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UTILIZATION OF RICE STRAW HYDROLYSATE BY *Aspergillus terreus* FOR MICROBIAL PROTEIN PRODUCTION.

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ABSTRACT

The crude rice straw hydrolysate (RSH) produced by dilute acid hydrolysis contains toxic substances, which are inhibitory to microbial growth. Removal of these toxic compounds was achieved by ethyl acetate extraction to produce the extracted rice straw hydrolysate (Ext -RSH). The utilization of the fermentable sugars of RSH as well as Ext -RSH by *A. terreus* was determined in batch culture fermentation. The Ext -RSH gave a marked improvement in fermentability over the crude RSH. The presence of toxic materials in crude RSH resulted in a long fermentation time (120 h) but this value was 60 h in the Ext -RSH medium. The productivity of biomass increased 3-4 times after extraction of the inhibitory substances from RSH. The effect of crude inhibitors extract on biomass production was tested by the standard inhibition assay and showed complete inhibition when it was added to the Ext -RSH culture medium at a concentration of 20 g/l. Monod model was developed to describe the cellular growth of *A. terreus* in RSH medium. The model included inhibition term for toxic agent in RSH. The agreement between the experimental data and the model predictions demonstrated the adequacy of the proposed models.

Keywords:*Aspergillus terreus*,rice straw hydrolysate,fermenration kinetic,microbial protein.

INTRODUCTION

More than 3.5 million tones of rice straw are annually generated in Egypt. Part of this amount besides sugarcane bagasse are being used properly in Egypt on industrial scale, e.g. pulp and paper industry. However, most of these wastes just used for fuel in villages causing health problems and environment pollution. Utilization of rice straw as fuel is no longer acceptable means of use. There fore, there is a great attention to recycle the rice straw as agricultural waste.

The hydrolysis of rice straw by diluted sulphuric acid has been proposed as a promising approach for converting this waste to fermentable sugars (Parisi, 1989). The rice straw hydrolysate can be used as an ideal substrate for fermentation process to produce valuable products (Philippidis and Wyman, 1992), However the acid hydrolysis of lignocellulosic materials generated in the hydrolysate a broad range of undesirable compounds (Martin *et al.,* 1992 and McMillan, 1994). The sources of these compounds are the individual lignocellulosic components, lignin, cellulose and hemicellulose (Nishikawa *et al,* 1988; Frazer and McCaskey, 1989) Sugar degradation is the most important consideration in dilute acid hydrolysate, which represents 30% loss of potential sugar yield. In hot dilute acid, sugars are degraded to furfural, laevulinic acid and formic acid (Clark and Mackie, 1984). Also, some reactive intermediates and 5-hydroxymethylfurfural are converted to compounds termed as humic substances. In addition, the lignin degradation releases phenolic compounds into the lignocellulosic waste

hydrolysates. These phenolic compounds may be generated also from carbohydrates (Ando *et al.,* 1986).

The presence of these inhibitory compounds in rice straw hydrolysate may be considered as potentially toxic for the most microorganisms utilizing the fermentable sugars in such hydrolysate. Several methods of detoxification, *i.e.,* the removal of the inhibitors from the lignocellulosic hydrolysates, have been proved to increase their fermentability. The addition of activated charcoal (Roberto *et al*, 1991), extraction with organic solvents (Wilson *et at,* 1989), ion exchange (Fein *et al.*, 1984), ion exclusion (Buchert *et al*., 1990), molecular sieves (Tran and Chambers, 1986) and steam stripping (Yu *et al,* 1987) have been investigated. In few studies, attempts have been made to obtain a more complete picture of these compounds (Marko-Varga *et al.*, 1994). This knowledge should enable not only the development of detoxification methods, but also modification of hydrolysis processes to minimize the formation of the most potent inhibitors (Baugh and McCarty, 1988).

The interest in the industrial application of rice straw hydrolysate fermentation necessitate the need to build growth and fermentation models design for application in fermentor. Therefore, the present study was undertaken in order to increase the fermentability of rice straw hydrolysate. The microbial biomass production by *Aspergillus terreus* was analyzed to develop a growth kinetic model to express the effect of the toxic compounds on the cellular protein production by this local fungal isolate.

MATERIALS AND METHODS

Microorganism: A local fungal isolate of *Aspergillus terreus* (Mansour 1988), was used throughout this work. The fungal isolate was maintained on slopes of potato dextrose agar (Difco).

Rice straw: Material was obtained from the Agric. Exper. Station, Agric. Res. Center, Giza - Egypt. Rice straw was ground to particle size of 60-100 mesh and washed with tap water then dried at room temperature.

Preparation of rice straw acid hydrolysate: Rice straw was hydrolysed by sulphuric acid (2%, *W/V)* at L:S ratio of 10:1 (W/W). The mixture was autoclaved at 121^0C for 30 min. After filtration the acid liquor was neutralized to pH 7.0 with calcium hydroxide and the resulting precipitate was removed by filtration. The hydrolysate was concentrated under vacuum at 60^0C to have reducing sugar concentration of 60 g/l. This preparation was considered as crude rice straw hydrolysate (RSH).

Ethyl acetate extraction: The crude (RSH) was reacidified with H_2S0_4 to pH 2.0 and subsequently extracted with ethyl acetate in a continuous extractor for 3h. The aqueous phase containing the fermentable sugars was collected and the residual ethyl acetate was removed by evaporation under vacuum at 40^0C. After neutralization to pH 7.0 the extracted hydrolysate was readjusted to reducing sugar concentration of 60 g/l. This hydrolysate preparation was claimed to be free of inhibitor materials and referred as (Ext -RSH).

Inhibitor substances extract: The organic solvent phase after extraction of RSH was evaporated under vacuum at 40^0C. *A* viscous dark brown extract was obtained after removing the solvent. The concentration of this extractable

material was found to be 8.7 g/l of the starting hydrolysate.

Inoculum: The inoculum was prepared by growing the fungal isolate on potato dextrose agar plates for 7 days at 30^0C. The conidia were harvested with isotonic NaCI solution. The spore suspension was diluted, vortexed to break up any clumps of spores and then microscopically counted. The spores suspension was standardized to 10^9 spores/mI.

Growth medium: The mineral salts of Czapek's medium (Difco,1985) was used for the fungal growth. It has the following composition (g/l): $NaNO_3$, 2.0; KH_2P0_4, 1.0; $MgSO_4$. $7H_20$, 0.5; KCI. 0.5; $FeSO_4.7\ H_20$, 0.01 and the desired fermentable sugar concentration from RSH or Ext -RSH was added as experimental requirements.

Batch culture fermentation: Fungal culture was grown in 250 ml Erlenmeyer flasks containing 50 ml of medium. Flasks were sterilized at 121^0C for 15 min. Inoculation was performed with spore suspension *(*1.0 ml) and the flasks were incubated at 30^0C in a rotary shaker (150 rpm). At the end of the cultivation period, samples were taken for biomass and reducing sugar determination.

Fungal biomass estimation: Fungal dry weight was determined in a known volume of the broth. The sample was pipetted into preweighed centrifuge tube and centrifuged (5000 rpm 10 min) The fungal pellets were washed twice with water. The washed pellets were oven dried (80^0C for 24h) to constant weight. The total dry biomass in the fermentation vessel was calculated.

Reducing sugar determination: The total reducing sugars in the culture supernatant were estimated using DNS method (Miller, 1959).

RESULTS AND DISCUSSION

1.Fermentability of the rice straw hydrolysate (RSH) and its ethyl acetate extraction product (Ext-RSH) by *Aspergillus terreus.*

The presence of many toxic compounds for microorganisms in the dilute acid hydrolysates of lignocellulosic wastes has long been recognized (Safi *et. al.,* 1986). The lack of information on specific inhibitors in these hydrolysates due primarily to the absence of any detailed analysis for the toxic substances in such hydrolysates (Clark and Markie, 1984). For this reason, the method used in the present work to detoxifying the rice straw hydrolysate to make it more fermentable by the fungal isolate *Aspergillus terreus* have only been developed on an empirical basis (Hajny, 1981).

For evaluating this organic solvent treatment, the relative fermentabilities of the crude rice straw hydrolysate (RSH) and its ethyl acetate extract product (Ext-RSH) were determined. The sugar consumption during the fermentation of these two hydrolysate preparations is shown in Figure (1). As expected, the extracted hydrolysate (Ext-RSH) gave a marked improvement in fermentability by *A. terreus.* The consumed fermentable sugar was 50% and 90%, from the amount originally present, in the RSH and Ext-RSH medium, respectively. From these results, it can be concluded that the fermentability of rice straw hydrolysate was almost doubled after the ethyl acetate extraction.

Fig (1): Relative sugar consumption (%) by *Aspergillus terreus* the batch culture fermentation of RSH and EXT-RSH.

The profile for the batch cultures fermentation of these two rice straw hydrolysate preparations by *A. terreus* was carried out with initial fermentable sugar concentration 30 g/l. Figure (2) shows the biomass production and sugar utilization during the time-courses of fermentation at 30^0C. Both (RSH) and (Ext-RSH) have similar profiles of change of the fermentation variables. However, the two preparations differ on the overall time of fermentation as well as their final results (Table 1). The long fermentation time in the RSH medium (120 h) was due to the presence of the inhibitory materials. The toxic substance in the crude hydrolysate affected the activity of cells and partially inhibited their growth and metabolic rates. A similar effect of the toxic substances on the fermentation dynamic of *Saccharomyces cerevisiae* in the waste sulphite liquor was reported (Rousseau *et al.,* 1992). Unlike the Ext-RSH, which demonstrated nearly a complete consumption of fermentable sugars (97.3%) after 60 h, in the RSH medium only 86.8% of the sugars were consumed after 120h. This observation was probably due to the presence and further accumulation of toxic substances in the RSH medium which inhibited the cellular activities. Additionally, a more significant difference was observed in the productivities of the biomass in the two hydrolysate preparations (Table 1). A three times increase in the biomass productivity can be reported for Ext-RSH compared with RSH medium.

Table (1): Final results of the batch cultures fermentation with *Aspergillus terreus* on RSH and Ext-RSH medium.

Parameters	RSH	Ext-RSH
Initial sugar conc. (g/l)	30	30
Fermentation time (h)	120	72
Residual sugar conc. (g/l)	3.95	0.82
Consumed sugars, **s** (g/l)	26.05	29.18
Fungal dry wt., **x** (g/l)	4.152	8.440
Biomass yield, $\mathbf{Y}_{x/s}$ (g/l)	0.159	0.289
Biomass productivity (g/l.h)	0.0346	0.1172

Fig (2): Time of cellular biomass and sugar utilization during batch culture fermentation of *Aspergillus terreus* on RSH and EXT-RSH preparations.

2. Inhibition extent of the ethyl acetate extract from rice straw hydrolysate (RSH).

After organic solvent extraction of RSH by ethyl acetate the concentration of the extracted toxic substances was determined. These inhibitory materials were found to be 8.7 g/l of the starting RSH hydrolysate. The effect of toxic substances on the fermentation rate and the growth of *A. terreus* was tested. For this purpose batch culture fermentation runs were carried out using Ext-RSH with initial fermentable sugar concentration 30 g/l provided with different toxic substance concentrations (0-20 *g/l).* The biomass production at different toxic substance concentrations were expressed as percentage relative to that in the control (Ext- RSH) without the toxic substances. The results shown in Fig. (3) indicated that complete growth inhibition of *A. terreus* occurred at toxic substances level of 20 g/l. Hence, this level was chosen as maximum toxic substances concentration (I_{max}) above which no measurable growth can be estimated.

Fig (3): Effect of toxic substances concentration on biomass production by *Aspergillus terreus using* EXT-RSH after 48 h cultivation.

Because of the presence of a large number of potentially toxic compounds in the RSH and the absence of complete information about its nature, the author could not relate this toxicity effect to carbohydrate or lignin degradation products. By comparison, the carbohydrate degradation products were found to be much less inhibitory (Clark and Mackie, 1984). The low molecular weight phenolics in the lignocellulosic hydrolysates derived from lignin degradation was recognized as extreme toxic substances in such hydrolysate (Lee and McCaskey, 1983) for most microorganisms.

3. Modeling of the batch culture fermentation of RSH for biomass production by *Aspergillus terreus*.

3.1. Biomass kinetic model

The kinetics of cellular biomass production often expressed by Monod relation (Monod. 1949), which causes a coupling between the increase of cell biomass and the substrate concentration. The maximum specific growth rate (μ_{max}, h^{-1}) and saturation constant (K_s, g/l) are specific to the microorganism and substrate and are taken as constant. The Monod relation for the fermentation on RSH medium may include an extra inhibition term to account for the effect of the toxic substance, which present in RSH, on the biomass production. The following equation can be proposed for this case:

$$(\mu_s)_1 = \mu_{max} \frac{S}{Ks + S} \left(\frac{\overline{I}}{I_{max}} \right)^n \quad \text{RSH medium ..... (1)}$$

The term $(I/I_{max})^n$ express the inhibitory effect of toxic substances in the RSH. For the hydrolysate which was extracted by ethyl acetate, this term should be eliminated from the above equation. Thereafter the model for Ext-RSH was formulated in the following simple form:

$$(\mu_s)_2 = \mu_{max} \frac{S}{K_s + S}$$ **Ext-RSH medium ……(2)**

The model parameters μ_{max} and K_s were first determined from the batch fermentation data on Ext-RSH with *A. terreus.* Thereafter these parameter values were used to develop a model for biomass production on the crude rice straw hydrolysate (RSH), which contains the toxic substances.

3.2. Estimation of (μ_{max}) and (K_S) values

These kinetics constants were determined from series of batch cultures fermentation on Ext-RSH. For this purpose, six experiments were performed for cultivation of *Aspergillus terrus* with different initial fermentable sugar concentrations 5,10,15,20,25 and 30 g/l. The data for the change of the biomass with cultivation time were first smoothed by cubic spline function. Figure (4) shows the semilog plotting for growth data obtained from the above experimental trials.

Fig (4): The modified of the cellular biomass in batch culture

fermentation of *Aspergillus terreus* on EXT-RSH at different fermentable sugar concentrations at 30°C

The specific growth rates $(\mu_s)_2$ were evaluated from these graphs (Fig. 4) for different initial sugar concentrations and recorded in Table (2). For estimation of μ_{max} and K_s, the $(\mu_s)_2$ values were plotted against their corresponding initial sugar concentrations as shown in Figure (5a). This hyperbolic function is unreliable for μ_{max} and K_s evaluation as given by Eq. (2). Therefore, a suitable rearrangement of Eq. (2) to a form that permits the experimental data to be plotted as straight line can be given as follows:

$$\frac{(\mu_s)_2}{s} = \frac{1}{K_s}(\mu_s)_2 + \frac{(\mu_{max})}{K_s} \quad \text{.....(3)}$$

Plotting $(\mu_s)_2/s$ against $(\mu_s)_2$ yields a linear relationship intercepting the abscissa at μ_{max} value and with slope equal to $(-1/k_s)$ as shown in Fig. (5b). The μ_{max} and K_s values for batch fermentation on the Ext-RSH medium were determined to be 0.212 (h^{-1}) and 4.76 (g/l), respectively. These parameter values were used to develop a model for biomass production on crude RSH as demonstrated in the next simulation studies.

3.3. Development of the biomass kinetic model.

In order to obtain a mathematical representation of biomass production in batch culture of *A. terreus* on crude RSH, the proposed relationship in Eq. (1) which described the cellular growth in the presence of the toxic substance was evaluated. The crude rice straw hydrolysate (containing: 60 g/l sugar and 8.7 g/l toxic substances) was used for estimation of the specific growth rates $(\mu_s)_1$ at different initial sugar concentrations (5-30 g/l). Table (2) shows the $(\mu_s)_1$ values which determined on crude RSH and the corresponding sugar and toxic substances concentrations. From equation (1), the inhibition term (I/Imax)n values were calculated and recorded in Table (2). To fit the proposed model (Eq.1), the toxic power (n) was calculated to be 0.433-0.370 with average (n=0.404). This (n) value was used to calculate the model predicted values of $(\mu_s)_1$ from Eq. (1) as recorded in Table (2).

Table (2) Numerical values for biomass production model with *Aspergillus terreus* on the rice straw hydrolysates.

Sugar Conc. (g/l)	Ext-RSH		RSH					
	$(U_s)_2$ h^{-1}	$(U_s)_2$ ------- s	Exper. $(U_s)_1$ h^{-1}	I (g/l)	$(\frac{1}{1_{max}})$	$(\frac{1}{1_{max}})^n$	N *	Calcu. $(U_s)_1$ h^{-1}

5	0.1092	0.0218	0.0260	0.725	0.0362	0.238	0.433	0.0285
10	0.1446	0.0245	0.0480	1.450	0.0725	0.332	0.420	0.0500
15	0.1621	0.0108	0.0675	2.175	0.1087	0.416	0.395	0.0661
20	0.1726	0.0086	0.0810	2.900	0.1450	0.469	0.392	0.0790
25	0.1795	0.0072	0.0880	3.625	0.1812	0.490	0.417	0.0899
30	0.1845	0.0061	0.1050	4.350	0.2175	0.569	0.369	0.0996

*** Average of (n) value = 0.404**

Fig (5): Estimation of Umax and Ks values a) the Monod relationship (Eq.2) between the specific growth rate (Us)2 and initial concentrations, b) Poltting of [μs)2/S} against (μs)2 as recorded in Eq.3 (the linear form of Monod relation). The data are recorded in Table 2.

Fig (6): Comparison between the experimental and the model-predicted values of the specific growth rate (Us)1 for biomass in the fermentation on the crude protein RSH with *Aspergillus terreus.*

The comparison of the experimental and the model predicted values of the fermentation variable $(\mu_s)_1$ are shown in Figure (6). The obtained results indicated that the proposed model could adequately simulate the batch culture fermentation of *A. terreus* on crude RSH. The close approximation of the experimental results by the mathematical model reflects that the same kinetic parameters can be used to represent the cellular growth of *A. terreus* on both rice straw hydrolysate preparations.

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إنتاج البروتين الميكروبى (Biomass) باستخدام ناتج التحليل المائى الحامضى لقش الأرز بواسطة فطرة *Aspergillus terreus*

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مركز البحوث الزراعية – معهد بحوث الأراضى والمياه والبيئة . الجيزة – ج . م . ع .

1- تم فى هذه الدراسة عملية التحليل المائى الحامضى لقش الأرز بواسطة حمض الكبريتيك (2%) على حرارة 121°م لمدة 30 دقيقة – ثم تمت عملية معادلة ناتج التحليل المائى الحامضى وتركيزة حتى 60 جم سكر / لتر . وقد سمى هذا المستحضر (محلول التحليل الخام).

2- تم عمل استخلاص مستمر لمحلول التحليل الخام بواسطة مذيب عضوى (خلات الايثايل) لمدة 3 ساعات وسمى هذا المستحضر (محلول التحليل المستخلص) .

3- تم عمل تبخير للمذيب العضوى الناتج بما يحتويه من المواد المثبطة الناتجة من هدم اللجنين والسكر وسميت هذه المادة المتخلفة بعد التبخير (المواد المثبطة) .

4- عند إجراء عملية تخمير لكل من محلول التحليل الخام وكذلك المستخلص وجد أن المحلول المستخلص ارتفعت قدرته التخميرية إلى الضعف حيث بلغ استهلاك السكر 90% بينما فى المستخلص الخام لم يزيد استهلاك السكر عن 50% .

5- عند عمل تخمير الدفعة الواحدة (batch fermentation) لكلا من المحلول الخام والمستخلص وجد أن تخمير المحلول الخام يستغرق وقت طويل (120) ساعة مع استهلاك 86.8% من السكر المستخدم فى البيئة بينما فى المحلول المستخلص لم يستغرق التخمير سوى (72 ساعة) مع وجود استهلاك تام تقريبا (97.3%) للسكر – وقد لوحظ أيضا زيادة إنتاجية البروتين الميكروبى (Biomass) ثلاثة أضعاف فى حالة المحلول المستخلص .

6- أوضحت التجارب أن تركيز المواد المثبطة الواجب إضافتها للمحلول المستخلص لكى تحدث تثبيط تام لنمو فطر *A. terreus* هو 20جم/لتر .

7- تم عمل نموذج رياضى للعلاقة بين المواد المثبطة الموجودة فى محلول التحليل المائى الحامضى الخام ونمو فطر الـ *A. terreus* وكانت النتائج كالتالى :

I- تم افتراض أن محلول التحليل المستخلص يوازى فى قدرته التخميرية محلول السكر النقى وبذلك يمكن حساب معدل النمو (specific growth rate , μ) تبعا للمعادلة الآتية :

$$(\mu_s)_2 = \mu_{max} \frac{S}{K_s + S} \quad \text{.....(1)}$$

وبالنسبة لمحلول التحليل الخام أضيفت للمعادلة السابقة جزء خاص بتأثير المواد المثبطة الناتجة من عملية التحليل المائى والموجودة فى هذا التحليل

$$(\mu s)_1 = \mu max \frac{S}{Ks + S} . \left(\overline{\frac{I}{I max}} \right) \quad \text{..... (2)}$$

ب- تم حساب القيم K_s , μ_{max} من سلسلة من تجارب التخمير لمحلول التحليل المستخلص وكانت القيم لهذه الثوابت هى $K_s = 4.76$ (g/l) U_{max} ,$= 0.212\ h^{-1}$

جـ- أمكن رياضيا حساب قيمة (n) فى المعادلة (2) حيث وجد أنها تساوى 0.404 مما يمكن معه حساب قيمة $(\mu_s)_1$ نظريا .

8- النموذج الرياضى المقترح يمكن عن طريقه حساب معدلات التخمير الحقيقية لمحاليل ناتج التحليل المائى للمخلفات السليلوزية فى وجود مواد التثبيط الناتجة من عملية التحليل المائى نتيجة هدم السكر واللجنين.