U. of K. J. Agric. Sci. 17(1),84-99, 2009

Production of Fuel Ethanol from Molasses by Thermotolerant Yeast

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Abstract: A thermotolerant strain of the yeast *Kluyveromyces marxianus,* isolated from Kenana sugar factory in the Sudan, was used for the production of ethanol from molasses. Fermentations were carried out in a bioreactor with 10-litre working volume at three temperatures and three sugar concentrations in batch and at one temperature and three feeding rates in fed-batch processes. In the batch fermentations, the best results were obtained at 40°C and 20% sugar, where a maximum of 9.2% (w/v) ethanol concentration was produced in 30 hours with a yield of 90% of the theoretical and a maximum ethanol specific productivity of 0.65 g per gramme yeast and hour. In the fed-batch process at 40°C, the best results were obtained at 0.5 l/h feeding rate of a substrate with 400 g/l sugar. Under such conditions, the yeast produced up to 9.34% (w/v) ethanol with 91.6% of the theoretical yield in 14 hours of fermentation and a maximum specific ethanol productivity of 0.9 g per gramme yeast and hour.

Key words: Ethanol production; thermotolerant yeast; cane molasses

INTRODUCTION

Due to problems of environmental pollution and high oil prices and for political reasons, many countries in the world started to pay more attention to renewable energy. Ethanol from renewable biomass is a main target as a liquid energy source. In 2005, the world production of ethanol was 35.3 million tons, with Brazil (12.6 millions) and the U.S. (11.7 millions) as the main producers (Shapouri *et al*. 2006). In 2006, the U.S. production reached about 14.5 million tons and was estimated to reach

about 20 million tons by early 2007 (Shapouri *et al*. 2006). It is estimated that 80% of ethanol produced worldwide is used as fuel (Berg 2004).

The net energy balance of making fuel ethanol from corn grain in the U.S. is 1.34, that is for every unit of energy that goes into growing corn and turning it into ethanol, we get back about one-third more energy as automotive fuel. This balance can be increased to 2.09 by improving farming and fermentation practices. On the other hand, the energy balance for cellulosic bioethanol is estimated at 2.6 (USDE 2007). Factors that affect ethanol productivity include control of the production process and improved yeasts. Innovations being tested include improved feedstock, more robust yeasts that tolerate higher temperatures and ethanol levels, and improved fermentation processes to increase ethanol concentrations (Novozymes and BBI International 2005).

Most ethanol fermentations are carried out at 30°C using the mesophilic yeast *Saccharomyces cerevisiae* (Hacking *et al*. 1984). Fermentations at temperatures of 40°C° and above bring many advantages such as decreased cost of cooling, easier handling in regions with warmer climate and fewer problems with yeast contamination (Einsele 1983) Many workers reported about thermotolerant yeasts used for ethanol production. *Kluyveromyces marxianus* var. *marxianus* 974 and 977 produced 6% to 6.5% (w/v) ethanol at 43°C from 19° Bx. cane syrup (Anderson *et al*. 1986). A strain of *Saccharomyces diastaticus* produced 7% (w/v) ethanol from 20% glucose at 40°C (D'Amore *et al.* 1989) and *Kluyveromyces marxianus* L.G. produced 3.76% (w/v) ethanol at 42°C on glucose medium (Ballesterose *et al*. 1991). *Kluyveromyces marxianus* DMKU 3-1042 produced 8.7% (w/v) ethanol at 40°C from sugar cane juice containing 22% sugar (Limtong *et al*. 2007). An overall yield above 90% of the theoretical is regarded as a target of improvement in ethanol productivity (Hettenhaus 1998).

Sugarcane is one of the crops that are grown in the Sudan with great success. The country has now five operating sugar factories that produce about 712 thousand tons of cane sugar annually, and two factories are under construction (MOI 2008).

This study was undertaken to investigate the optimal conditions for the production of ethanol from cane molasses using a strain of thermotolerant yeast isolated from the mills in a sugar factory in the Sudan.

MATERIALS AND METHODS

The yeast strain

The yeast strain used in this study was isolated from the clarified juice in Kenana sugar factory (Sudan). It was selected among 200 yeast isolates made in a screening programme that covered different locations in the country, and was identified as *Kluyveromyces marxianus* (unpublished work). The yeast was kept in the refrigerator on PDA slants.

Fermentation experiments

The fermentation experiments were carried out in a bioreactor with 10-litre working volume (Bioengineering AG, Switzerland), equipped for measurement and control of temperature, pH, foam level, stirring rate and aeration rate.

Mineral medium and molasses substrate

The mineral medium used in all fermentations contained 4.0 g/l $(NH_4)_2SO_4$ and 0.4 g/l $(NH_4)_2PO_4$. After adjusting the pH to 4.5, the medium was autoclaved at 121°C, either in 1-litre flasks for 15 minutes or in the bioreactor for 30 minutes. Molasses was obtained from Kenana Sugar Factory in the Sudan. The molasses substrate was clarified by diluting molasses with tap water (2:1), lowering the pH to 4.5 by H_2SO_4, heating to boiling, filtration over filter paper (Schleicher and Schuell, Nr. 589) with a vacuum pump and autoclaving at 121°C for 15-30 minutes according to volume.

Seed culture preparation

The seed culture of the yeast was first propagated for 24 hours in one litre shake flasks (containing 250 ml mineral medium + 250 ml molasses substrate) inoculated with loopfuls from slant cultures. The contents of 4

flasks were used to inoculate a 10-litre bioreactor to produce about 300 g yeast dry matter per run. Propagation was at 40°C, in fed-batch processes; the yeast was harvested by filtration, and the cake (with about 20% dry matter) was kept in the refrigerator till used.

Ethanol production in batch or fed-batch fermentations

The batch fermentations were carried out at 35°C, 40°C and 45°C and 15%, 20% and 25% sugar concentrations in 10-litre broth inoculated with 100 g yeast dry matter. The fed-batch processes were started with 5-litre mineral medium inoculated with 100 g yeast dry matter, and the molasses substrate (5 to 5.25 liters) containing 400 g/l sugar (1 kg molasses + 550 ml water) was fed to the bioreactor by pumping. All fed-batch fermentations were carried out at a temperature of 40°C and three substrate-feeding rates; namely, 0.25, 0.5 and 0.75 litres per hour. In both the batch and fed-batch processes, the bioreactor was aerated (0.5 vvm) at the beginning of fermentation for one hour to activate the yeast and stirred throughout at 200 rpm. Samples (10 ml) were taken at zero time and then every 1 to 2 hours to measure the concentrations of ethanol, sugars and the yeast biomass, and the fermentation was stopped when the sugar concentration reached zero or when ethanol concentration remained constant or decreased**.**

Chemical analysis

Ten milliliter samples were drawn from the bioreactor for chemical analysis. Yeast dry matter was determined by centrifugation of the samples and drying overnight in an oven at 105°C. The supernatant from the centrifugal tubes was used for ethanol and sugar determination, which were measured enzymatically using the UV-method (Boehringer, Mannheim) and a spectrophotometer (type PMG, Zeiss, Germany) at 340 nm.

RESULTS AND DISCUSSION

Ethanol production in batch fermentations

At the fermentation temperature of 35°C and sugar concentrations of 15% and 20%, the maximum ethanol concentrations produced were 7.2% and

9.3% (w/v), representing 94% and 91% of the theoretical yields, respectively (Fig. 1). At 40°C, maximum ethanol concentrations and yields at the same sugar concentrations were 7.2% and 9.2% (w/v), and 94% and 90%, respectively (Fig. 2). At both temperatures, no sugar was detected in the bioreactor by the end of fermentation, indicating that the yeast was able to consume it completely. At 25% sugar, the yeast produced a maximum of 8.9% (w/v) ethanol at 35°C and 8.8% (w/v) at 40°C, representing 69.8% and 69.0% of the theoretical yield, respectively (Figs. 1 and 2), and about 60 g/l sugar remained unconsumed in the fermentation broth (results not shown). The relatively high osmotic pressure at this sugar concentration seemed to have negatively affected ethanol production and tolerance of the yeast. At the fermentation temperature 45°C, maximum ethanol concentrations were 6.6%, 6.4% and 6.2% (w/v), representing 86.3%, 62.7% and 48.6% of the theoretical yields at the sugar concentrations 15%, 20% and 25%, respectively (Fig. 3), and about 10, 70, and 110 g/l sugars, respectively, remained in the broth (results not shown). This result indicated that the yeast under study can produce a maximum of up to 6.6% (w/v) ethanol at this high temperature.

Ethanol specific productivity decreased with time as ethanol concentration in the broth increased (Table 1). The initial specific productivity was highest at 40°C with values of 0.65, 0.62 and 0.55 g ethanol per gramme of yeast and hour for the sugar concentrations 15%, 20%, and 25%, respectively. At 35°C, the initial specific productivities were 0.59, 0.57 and 0.50 g/g.h and at 45°C, they were 0.52, 0.48 and 0.43 g/g.h for the three sugar concentrations, respectively. Fermentation times to reach maximum ethanol concentration at 35°C were 24 hours at 15% sugar, 34 hours at 20%, and 38 hours at 25%, whereas at 40°C, the times were 20, 30, and 36 hours, and at 45°C, they were 28, 30, and 32 hours, respectively. Fermentation times at 45°C were actually longer than both other temperatures, because the yeast had less ethanol specific productivity and hence similar concentrations were reached in longer times. These results indicate that ethanol yields at the fermentation temperatures 35°C and 40°C were about the same, while the yields at

45°C were much lower, but fermentation times at 40°C were shorter. Fermentation temperature 40°C can be regarded as the best.

Ethanol production in fed-batch fermentations

The fed-batch fermentations were run at 40°C, which was the best temperature as indicated by the results of the batch experiments. The substrate feeding rates were 0.25, 0.50, and 0.75 litres per hour from a substrate with 400 g/l sugar.

At the feeding rate 0.25 l/h, the maximum ethanol concentration obtained was 92.9 g/l from a total of about 2000 g sugar fed to the bioreactor (5 litres substrate) making about 91% of the theoretical yield (Fig. 4). Ethanol productivity was constant throughout the fermentation time ranging between 45 and 49 g ethanol per hour and between 0.41 and 0.45 g ethanol per gramme of yeast and hour (Table 2). The maximum ethanol concentration was reached, at the end of the substrate feeding, after 20 hours and sugar concentration in the bioreactor was about zero throughout the fermentation time (results not shown). The yeast biomass increased in the first 7 hours to 118 g, then decreased slightly and gradually until it reached 103 g at the end of fermentation (Table 2).

At the feeding rate 0.5 l/h, the maximum ethanol concentration was 93.4 g/l after 14 hours of fermentation from a total of about 2000 g sugar fed to the bioreactor (5 litres substrate) making about 91.6% of the theoretical yield (Fig. 4). The maximum ethanol specific productivity was 0.90 g per gramme of yeast and hour, which decreased gradually and continuously as ethanol concentration in the bioreactor increased (Table 2). Total ethanol productivity per hour remained constant in the first 5 hours of fermentation at 94 to 95 g, because yeast biomass increased during this time, then it decreased towards the end of fermentation as yeast growth stopped (Table 2). Sugar concentration was about zero in the first 4 hours of fermentation, then started to increase, reaching a maximum of about 20 g/l at the end of substrate feeding after 10 hours, and then decreased again to undetectable amounts by the end of fermentation (results not shown).

Yeast biomass increased to 119 g in the first 5 hours, and then decreased to 100 g at the end of fermentation (Table 2).

At the feeding rate 0.75, the maximum ethanol concentration reached was 91.9 g/l after 15 hours of fermentation from a total of about 2100 g sugar fed to the bioreactor (5.25 litres substrate) making about 85.8% of the theoretical yield (Fig. 4). The maximum ethanol specific productivity was 0.92 g per gramme of yeast and hour, which decreased gradually and continuously as ethanol concentration in the bioreactor increased (Table 2). The specific productivity of 0.92 g/g.hr seems to be the absolute maximum for this yeast under these conditions since some of the sugar fed to the bioreactor remained unassimilated. Total ethanol productivity per hour remained constant in the first 3 hours of fermentation at 97 to 98 g, because yeast biomass increased during this time, then it decreased towards the end of fermentation as yeast growth stopped (Table 2). Sugar started to accumulate from the beginning of fermentation and reached a maximum of about 72.2 g/l at the end of substrate feeding after 7 hours, and then decreased again to 14.7 g/l by the end of fermentation (results not shown). Yeast biomass increased to 120 g in the first 7 hours, and then decreased to 100 g at the end of fermentation (Table 2).

It is obvious that the feeding rate of 0.25 l/h was low, the yeast did not reach its maximum ethanol specific productivity and the fermentation time was long compared to the other two feeding rates. At the feeding rate 0.5 l/h, the yeast reached a specific productivity near maximum, the fermentation time was shortest and the yield highest. At the feeding rate 0.75 l/h, the absolute maximum specific productivity was reached, but the fermentation time was longer than at 0.5 l/h and the yield was much lower. These results indicate that the feeding rate of 0.5 l/h was the best.

It can be concluded that the thermotolerant yeast under study has an optimum ethanol fermentation temperature around 40°C. It produced up to 9.3% (w/v) ethanol in batch and fed-batch fermentations representing more than 90% of the theoretical yields. Fed-batch fermentations took

shorter time, because the specific ethanol productivity was highest. The feeding rate of 0.5 l/h with a substrate of 400 g/l sugar concentration was the best. At 45°C, still up to 6.6% (w/v) ethanol was produced by this yeast.

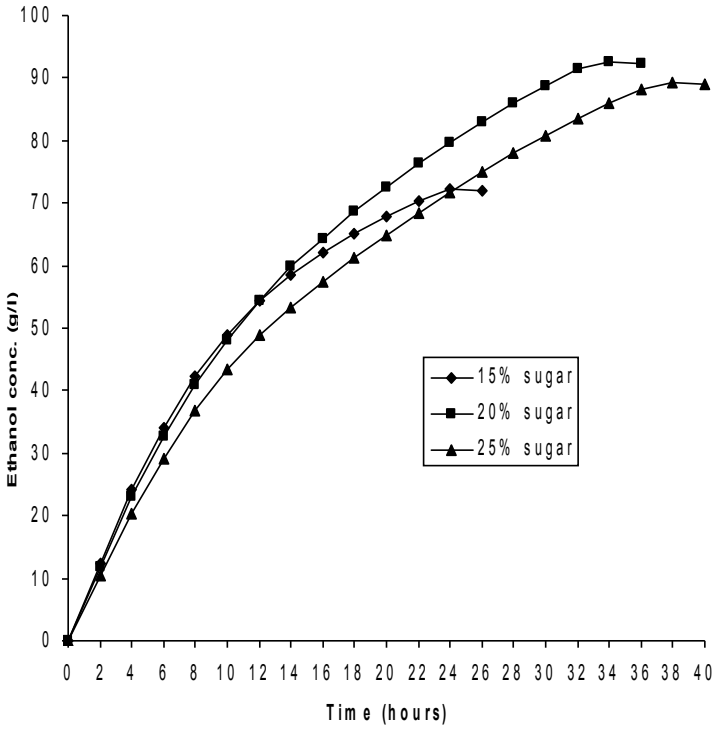

Fig. 1: Ethanol production at 35°C in batch fermentation

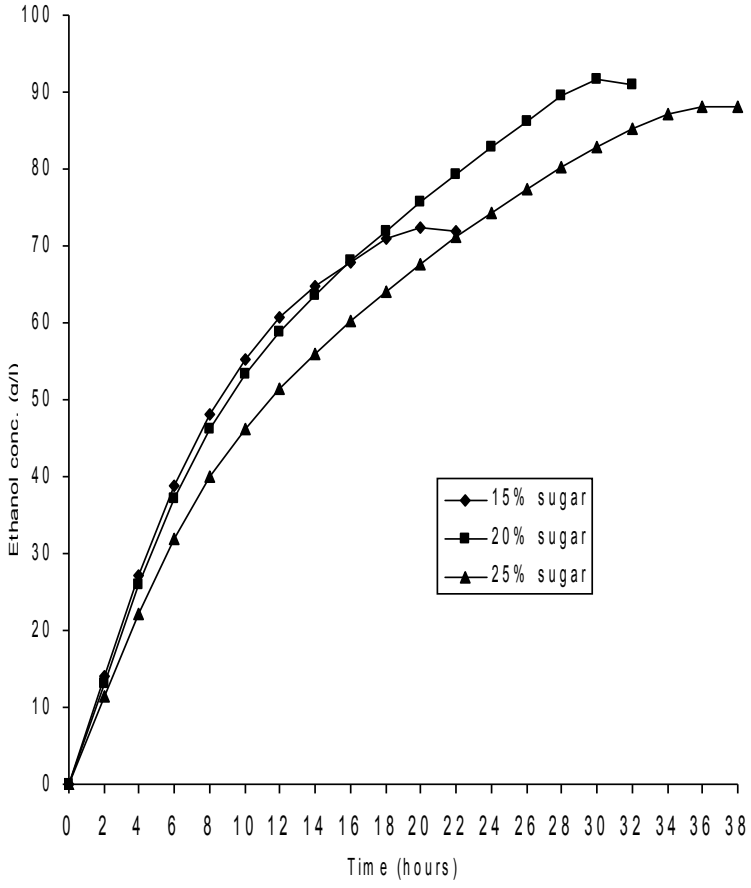

Fig. 2: Ethanol production at 40°C in batch fermentation

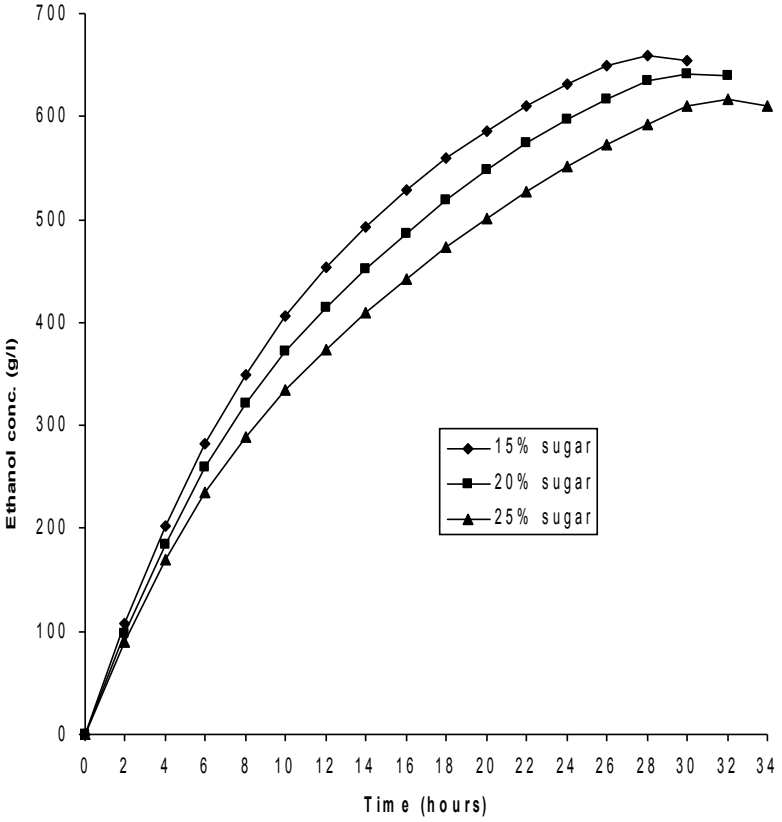

Fig. 3: Ethanol production at 45°C in batch fermentation

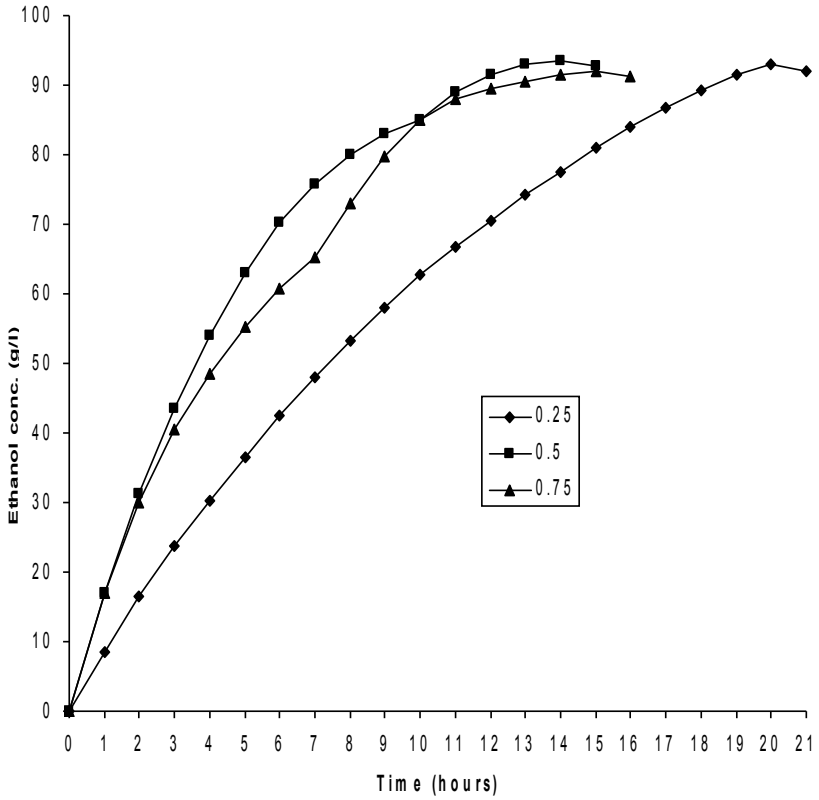

Fig. 4: Ethanol production at 40°C in fed-batch fermentation

Table 1. Ethanol specific productivity of the thermotolerant yeast at three temperatures in batch fermentations

Time (hr)	Ethanol specific productivity (g/g.hr)								
	35°C			40°C			45°C		
	15%	20%	25%	15%	20%	25%	15%	20%	25%
2	0.59	0.57	0.5	0.65	0.62	0.55	0.52	0.48	0.43
4	0.53	0.51	0.46	0.6	0.59	0.5	0.45	0.42	0.38
6	0.46	0.44	0.42	0.54	0.53	0.47	0.39	0.36	0.32
8	0.39	0.4	0.37	0.44	0.43	0.38	0.33	0.31	0.27
10	0.33	0.35	0.33	0.37	0.35	0.31	0.28	0.26	0.22
12	0.27	0.3	0.27	0.27	0.28	0.27	0.23	0.21	0.2
14	0.21	0.27	0.23	0.2	0.24	0.23	0.2	0.19	0.18
16	0.18	0.23	0.2	0.16	0.22	0.22	0.18	0.18	0.17
18	0.16	0.21	0.19	0.16	0.21	0.2	0.16	0.16	0.16
20	0.14	0.2	0.19	0.08	0.19	0.19	0.14	0.15	0.15
22	0.13	0.19	0.18		0.18	0.18	0.13	0.14	0.14
24	0.09	0.18	0.18		0.18	0.18	0.12	0.12	0.13
26	0	0.16	0.17		0.18	0.16	0.1	0.11	0.12
28		0.15	0.16		0.17	0.15	0.05	0.09	0.11
30		0.15	0.15		0.1	0.14	0	0.04	0.09
32		0.14	0.14			0.13		0	0.04
34		0.07	0.13			0.11			0
36		0	0.12			0.05			
38			0.07						
40			0						

* Sugar concentrations

Table 2. Ethanol production (EtOH), total yeast biomass and ethanol specific productivity (Ps) of the thermotolerant yeast in fed batch fermentations at three different substrate-feeding rates

Time (hr)	0.25[1]			0.5[1]			0.75[1]		
	EtOH (g/hr)	Yeast biomass (g)	Ps (g/g.hr)	EtOH (g/hr)	Yeast biomass (g)	Ps (g/g.hr)	EtOH (g/hr)	Yeast biomass (g)	Ps (g/g.hr)
0	0	100	0	0	100	0	0	100	0
1	45	104	0.43	94	105	0.9	98	106	0.92
2	46	107	0.43	94	110	0.85	97	112	0.87
3	46	110	0.42	95	114	0.83	98	115	0.85
4	45	114	0.39	95	117	0.81	96	120	0.8
5	47	116	0.41	94	119	0.79	95	121	0.79
6	47	118	0.4	90	118	0.76	94	120	0.78
7	48	118	0.41	82	118	0.69	90	120	0.75
8	49	117	0.42	75	116	0.65	80	118	0.68
9	48	117	0.41	70	117	0.6	70	118	0.59
10	49	115	0.43	60	117	0.53	54	116	0.47
11	48	113	0.42	40	115	0.36	30	116	0.26
12	47	110	0.43	25	110	0.23	15	114	0.13
13	48	110	0.44	15	108	0.14	10	115	0.09
14	47	107	0.44	5	105	0.05	10	114	0.09
15	48	108	0.44	0	100	0	5	110	0.05
16	49	109	0.45				0	106	0
17	46	106	0.43				0	100	0
18	45	106	0.42						
19	43	105	0.41						
20	38	105	0.36						
21	0	103	0						

1 = substrate feeding rates (l/h)

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مجلة جامعة الخرطوم للعلوم الزراعية: المجلد السابع عشر– العدد الاول، 2009م

انتاج الكحول الايثيلى من المولاس باستعمال خميرة متحملة للحرارة

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موجز البحث: استعملت فى هذه الدراسة سلالة من خيرة *Kluyveromyces marxianus* لانتاج الكحول الايثيلى من المولاس، وهى خميرة متحملة للحرارة، عزلت من مصنع سكر كنانه فى االسودان. أجريت تجارب التخمير فى مخمر سعة 10 لتر على ثلاث درجات حرارة و ثلاثة تركيزات سكر فى حالة التخمير المتقطع، وعلى درجة حرارة واحدة وثلاثة معدلات تغذية بالوسط فى حالة التخمير شبه المستمر. فى حالة التخمير المتقطع، كانت أفضل النتائج عند 40°C و 20% تركيز سكر، حيث بلغ التركيز الأعلى للكحول (w/v) 9.2% فى مدة تخمير حوالى 30 ساعة، و كانت كمية الحصاد 90% من القيمة النظرية و أقصى انتاجية نوعية من الكحول كانت 0.65 جرام من جرام خميرة واحد فى الساعة الواحدة. فى حالة التخمير شبه المستمر على 40°C، كانت أفضل النتائج على معدل تغذية 0.5 لتر فى الساعة من وسط بتركيز 400 جرام سكر فى اللتر فى هذا الوضع، كان أعلى تركيز كحول أنتجته الخميرة (w/v) 9.34% ، وحصاد يعادل 91.6% من النظرى فى مدة نخمير 14 ساعة وأعلى انتاجية نوعية 0.9 جرام. كحول من جرام خميرة فى الساعة الواحدة.