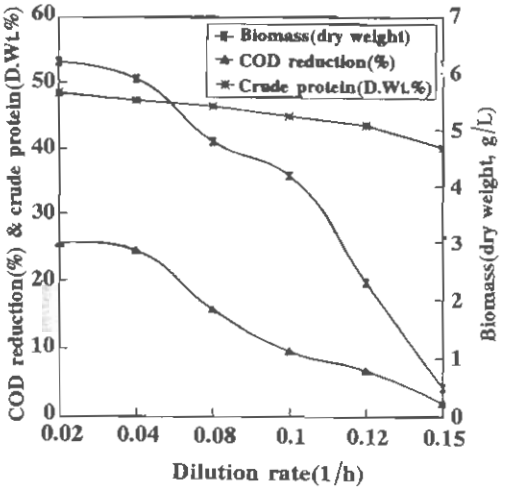


Fig. 3: The effect of dilution rate on biomass production, COD reduction and crude protein content of Hansenula sp. grown in sugar beet stillage (35°C, 500 rpm, 1 vvm, initial COD= 59900 mg dm^{-3}, initial pH=4.5)

and crude protein content of *Hansenula sp.* grown in sugar beet stillage. The washout of the culture was started at D= 0.15 h^{-1}, Therefore, D= 0.12 h^{-1} was considered a suitable dilution rate for optimizing the growth conditions where it is close to the critical dilution rate.

As can be seen from Fig. 3, the crude protein content decreased with the increase in the dilution rate, which may be due to the increase in growth rate which is in agreement with the results reported by *Silva* et al. [13].

Temperature

The temperature is an important factor in the growth of microorganisms. It affects the growth rate, metabolism, nutritional requirements, composition of the biomass, regulation mechanisms of the enzymatic reactions,and the cell permeability [18].

The control of temperature under industrial conditions can be a costly operation. It is therefore importatnt to determine the range of temperature at which a given microorganism is able to retain its maximal growth capacity or product formation. *Hansenula sp.* was cultured continuously (D= 0.12 h^{-1}) in sugar beet stillage at different temperatures (Fig.4). The results show that the activites of the organism at 32-36°C remained almost unchanged. Small deviations beyond these range caused a sharp decrease in growth. The slight differences exhibited in the protein content is due to the effects of temperature on the cell structure compounds, especially on the proteins and lipids [18].

Agitation

One of the most important parameters in the production of yeasts is the availability of oxygen. Agitation and aeration rates are two parameters that can change the oxygen transfer rate in a culture medium. Agitation affects the relationship between cell biomass production, oxygen and substrate uptake, heat evolved and the power consumed for aeration and temperature control. It affects overall production costs [11].

Agitation also affects gas-liquid mass transfer in three ways: increasing the contact area by improving the dispersion of the gas as small bubbles,increasing the gas-liquid contact time through the whirling movement set-up by agitation and reducing the stagnancy of liquid film by increasing the turbulence [18]. Therefore the effect of the agitation rate was investigated. The effect of agitation rate on the process is shown in Fig. 4. No attempt was made to measure the effect of agitation on the oxygen transfer rate, since the dynamics of a laboratory fermenter is very different from that of enlarged systems, the results cannot be directly used in the scale-up of the process.

As can be seen in Fig. 5, the biomass production and COD reduction increased steeply with the in-

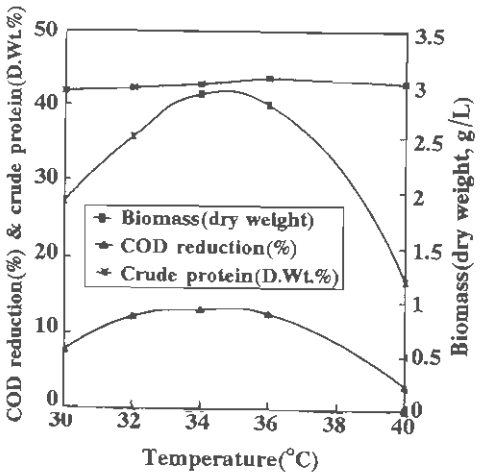


Fig .4: The effect of temperature on biomass production, COD reduction and crude protein content of Hansenula sp. grown in molasses stillage (D = 0.12 h^{-1} , 500 rpm, 1 vvm , initial COD = 75000 mg dm^{-3} , initial pH = 4.67)

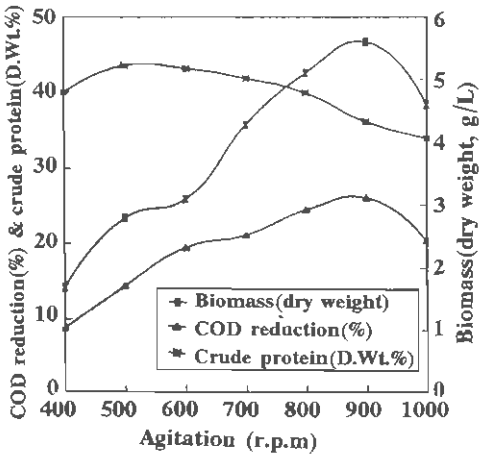


Fig.5. The effect of agitation rate on biomass production, COD reduction and crude protein content of Hansenula sp. grown in sugar beet stillage (D = 0.12 h^{-1}, 35°C, 1 vvm, initial COD= 75000 mg dm^{-3}, initial pH = 4.67)

crease of agitation speed reaching a maximum at 900 rpm. The decrease in performance at higher agitation speeds could be due to disruption of fungal cell walls. A slight and continuous decrease in crude protein content can be observed which could be related to the increase in the growth rate which is consistent with the results of the previous stage.

Aeration

Aeration rate affect the oxygen transfer rate by increasing the partial oxygen pressure. The effect of aeration on the oxygen transfer rate is less than that of the agitation rate [22]. The effect of aeration rate is shown in Fig. 6. As can be seen, the optimal aeration rate for maximum biomass production and COD reduction is 1.5 vvm. The aeration rate above 1.5 vvm has an adverse effect on the performance of the process.

It is thus clear that the aeration rate does note affect the protein content which is in agreement with the results reported by *Silva* et al. [13].

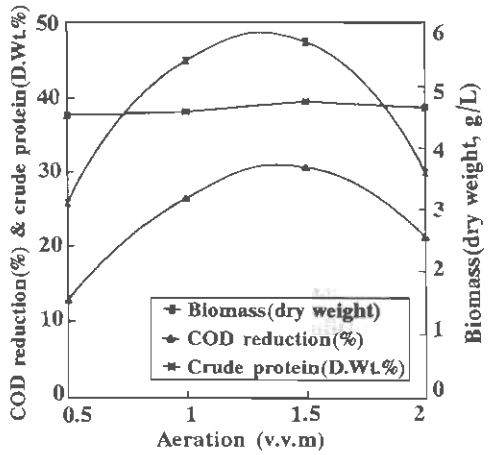


Fig. 6: The effect of aeration rate on biomass production, COD reduction and crude protein content of Hansenula sp. grown in sugar beet stillage ($D = 0.12\ h^{-1}$, 35°C, 900 rpm, initial COD = 75000 mg dm^{-3}, initial pH = 4.67)

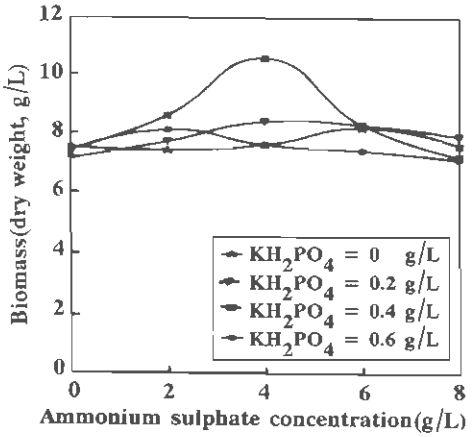
Addition of nitrogen and phosphorus sources

Nitrogen is essential in protein synthesis and is used as a component of cell wall polymers. Ammonium is most commonly used because of its price and ease of application. Phosphorus is taken up only as dehydrogeno-phosphate ions(orthophosphate), $H_2PO_4^-$ [18].

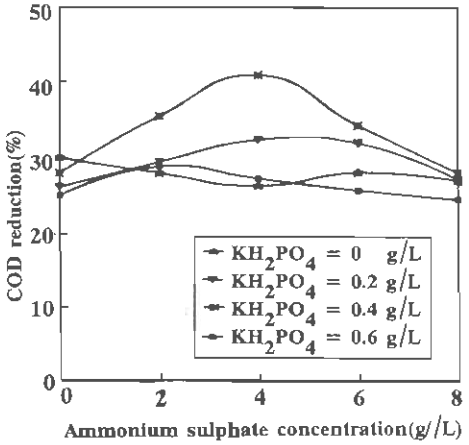
Previous studies have demonstrated that nitrogen and phosphorus supplementation of stillage can increase the biomass and protein content of the yeasts and filamentous fungi [2,5,8,10-13].

The effect of addition of nitrogen and phosphorus sources in the batch culture is shown in the Fig. 7. It is apparent from these results that addition of nitrogen source or phosphorus source alone does not affect the biomass production and COD reduction levels. The results show that the addition of 4 g dm^{-3} ammonium sulphate and 0.4 g dm^{-3} KH_2PO_4 to the sugar beet stillage gives the best results.

The effect of nitrogen and phosphorus sources on



(a)



(b)

Fig.7 : The effect of additing nitrogen and phosphorus to the sugar beet stillage on biomass production (a), and COD reduction (b), by Hansenula sp. in the batch culture (35°C, 200 rev min^{-1}, incubation time = 48 h, initial COD= 64000 mg dm^{-3}, initial pH =4.8)

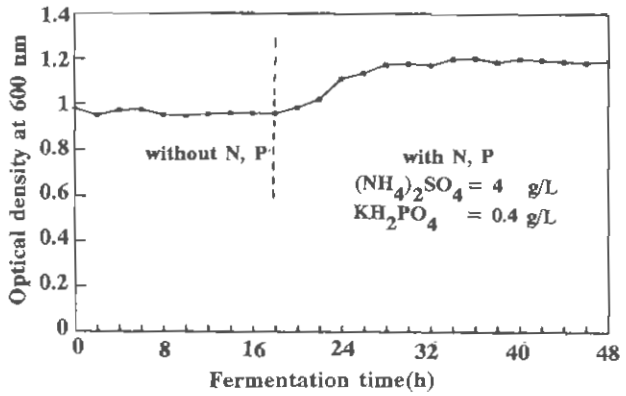


Fig.8: The effect of addition of nitrogen and phosphorus to the sugar beet stillage on the concentration of Hansenula sp. in the continuous fermentation ($D=0.12\ h^{-1}$, 35°C, 900 rpm, 1.5 vvm, initial COD=64000 mg dm^{-3}, initial pH =4.8, $(NH_4)_2SO_4$= 4 g dm^{-3}, KH_2PO_4 = 0.4 g dm^{-3}).

the continuous fermentation of *Hansenula sp.* are shown in the Fig. 8 and Table 1. These results reveal that the addition of nitrogen and phosphorus sources cause considerable increase in the protein content of biomass (from 39.6% to 50.6%). The biomass production and COD reduction also increases from 5.7 to 8.5 g dm^{-3} and 31% to 35.7% , respectively.

Although supplementation of the stillage medium increase the biomass production and COD reduction levels, but its application depends on the cost of the chemicals and on the value of the biomass to be obtained.

Table 1. The effect of addition of nitrogen and phosphorus to the sugar beet stillage in the continuous culture of Hansenula sp. ($D= 0.12\ h^{-1}$, 35°C, 900 rpm, 1.5 vvm, initial COD = 64000 mg dm^{-3}, initial pH = 4.8)

Medium	Biomass dry weight (g dm^{-3})	COD reduction (%)	Protein content (N%×6.25)
nonsupplemented stillage	5.7	31	39.6
supplemented stillage*	8.5	35.7	50.6

* with $(NH_4)_2SO_4$ =4 g dm^{-3} and KH_2PO_4=0.4 g dm^{-3}

Dilution rate

Two parameters must be taken into account for SCP production: first the biomass yield (Yx/s), which shows the efficiency of the substrate to biomass transformation and second the productivity, which shows the quantity of biomass produced per unit volume in a given time. Productivity depends on the cell concentration in the fermenter and on the specific growth rate (μ). Since in the continuous culture the specific growth rate is equal to the dilution rate ($D=\mu$) , therefore the effect of dilution rate on productivity was investigated.

Fig. 9 shows the effect of increase in the dilution rate on biomass production, COD reduction, crude protein content and productivity of *Hansenula sp.* grown in sugar beet stillage supplemented with 4 g dm^{-3} ammonium sulphate and 0.4 g dm^{-3} KH_2PO_4. As it can be seen, the highest productivity is obtained at the dilution rate of 0.12 h^{-1}.

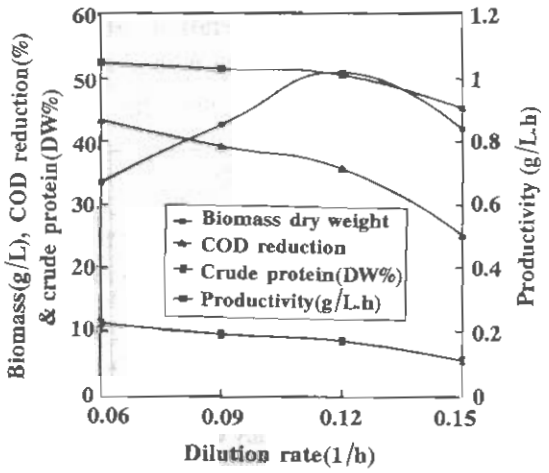


Fig. 9: The effect of dilution rate on biomass production, COD reduction, crude protein content and productivity of Hansenula sp. grown in sugar beet stillage (35°C, 900 rpm, 1.5 vvm, initial COD= 64000 mg dm^{-3}, initial pH=4.8, $(NH_4)_2PO_4$= 4 g dm^{-3}, KH_2PO_4=0.4 g dm^{-3}).

From the above results, a decrease in protein content with increase of dilution rate was observed which could be related to the increase in the growth rate and agreed with results of previous stages.

Nutritional Value of SCP

The overall analysis of SCP produced from *Hansenula sp.* grown on the supplemented sugar beet stillage was compared with several important fungi

Table 2: Composition of Hansenula sp. and several important fungi for SCP production (Olsen and Allermann,[20])

Analysis	*Hansenula sp.*	*Candida utilis*	*Fusarium graminearum*	*Paecilomyces varioti*
Dry matter(%)	97.3	91	94.2	96
Crude protein (6.25×N%)	50.6	48	54.1	55
Carbohydrates(%)	24	—	—	—
Lipids(%)	1	1.35	1	1
Total nucleic acid (%)	8.7	—	—	—
Total ash (%)	3	11.2	6.1	5

and the results are shown in Table 2.

The essential amino acid profiles of the produced SCP are in agreement with the FAO reference protein and are comparable with some other food proteins such as soya bean and fish meal as it is shown in Table 3.

Comparison of the amino acids profile of different yeast species and the profile of egg and soya cake shows that there is a deficit in sulfurous amino acids (especially methionine) but with a good balance for other essential amino acids (Lysine, tryptophan).

During this procedure a valuable by-product is produced which could be very important, specially for the countries that are in need of importantion of protein. The production of SCP could be as one of the steps needed for the complete treatment of the stillage. In addition, the yeast consumes the inhibitory components in the ethanol production, such as lactate and acetate, consequencely the effluent could be recycled as diluting water in the fermenter. Thus will reduce water consumption in dilution of molasses and residual stillage volume, making for futher treatments, more economical.

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Table 3: Some essential amino acids content of Hansenula sp., soya bean meal, fish meal and the FAO reference protein

Amino acids (g/100 g)	*Hansenula sp.*	soya bean meal	fish meal	FAO reference
Isoleucine	4.1	5.4	4.6	4.2
Tyrosine	3.7	2.7	2.9	2.8
Threonine	3.3	4.0	4.2	2.8
Valine	5.5	5.0	5.2	4.2
Lysine	6.1	6.5	7.0	4.2

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