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BATCH AND REPEATED FED BATCH CULTIVATION OF *Candida utilis* ON CORN MEAL HYDROLYSATE FOR CELLULAR BIOMASS PRODUCTION.

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ABSTRACT

A Fermentation medium based on corn meal hydrolysate was developed for the production of single cell protein (SCP)using the yeast strain *Candida utilis* . The corn meal was gelatinized for 1h at 80^{o}C then liquefied with a thermostable α-amylase at 70^{o}C. The liquefied starch was saccharified with glucoamylase at 55 ^{o}C for two hours . Over 98% of the starch was hydrolyzed, about 80% of the hydrolysate was glucose . The hydrolysate was formulated in a suitable medium for supporting the requiste growth rate and yield of *Candida utilis* . The growth parameters of *Candida utilis* grown on corn meal hydrolysate in a simple batch fermentation mode are presented .A strategy for utilization of repeated fed batch fermentation was demonstrated. The data showed that the repeated fed batch mode was a suitable fermentation technique for biomass production.

Keywords: Repeated Fed Batch Cultivation, *Candida utilis*, corn Meal Hydrolysate, Cellular Biomass.

INTRODUCTION

Various culture modes have been suggested for biomass production (Pirt, 1975). Open growth system differ from closed system in that there is a continuous input of nutrient substrate (s) and removal of cellular biomass and unused substrate(s). Therefore, the open system known as continuous flow culture and many different forms of this cultivation mode have been described and applied (Slater and Hardman, 1982). Continuous culture enable exponential growth phase to be prolonged indefinitely, establishing steady state condition. Hence, the continuous cultivation mode provide high productivity if it can be operated with no trouble and the activity of microorganism is still constant (Pirt, 1972). However, only few continuous cultures have been carried out successfully in industry due to various reasons.

Several other cultivation modes have been also suggested by many investigators for improving the biomass productivity such as fed batch and repeated fed batch cultures (Bull, 1974). In general, the fed- batch is superior to conventional batch operation especially when changing of nutrient concentrations affect the yield or productivity of the cellular biomass (Anthony *et al.,* 1996). Other advantages for fed batch cultivation system were reported (Yamane and Shimizu, 1984).

When a portion of a batch culture is withdrawn at intervals and the residual part of the culture is used as an inoculum for the next batch culture, the system of operation is called repeated fed batch culture (Burns and Slater, 1982). In addition to increased productivity, repeated fed batch culture has the advantage that inocula need not be prepared for the second subsequent cycles; the chances of contamination are also lower than in the

continuous culture (Ejiofor *et. al.,* 1994). Thus, repeated fed batch cultivation mode considered one of the useful system for economical production of single cell protein.

Many yeast strains were used for the production of cellular protein in batch mode. These yeasts including *Candida utilis* which was authorized for use in food products for its amino acids composition and vitamins content (Bajpai and Bajpai, 1986 & 1987).

For commercial success of SCP production, the current investigation focused on two important aspects:

1. The fermentation raw materials are the major contributors to the production cost of low value products such as single cell protein **,** search for cheaper and locally available fermentation substrate is' essential.
2. Adapting the select microorganism strain for single cell protein production on cultivation mode as repeated fed batch would decreased fermentation time for growing the microorganism. The result of this process would reduce the capital and operating cost.

For these reasons, the goal of the present work was to evaluate the cheaper nutrient corn meal hydrolysate as fermentation substrate for single cell protein production using the yeast strain *Candida utilis*. Batch and repeated fed batch cultures were compared to known the strengths and weaknesses of each cultivation mode.

MATERIALS AND METHODS

Enzymes: Amylase (EC 3.2.1.1., Termamyl- 60) from *Bacillus Licheniformis* and glucoamylase (EC 3.2.1.3., AMG- 200) from *Aspergillus niger* were obtained from NOVO Industri. The activities of the two enzymes were 60 KNU/g and 200 AGU/ml, respectively, as defined by the manufacture (NOVO Information, 1972, File No. A5170-GB).

Corn Meal: The ground corn (maize) was purchased locally form a feed store, Giza- Egypt.

Microorganism: Yeast strain of *Candida utilis*, was provided from the Yeast Culture Collection Unit, Agric. Microbiol. Res. Dept., Agric. Res. Center, Giza - Egypt. It was maintained on nutrient agar slants by subculturing at monthly intervals, incubated at 28 ^{0}C for 48h and then stored at 4 ^{0}C.

Enzymatic corn meal hydrolysate: A corn meal slurry (20 % W/V*)* was prepared and a low temperature long cooking procedure was applied for the liquefaction and saccharification processes (Fig. 1). The method involving cooking for I h in the presence of Ca^{++}ion (0.001%, $CaCl_2$. $2H_2O$) at 80 ^{0}C under stirring. The gelatinized starch was Liquefied with 0.3 mL of the thermostable α-amylase (Termamyl) per 100g of corn meal. The process was allowed to continue for I h at 70 ^{0}C. The liquefied broth (1 liter) was then saccharified for 2h at 55 ^{0}C with 0.25 g glucoamylase enzyme. Samples (2 ml) were taken every 10 min. and the enzyme action was stopped by HCl (0.1 M, I mL). After centrifugation the reducing sugars were determined in the clear supernatant.

Fermentation medium : Liquid nutrient medium for cultivation of *Candida utilis* and SCP production was used. The liquid medium composition was :

3.5% sugars (hydrolysate), 0.5% peptone, 0.3% yeast extract, 0.2% KH_2PO_4 and 0.1% Mg SO_4. The hydrolysate sugars were sterilized separately at 121 ^{0}C for 15 min and mixed with the sterilized medium components.

Fig.(1):Hydrolysis profile of corn meal saccharification by low temperature long cooking process.

Preparation of inoculum: It was prepared by subculturing from a maintenance slope into a synthetic medium (50 mL) containing glucose (30 g/L), yeast extract (5 g/L) and peptone' (3 g/L). The medium was adjusted to pH 5.0 and sterilized at 121 ^{0}C for 15 min. After incubation for 24 h at 30 ^{0}C on rotary shaker (175 rpm), the broth (0.044 g cell / 5 ml) was used as inoculum.

Repeated fed batch operation. Erlenmeyer flask (500 mL) was used as batch fermentation vessel with working volume of 100 mL. The vessel was fitted with CO_2 exhaustion port and an aseptic sampling system. All the fermentation runs were carried out at 30^{0}C. in a rotary shaker (200 rpm). Repeated fed. batch fermentation was carried out at V_{out}/V_t (ratio of the volume drawn out to the total volume) values of 0.25, 0.5 and 0.75. The culture broth was aseptically withdrawn from the vessel and the sterilized fresh medium equal to the volume drawn out was added aseptically to the vessel from the inoculation port. The culture volume kept constant in the vessel as 100 mL. Samples of culture broth were taken at definite intervals from the fermentation vessel in batch and repeated fed batch runs and the reducing sugars and biomass concentration were determined.

Analytical methods: Total reducing sugars were estimated using the DNS method (Miller, 1959). Cell concentration was determined by measuring the absorbance at 600 nm and calculated in g dry weight per liter by means of a calibration plot which was previously established.

RESULTS AND DISCUSSION

1. Corn meal hydrolysis process

The time course of the liquefaction and saccharification stages for the conversion of corn meal starch to reducing sugars is shown in Fig. (2). The production of sugars from starch followed the expected pattern: α-amylase liquefied the starch by random hydrolysis of the α-1.4 glycosidic linkages

yielding some glucose but mostly maltose and oligosaccharides. Addition of glucoamylase converted the maltose and maltooligosaccharides rapidly to glucose. The hydrolysis data indicated that the conversion of starch to reducing sugar exceeded 98% . Glucose constituted 80.7% of the converted materials; the rest was maltooligosaccharides comprising essentially maltose and isomaltose.

Fig.(2): Time-course of enzymatic Hydrolysis of corn meal starch

The long low-temperature cooking procedure (Fig.2), which used in the present work, was considered beneficial in that high pressure steam was not required to attain the high temperature needed in the alternative short high temperature procedure (Atthasampunna *et al.,* 1987) . Consequently, the technology requirements for steam generation were simplified.

The cost of enzymatic hydrolysis can be minimized by substitution of pure enzymes with amylase-producing microorganisms (Okafor and Ejiofor, 1990) in alternative processing schemes . The corn meal hydrolysate contains 160 g of reducing sugars per liter which was higher than the value used for the simulation. Concentration of the hydrolysate would not have been necessary and a higher concentration of corn meal than 20% could have been used while ensuring thorough mixing at a lower agitation rate of about 100 rpm.

2. Batch Culture Fermentation of *Candida utilis*:

The sugars (glucose and maltooligosaccharides) produced from corn meal starch can be easily transported across the cell membrane and metabolized by yeasts (Kristiansen, 1994). Therefore, the corn meal hydrolysate could provide the fermentation industry with inexpensive and locally available fermentable raw material . Utilization of corn meal as a rich source of hydrolysable carbohydrate was expected to reduce the expenditure on raw material procurement and relax the dependence on molasses in corn producing regions, like Egypt.

The simple batch cultivation mode of *C. utilis* with corn meal hydrolysate was performed on rotary shaker (175 rpm) at 30^{o}C. Figure (3) is an illustration of a typical set of batch fermentation at initial sugars concentration of 3.5%.

Fig.(3): Plots of cell biomass concentration and sugar consumption during simple blatch fermentation runs of *C. utilis.*

The graph shows the transient profiles of reducing sugars and cellular biomass during 48h of fermentation time. It can be seen that the batch fermentation divided into three stages. In the first stage (0-2h) there was a lag period, in which the initial biomass remained constant. However, slow decrease in reducing sugars (2%) was observed during this period. In the second stage (2-12h) the biomass production rate increase exponentially and the reducing sugars concentration decreased. At the last stage (12-36h), the biomass production decreased slowly and reach a constant value of 1.34 g cell / 100 ml after 48h cultivation period.

The batch growth parameters are given in Table (1) . The maximum biomass concentration after 48h was found to be 1.34 g cell/100 ml and the total consumed sugar was 2.82 g /100 ml. The growth yield coefficient (Yx/s) was calculated to be 0.475 g cell/g sugars (-). The overall biomass productivity was 0.0279 g cell / h.

Table (1): Parameters of batch culture of *Candida utilis* grown on corn meal hydrolysate

Parameters	Value
Maximum biomass, X (g/100 ml)	
a) in 24 h	1.104
b) in 48 h	1.340
Sugar consumption, s (g/l)	
a) in 24 h	1.635
b) in 48 h	2.820
Growth yield coefficient, $Y_{x/s}$	
(g cell / g sugars)	0.475
Biomass formation rate, r_x	
(g cell / h)	0.0279
Sugar uptake rate, $r_{x/s}$	
(g sugars / h)	0.0587

The growth kinetics was simply expressed as :

$$\frac{dx}{dt} = \mu X \qquad \text{when} \qquad X < X_{max}$$

$$dx / dt = 0 \quad \text{when} \quad X = X_{max}$$

By integration this equation

$$X = X_o e^{\mu t}$$

so,

$$Ln (X / X_o) = \mu t$$

The dynamic of corn meal hydrolysate by *C.utilis* was illustrated in Fig.(4). Plotting of L n (X / X_o) versus time (t) showed that the exponential phase ended within 10-12 h.

The parameter (μ) was obtained from the slope of Ln (X/Xo) versus (t) data . This specific growth rate (μ) value was found to be 0.216 h^{-1} . Metabolism of the yeast cell is affected by the concentrations of the various medium components. Preferential uptake of these components may also affect the specific growth rate (μ) value.

3. Pattern of sugars utilization in batch culture by *Candida utilis* .

A substrate (sugars) consumption expression with substrate dependent growth yield (Y) was developed as :

$$ds / dt = [1 / Y (s)] \; [dx / dt]$$

This equation was integrated for a short time interval, by assuming the growth yield (Y) a constant, so

$$S_{n+1} - S_{n-1} = - (1 / Y_n) (X_{n+1} - X_{n-1})$$

Where subscript (n) indicates the value of the subscripted parameter at time t_n. For this purpose, the experimental values of consumed sugars(s) and the produced biomass (x) were evaluated from Fig.4 with 30 min of intervals and Y_n was calculated. The values of growth yield (Y_n) as a function of consumed sugars(s) was plotoed in Fig. (5). This graph implied a relation of the following for :

$$Y = Y_0 - Ks (S - S_c)$$

The parameter S_c was referred to as critical substrate concentration. The above equation may indicate two substrate dependent metabolic ranges during the growth of *C. utilis* as shown in Fig. (5) . In the first metabolic range, growth yield (Y) may decrease sharply with increasing the consumed sugar. The growth yield was decreased to extent 70% with increasing the consumed sugars to release of the energy metabolism byproducts or intermediates at the expense of growth yield. Release of such energy metabolism byproducts has been detected by Ozilgen (1985). In the second metabolism range, where growth yield decreased slowly, the yeast strain *C. utilis* may employ the complex medium constituents or the previously released energy metabolism byproducts as an energy source, together with the sugars .

Variation of growth yield with substrate concentration is not a new observation. The best known example of it , is the substrate effect on growth yield of baker's yeast. At low substrate concentration, the baker's yeast has higher biomass yield (Reed, 1982) . Fed batch fermentation processes are employed in commercial baker's yeast producing plants to prevent the yeast to be subjected to high substrate concentrations.

The critical substrate concentration (S_c) was determined to be 2.62 g sugars/100 ml. The time when S_c was reached was referred as t_c and calculated to be 18 h.

4. Repeated fed batch fermentation of *Candida utilis.*

For increasing the biomass productivity with C. utilis on corn meal hydrolysate, the repeated fed batch cultivation mode was investigated. This batch mode was not modeled but was designed simply using the corn meal hydrolysate with an initial reducing sugar concentration of 3.5%. After 48 h of cultivation time, the rate of biomass production was decreased and the concentration of the cellular growth was 1.34 g cell/100 ml. At this stage, the batch fermentation mode was switched over to repeated fed batch mode. A portion of the fermentation vessel contents was emptied out and replaced with the same volume of fresh medium.

7

Fig.(4): The modeling of the growth of *C. utilis* in a batch fermentation with corn meal hydrolysate as the limiting substrate.

Fig.(5): Plots of variation of growth yield (Y) with sugar concentration

The concentration of the added medium was adjusted to ensure that upon dilution with old culture, the sugars concentration attained the value it had at the beginning of the old run. Hence, each repeated batch cycle was conducted with an identical initial glucose concentration. The second and subsequent fed batch cultivation were carried out with the residual cells in the fermentation vessel as seed . The repetition of batch cycles were continued for a total of 6 , 12 . 24 batch runs in repeated mode at V_{out}/V_t values of 0.75 , 0.50 and 0.25 , respectively . The total cultivation time for batch cycles was kept constant at 192 h in all repeated fed batch experiments.

The repeated batch run profiles which obtained at V_{out}/V_t of 0.75 are shown in Figure (6A). It could be seen from this graph that the time course of biomass production was almost the same for all six repetitions. The repeated fed batch results which obtained at V_{out}/V_t ratios of 0.50 and 0.25 were illustrated in Figures. (6B & 6C). A comparison between the batch and repeated fed batch fermentation results are summarized in Table (2). Analysis of the data in Table (2) indicated that the consumption of the sugars for biomass production in batch culture is higher (82%) than the repeated fed

Table (2): Results of batch and repeated fed batch fermentation of *Candida utilis* on corn meal hydrolysate

Parameters	Batch culture system	Repeated fed batch system		
		V_{out}/V_f 0.75	V_{out}/V_f 0.50	V_{out}/V_f 0.25
Fermentation time (h)	192	192	192	192
Total volume (ml)	400	550	700	700
Sugars used (g)	14	22.6	34.3	42.7
Consumed sugars (-) , s (g)	11.4 (82%)	16.7 (74%)	23.3 (68%)	27.2 (60%)
Residual sugars (g)	2.6 (18%)	5.9 (26%)	11 (32%)	15.5 (40%)
Max. biomass in 192 h, x (g)	5.4	7.4	9.3	10.1
Biomass productivity (%)	(100%)	(137%)	(172%)	(187%)
Growth yield coefficient $Y_{x/s}$, g cell / g sugars (-)	0.474 (100%)	0.443 (93%)	0.339 (84%)	0.371 (78%)
Formation rate of Biomass, r_x g cell / h	0.0281	0.0385	0.0484	0.0526
Sugar uptake for biomass, r_{xs} g sugars / h [$r_{xs} = r_x / Y_{x/s}$]	0.0593	0.0869	0.1213	0.1418

Fig.(6):Repeated fed batch fermentation of *C. utilis* with different V_{out}/v_t ratios.

batch system (60-74%). In repeated fed batch culture, the sugars consumption decreased as the Vout/Vt ratio was decreased. Only 60% of the added sugars were consumed at Vout/Vt = 0.25. Thus, the residual sugar was low (18%) in batch culture and high (26-40%) in repeated fed batch system. At Vout/Vt = 0.25 the residual sugars were 40% of the total sugars used in the fermentation.

The growth yield coefficient ($Y_{x/s}$) in batch culture was higher (0.474 g/g) than this value in repeated fed batch system (0.443 – 0.371 g/g). Also, the lowest value of ($Y_{x/s}$) (0.371 g/g) was obtained at Vout/Vt = 0.25 which was 22 % lower than the ($Y_{x/s}$)value in batch culture. On the other hand, the rate of the sugars uptake for biomass production in batch culture was lower (0.0593 g/h) than the repeated fed batch system (0.0869 – 0.1418 g/h). It may be noted that the sugars uptake at Vout/Vt ratio = 0.25 was 2.4 times higher over this value in batch culture.

The biomass productivity in repeated fed batch at Vout/Vt = 0.75 was about 37% higher over the batch culture productivity. Similary, the overall cell productivity were 72% and 87% higher over batch culture at Vout/Vt values of 0.50 and 0.25, respectively. In the case of simple batch culture, the biomass production would be even lower than those given in Table (2) since an allowance would have to be made for the down time for harvesting, cleaning, filling and sterilization after each run. On taking this into account, the percent increase in productivities would be even higher with repeated fed batch culturing compared to simple batch productivities.

It can be concluded, a strategy for utilization of the corn meal starch in production of cellular biomass was demonstrated. Corn meal starch could be liquefied, saccharified and formulated into a suitable medium that was equivalent to glucose medium in supporting the requisite growth rate and yield of cellular biomass. With the demonstration of corn meal as a rich source of hydrolysable carbohydrate and the low level of its current utilization for other purposes, suggest a strong potential for its large-scale use in the fermentation industry.

For accurate parameters estimation, screening for another yeast strains and media as well as the design of production-scale operations, processes should be based on simple and robust models capable of implementation in a low technology environment .This work has shown that the repeated fed batch fermentation can be used for single cell protein production .

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تنمية خميرة *Candida utilis* بطريقة تخمير الدفعة الواحدة (Batch) وتخمير الدفعات المتكررة Repeated Fed Batch على ناتج التحليل الإنزيمى لدقيق الذرة لإنتاج الكتلة الحيوية (Biomass)

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1) تم في هذه الدراسة إجراء تحليل مائي إنزيمى لدقيق الذرة باستخدام أسلوب جديد عن طريق التعريض لحرارة غير مرتفعة لمدة طويلة وفي وجود إنزيمات الاميليز والجلوكواميليز .
2) أمكن إحداث عملية ال gelatinization بالتعريض لحرارة 80 م لمدة ساعة ثم إضافة إنزيم الاميليز الثابت حراريا thermostable على حرارة 70م لمدة ساعتين لإحداث عملية الـ liquefcation – وأجريت عملية التحليل المائي الكامل saccharification بواسطة إنزيم الجلوكواميليز على 55م لمدة ساعتين .
3) بلغت نسبة التحليل المائي لدقيق الذرة 98% وكانت نسبة الجلوكوز به حوالى 80% مما يجعله مصدر جيد للسكرات القابلة للتخمر
4) تم استخدم السكرات الناتجة من التحليل الإنزيمي في بيئة تحتوي على إضافات أخري هي 5,% بيتون ، 3,% مستخلص خميرة ، 2,% فوسفات البوتاسيوم ثنائى القاعدية و1,% سلفات المغنيسيوم .
5) باستخدام البيئة السابقة تم تنمية خميرة لـ *C.utilis* بطريقة تخمير الدفعة الواحدة (Batch) وفى وجود 3.5% وتم تقيم هذه الطريقة حيث وجد أن كمية الكتلة الحيوية (Biomass) بعد 48 ساعة هو 1.34جم/مللى لتر وبلغ استهلاك السكر 2.82جم/100مللى لتر بنسبة 80% .
6) وبتقدير الثوابت الديناميكية لعملية التخمر بطريقة تخمير الدفعة الواحدة(Batch) وجد أن :

Growth Yield ($Y_{x/s}$) = 0.475 g/g
Specific growth rate (μ) = 0.224 h^{-1}
Critical Substrate conc. (Sc)=2.62 g/100ml

7) أجريت في هذا البحث عملية دراسة تنمية الخميرة الـ *C . utilis* عن طريق التخمر بطريقة الدفعات المتكررة (Repeated Fed Batch) حيث تتم عملية سحب ناتج التخمر وإضافة بيئة جديدة إلى نفس وعاء التخمر. ثم تكملة عملية التخمر وذلك عدد مرات وقد تم تطبيق هذه الطريقة على ثلاث معدلات من السحب والإضافة هي 75% ، 50% ، 25% من حجم وعاء التخمير مما يعطي 24،12،6 عملية تخمير متتالية وقد أوضحت النتائج ما يلي :
   1) زيادة إنتاجية الكتلة الحيوية (Biomass) في هذه الطريقة مقارنة بطريقة تخمير الدفعة الواحدة (Batch) بمقدار يتراوح بين 37.5-87%.
   2) تزايد إنتاجية الكتلة الحيوية (Biomass) ويقل كمية السكر المتبقي كلما ذادت معدلات عمليات السحب والإضافة المتتالية وكانت أعلى إنتاجية عند 25% (سحب / إضافة) من حجم المخمر .
   3)تمتاز طريقة تخمير الدفعات المتكررة (Repeated Fed Batch) عن طريقة تخمير الدفعة الواحدة (Batch) بزيادة إنتاجية الكتلة الحيوية (Biomass) عن القيم المقدرة عمليا إذا وضعنا فى الاعتبار الزمن اللازم لتفريغ المخمر وإعادة التعبئة والتعقيم عند إجراء التخمير بطريقة تخمير الدفعة الواحدة (Batch) عدة مرات متتالية.