STUDIES ON FERMENTATION, ALCOHOL PRODUCTION AND VIABILITY IN INDUCED MUTANTS OF THE BREWING YEAST *SACCHAROMYCES CEREVISIAE*.

G. A. AKPAN, LUM AYEOFFE FONTEM and I. R. UDOTONG

ABSTRACT

Brewing yeast, *Saccharomyces. cerevisiae* cultured on malt extract agar was UV-irradiated. Eighteen mutant yeasts (SCM 01 – SCM 18) selected (after visual examination) were tested for fermentation, alcohol production and viability by acid and gas production, reduction in specific gravity and turbidity respectively, with sugars and delignified sugarcane bagasse. The yeasts had varied fermentation profiles in glucose, sucrose, galactose, dextrose and mannitol but did no ferment lactose. Glucose, sucrose and galactose were fermented strongly with acid and gas production. Dextrose was fermented weakly by SCM 01, SCM 06, SCM 09 and SCM 10 (with acid production only) but strongly by all other yeasts. The wild type yeast did not ferment mannitol but SCM 04 did so strongly and SCM 02, SCM 03, SCM 06, SCM 07, SCM 09, SCM 10 and SCM 13 did so weakly. Specific gravity fell sharply within 36h after inoculation and slightly thereafter. Alcohol production varied among the yeasts. Some mutants produced more alcohol than the wild type yeast. Viability was generally lower in the mutants than in the wild-type yeast in the sucrose medium. The reverse was true in the sugarcane bagasse medium. Yeasts with high viability tended to have high alcohol production ability in the sucrose medium and vice-versa.

KEYWORDS: Alcohol production; fermentation; induced mutants; *Saccharomyces cerevisiae;* viability.

INTRODUCTION

Saccharomyces cerevisiae is perhaps the most economically important yeast species and has been extensively studied (Stewart, 1981). The yeasts have been much exploited by man to produce foods, beverages and medicines. Over one million metric tones of yeast are produced annually and over two million metric tones of alcohol are produced from all-purpose fermentations involving yeasts (Sikyta *el al.,* 1986). Apart from alcohol production, yeasts also play an important role in the fermentation processes of many African foods (Faparusi et al., 1973; Okafor, 1977; Okagbue, 1988; Oyewole and Odunfa, 1988; Sanni, 1985).

As the need to develop the science of fermentation gains significance, there has been a call for the exploration of ways to improve the capabilities of the yeasts (Stewart, 1981). Classical genetic techniques include the isolation of spontaneous and induced mutants. Latter day techniques include recombinatinant DNA and protoplast fusion. In keeping with the objective of improving the capabilities of the yeasts, the brewing yeast, *Saccharomyces cerevisiae* was exposed to ultra-violet radiation and the resultant mutants were selected and tested for fermentation, alcohol production and viability.

MATERALS AND METHODS

Brewing yeast, *Saccharomyces cerevisiae* from the Champion Brewery. Plc Uyo, Nigeria was collected into a sterile bottle from the Champion Brewery. Plc Uyo, Nigeria and stored at 5°C.

One milliliter of the yeast was aseptically transferred into a test-tube containing 9ml distilled water and was serially diluted down to 10^{-5}. Exactly 0.1 ml of the aliquot from 10^{-3}, 10^{-4} and 10^{-5} dilutions were pipetted and inoculated into sterile plates of malt extract agar (Oxoid UK Ltd.) by the spread plate method. The plates were incubated at 28°C for 48h to form the primary culture. The yeasts were than propagated in test-tubes containing yeast extract glucose broth (Oxoid) and incubated at 28°C for 48h.

Serial dilutions of the culture in the yeast extract glucose broth medium were made down to 10^{-7} and then 0.1ml aliquot from 10^{-7} was pipetted into two sets of petri-dishes. One set was placed in an irradiation chamber and UV-irradiated for 10min following a modification of the method reported by Harm. (1968). Molten malt extract agar was immediately poured into the petri-dishes and these were incubated in the dark at 28°C for 48h. The un-irradiated set was the wild-type yeast, which served as the control. After the incubation period, the colonies were visually evaluated and 18 mutant colonies (SCM 01 – SCM 18) were randomly selected and their fermentation, alcohol producing abilities as well as viability were tested.

G. A. AKPAN, Department of Botany, Horticulture and Microbiology, University of Uyo, Uyo - Nigeria
LUM AYEOFFE FONTEM, Department of Botany, Horticulture and Microbiology, University of Uyo, Uyo - Nigeria
I. R. UDOTONG, Department of Botany, Horticulture and Microbiology, University of Uyo, Uyo - Nigeria

Fermentation tests

The basal medium for the fermentation tests was prepared by dissolving 50g of the sugar (analar grade), 3g of yeast extract agar (Oxoid) and 5g of peptone (Oxiod) in 1000 ml of distilled water. The sugars used for the test were glucose, sucrose, galactose, lactose, mannitol and dextrose, the hydrated form of glucose.

Ten milliliters of the basal medium were pipetted into test tubes and inverted Durham's tubes were placed in them. This set-up was sterilized at 121°C for 15min. After cooling, two drops of phenol red indicator were added and the medium was inoculated with 1.0ml of the yeasts and incubated at 28°C. It was then observed for acid and gas production. A change in colour indicated acid production while gas production was indicated by the displacement of the liquid in the Durham's tube.

Alcohol production and viability test

Alcohol production was tested with sucrose and delignified sugarcane bagasse. The sugarcane bagasse was being tried out as a new material for alcohol production by the yeast; consequently only the wild type and two mutant yeasts were tested with it. The medium for sucrose was prepared as described for the fermentation tests. Delignified sugarcane bagasse was prepared as follows: The sugarcane bagasse was thoroughly washed with tap water and tested for the absence of sugar with iodine. It was then soaked in 1.0% sodium hydroxide and autoclaved at 121°C for 15minutes. It was further washed thoroughly with tap water and tested for the absence of lignin with phenolthalein. The delignified bagasse was then dried in the oven at 50°C. For the preparation of the delignified sugarcane bagasse medium, 3g of the dried delignified sugarcane bagasse was added to 100ml of the mineral salts medium in a 250ml conical flask. The mineral salt medium was prepared by mixing 0.11 KH_2PO_4, 0.65g K_2HPO_4, 0.5g NH_4NO_3, 0.2g $MgSO_4$. $7H_2O$, 0.025g $CaCl_2$. H_2O, 2.5mg $FeCl_2$. $6H_2O$, 1.0mg $MnCl_2$. $4H_2O$ a..d 3.5mg$ZnSO_4$. $7H_2O$ in 1000ml of distilled water.

Both media were inoculated with 1.0ml of the 48h old broth cultures of the yeasts and incubated for 48h. Their specific gravity was measured at the beginning and at 12h, 18h, 24h, 42h and 48h after inoculation and autoclaved at 121°C for 15minutes. The percentage reduction in specific gravity was calculated 48h after inoculation. Percentage reduction in specific gravity was calculated as:

$$\frac{\text{Initial specific gravity-Final specific gravity}}{\text{Initial specific gravity}} \times 100\%$$

The specific gravity was measured using the density gravity bottle method at 20°C. The specific gravity was calculated as:

$$\frac{W3 - W1}{W2 - W1}$$

Where W1 = weight of dry empty bottle

W2 = weight of bottle filled with 50ml of water

W3 = weight of bottle filled with 50ml of broth culture.

The turbidity of the media was measured using an EIL6010 turbidometer at the beginning and at the end of 48h of inoculation. The data obtained was used to calculate the difference in turbidity of the media within the period. The experiments were replicated three times.

The data obtained were exposed to one way analysis of variance and where this was significant, the means were separated using the least significant difference (L.S.D) procedure. A correlation analysis was done for mean percentage reduction in specific gravity and mean difference in turbidity.

RESULTS

Fermentation

The wild-type and mutant yeasts were all able to ferment glucose, sucrose and galactose with the production of acid and gas. They also produced acid and gas from dextrose except SCM 01, SCM 06, SCM 09 and SCM 10, which produced acid only. The wild-type yeast did not ferment mannitol whereas SCM 04 fermented it with the production of acid and gas and SCM 01, SCM 02, SCM 03, SCM 06, SCM 07, SCM 09, SCM 10 and SCM 13 fermented it with the production of acid only. Lactose was not fermented by the wild-type and mutant yeasts (Table 1).

The yeasts fermented the sugars after different lengths of incubation time and thus exhibited varied fermentation profiles for the different sugars (Table 2). The fermentation profiles in glucose and sucrose were the same and fermentation was begun within 24h after inoculation with the wild-type and mutant yeasts. Their fermentation profiles in galactose, dextrose and mannitol were different from each other and from that in glucose and sucrose. Some yeast mutants did not begin to ferment galactose and mannitol until more than 36h after inoculation. SCM 02 began to ferment mannitol within 12h after inoculation. Dextrose was fermented by all the yeasts within 24h after inoculation.

Alcohol production and viability

There was a general fall in specific gravity of the media within 12h after inoculation of the wild-type and mutant yeasts. The fall was slight from about 36h after inoculation. (Fig. 1 and 2).

In the sucrose medium, the gradient of fall in specific gravity for the mutant yeasts SCM 03, SCM 04, SCM 15 and SCM 16 was steeper than for the other yeasts (Table 3). In the sugarcane bagasse medium, the gradient of fall in specific gravity for the mutant yeast SCM 04 was steeper than for SCM 18 and the wild-type yeast (Table 3).

The one-way analysis of variance for the percentage reduction in specific gravity 48h after inoculation in both the sucrose medium and the sugarcane bagasse medium indicated very highly significant differences between the yeasts (p<0.001). When the mean values for the mutant yeasts were compared with that of the wild-type yeast in the sucrose medium, those of the mutant yeasts SCM 02, SCM 04, SCM 07, SCM 08, SCM 11, SCM 12, SCM 13, SCM 15 and SCM 16 were significantly greater than that of the wild-type yeast. Those of the other mutants were significantly less than that of the wild-type yeasts, except SCM 09 and SCM 10, which were the same (Table 3
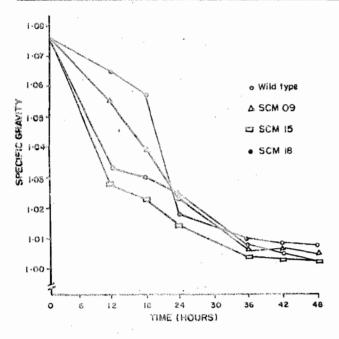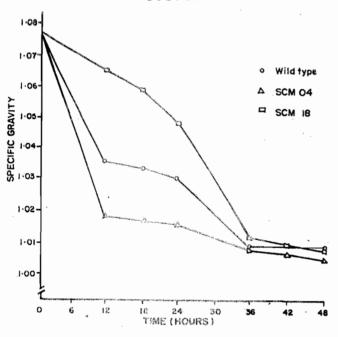
FIG. 1:

FIG. 2:

Table 1: Summary of the fermentation activities of the wild-type and mutant yeasts in the different sugar media.

Sugar	Wild-type	SCM01	SCM02	SCM03	SCM04	SCM05	SCM06	SCM07	SCM08	SCM09	SCM10	SCM11	SCM12	SCM13	SCM14	SCM15	SCM16	SCM17	SCM18
Glucose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Sucrose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Galactose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Dextrose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Mannitol	O	A	A	A	O	O	A	A	O	A	A	O	A	O	O	O	O	O	O
Lactose	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

AG = fermentation with the production of acid and gas
A = fermentation with the production of acid only
O= no fermentation.

(a)). In the sugarcane bagasse medium the mean percentage reduction in specific gravity of the mutant yeasts, SCM 04 and SCM 18 was significantly greater than that of the wild-type yeast (Table 3 (b)).

Table 4 shows the mean turbidity at 0h and 48h after inoculation and the mean difference in turbidity of the yeasts in the sucrose and sugarcane bagasse media. The on-way analysis of variance for the mean difference in the turbidity of the medium 48h after inoculation indicated very highly significant differences between the yeasts ($p < 0.001$). In the sucrose medium, the mean values for the mutant yeasts were significantly less than that of the wild-type yeast (Table 4(b) (i). In the

Table 2: Fermentation profiles of the wild-type and mutant yeasts in the different sugar media.

Sugar	0 – 12	13 – 24	25 - 36	Over 36
		Time (hours) after inoculation		
Glucose	.	SCM 16, SCM 15, SCM 11, SCM 10, SCM 13, SCM 04, Wild-Type. SCM 06, SCM 18, SCM 02, SCM 01, SCM 14, SCM 05, SCM 17. SCM 08, SCM 07, SCM 12, SCM 03, SCM 09		
Sucrose	-	SCM 16, SCM 15, SCM 11, SCM 10, SCM 13, SCM 04, Wild-Type, SCM 06, SCM 18, SCM 02, SCM 01, SCM 14, SCM 05, SCM 17, SCM 08, SCM 07, SCM 12, SCM 03, SCM 09		
Galactose	-	SCM 03, SCM 06, SCM 07, SCM 08, SCM 10, SCM 12, SCM 18, Wild-Type. SCM 05, SCM 15, SCM 02, SCM 09	SCM 13, SCM 04, SCM 01, SCM 11, SCM 14, SCM 17,	SCM 16
Dextrose	-	SCM 14, SCM 16, SCM 18, SCM 09, SCM 07, SCM 04, Wild-Type, SCM 03, SCM 11, SCM 08. SCM 02, SCM 17, SCM 06, SCM 12, SCM 15. SCM 13, SCM 10. SCM 01, SCM 05.		
Mannitol	SCM02	SCM 04	*SCM 03, SCM 06. SCM 07. SCM 09, SCM 10.	*SCM01, SCM13
Lactose	-	.		

*The first recorded yeast in each time grouping was the fastest in starting fermentation followed consecutively by the others

sugarcane bagasse medium, both mutant yeasts, SCM 04 and SCM 18, had a greater mean difference in turbidity than the wild-type yeast. (Table 4(b) (ii)).

The correlation between the mean percentage reduction in specific gravity and the mean difference in turbidity of the yeast in the sucrose medium was positive and not significant ($r = 0.0158$ at 17 degree of freedom).

DISCUSSION

The wild-type and mutant yeasts fermented glucose, sucrose, dextrose and galactose with acid and gas production with varied fermentation profiles but did not ferment lactose (Table 1 and Table 2). The wild-type yeast and the yeast mutants, except SCM 01, SCM 02, SCM 03. SCM 04, SCM 07, SCM 09 and SCM 13 did not ferment mannitol; SCM 04 fermented it with acid and gas production. Acid production alone indicates weak fermentation while acid and gas production together indicate strong fermentation. It is probable that the effect of UV-irradiation created a new pathway or enzyme system that enabled these mutant yeasts to ferment mannitol. The observed differences in the fermentation profile of the mutants is probably due to genetic differences among them.

The observation that the wild-type yeast had a steep gradient of fall in specific gravity soon after inoculation (Fig 1 and Fig. 2) suggests that the behaviour of those yeast mutants which had such a fall in specific gravity within 12h after inoculation was normal. Those yeast mutants in which the steep gradient of fall in specific gravity was delayed beyond 24h after inoculation exhibited abnormal behaviour. Mutant yeast SCM 04 behaved normally in both the sucrose and sugarcane bagasse media while mutant yeast SCM 18 behaved abnormally in both media.

The slight fall in specific gravity of the media 36h after inoculation (Fig. 1 and Fig. 2) was probably due to loss of viability of yeast cells as the alcohol content of the media increased.

UV-radiation like the other ionizing radiations has similar effects as the chemical mutagenic compounds (Amer and Ali, 1968; Crocker 1953; Darlington and Mcleish, 1951; George and George, 1978; Sach and Lang, 1960). Chromatin assembly is changed in some way in yeast mutants and causes increased levels of DNA damage. Such cells do not complete mitosis until the damage is repaired (Turner, 1995) In the cells carrying such damage, check points or mechanisms for making sure that all the necessary

Table 3: Fall/reduction in specific gravity at various times during 48h after inoculation with the wild-type and mutant yeasts in (a) sucrose medium (b) Sugarcane bagasse medium.

Hours after inoculation	Wild-type	SCM01	SCM02	SCM03	SCM04	SCM05	SCM06	SCM07	SCM08	SCM09	SCM10	SCM11	SCM12	SCM13	SCM14	SCM15	SCM16	SCM17	SCM18	LSD (P=0.05)
(a) 0	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	1.073	
12	1.033	1.042	1.047	1.025	1.025	1.032	1.043	1.038	1.052	1.055	1.052	1.058	1.062	1.054	1.028	1.028	1.043	1.065		
18	1.030	1.038	1.044	1.017	1.017	1.028	1.024	1.033	1.047	1.039	1.050	1.049	1.057	1.043	1.027	1.023	1.037	1.054		
24	1.025	1.034	1.033	1.016	1.015	1.027	1.022	1.027	1.024	1.023	1.047	1.047	1.055	1.035	1.026	1.022	1.027	1.018		
36	1.008	1.009	1.010	1.010	1.010	1.011	1.008	1.010	1.008	1.007	1.007	1.010	1.013	1.010	1.005	1.005	1.005	1.016		
42	1.005	1.009	1.010	1.007	1.007	1.009	1.007	1.007	1.006	1.006	1.006	1.008	1.006	1.006	1.005	1.004	1.004	1.010		
48	1.005	1.006	1.004	1.007	1.004	1.0007	1.007	1.006	1.005	1.006	1.003	1.010	1.006	1.009	1.004	1.004	1.004	1.010		
% Reduction	6.34	6.24	6.43	6.15	6.43	6.15	6.24	6.34	6.24	6.24	6.52	5.87	6.24	5.96	6.43	6.43	5.87	6.06		0.03
(b) 0	1.075				1.075														1.075	
12	1.035				1.018														1.065	
18	1.033				1.017														1.059	
24	1.030				1.016														1.049	
36	1.009				1.008														1.012	
42	1.009				1.006														1.009	
48	1.009				1.006														1.008	
% Reduction	6.14				6.42														6.23	0.10

events for cell cycle progression occur in the set order and the satisfactory completion of earlier events before later ones begin, usually operate (Weinert et al., 1994). For example, Yakovenko and Formazyuk (1993) demonstrated that the onset of cell division is associated with an increase in diadenosine oligophosphates which peak at the beginning of the synthetic phase, acting as triggers of DNA replication. Also, in heat-stressed yeasts Jenkins et al (1997) demonstrated the accumulation of spingolipids during heat stress adaption.

Change in specific gravity can be used as a measure of alcohol content of the medium. During fermentation, the specific gravity is reduced indicating an increase in the alcohol content of the medium. The greater the reduction in specific gravity, the greater is the amount of alcohol produced (Hough et al., 1975). In the sucrose medium the mutant yeasts SCM 02, SCM 04, SCM 07, SCM 08, SCM 11, SCM 12, SCM 13, SCM 15 and SCM 16 had significantly greater percentage reduction in specific gravity than the wild-type yeast (Table 3(a)). These mutants therefore produced more alcohol than the wild-type yeasts. The other mutant yeasts that had significantly less percentage reduction in specific gravity produced less alcohol than the wild-type yeast. Similarly in the sugarcane bagasse medium, mutant yeast SCM 04 and SCM 18 produced more alcohol than the wild-type yeast (Table 3 (b)).

The change in the turbidity of the medium was used as a measure of the budding ability and hence the viability of the yeasts. As the yeasts degraded and utilized the sugar in the medium, they budded and multiplied in number and caused an increase in turbidity. In the sucrose medium, all the mutant yeasts, except SCM 15, had significantly less turbidity than the wild-type yeast and therefore had lower viability than the latter. Their viability was therefore impaired by the UV-irradiation. In the sugarcane bagasse medium both mutant yeasts SCM 04 and SCM 18 had significantly higher turbidity than the wild-type yeast and therefore had better viability than the latter (Table 4).

There were differences in the fermentation profile, in the mean percentage reduction in specific gravity and in the mean difference in turbidity of the yeasts (Table 2, Table 3 and Table 4). These suggest the existence of genetic variability among them. Such variability may be due to the fact that at the time of UV-irradiation the yeast cells may have been in different phases of the cell cycle since yeasts continually bud when growing in optimum conditions (Frobisher et al., 1974). Normally yeasts have an array of sucrase genes (Carlson & Bolstein 1983; Stewart et al., 1983). These genes may have been altered in the mutants leading to their lower viability in the sucrose medium: The altered genes enhance the viability of the mutants in the de-lignified sugarcane bagasse medium. These observations support the fact that wild type organisms are better adapted to their environment than mutants (Smith 1978, Olaoye, 1999), which may exploit novel environments better than the wild-type (Kwong-Ndung and Ifenkwe, 1999).

The correlation between the mean percentage

Table 4: (a) Mean values of turbidity in (i) Sucrose medium, and (ii) Sugarcane bagasse medium of the wild-type and mutant yeasts at 0h and 48h after inoculation. (b) Mean values of difference in turbidity in (i) Sucrose medium, and (ii) Sugarcane bagasse medium of the wild-type and mutant yeasts 48h after inoculation.

		Yeasts																		
		Wild-type	SCM 01	SCM 02	SCM 03	SCM 04	SCM 05	SCM 06	SCM 07	SCM 08	SCM 09	SCM 10	SCM 11	SCM 12	SCM 13	SCM 14	SCM 15	SCM 16	SCM 17	SCM 18	LSD (p<0.05)
(a) Turbidity	(i) 0h	0.38	0.85	0.42	0.85	0.85	1.00	0.45	0.90	0.90	0.75	1.00	0.68	0.68	0.60	1.10	0.50	0.50	0.54	0.75	
	48h	1.50	1.40	1.40	1.25	1.75	1.50	1.50	1.50	1.40	1.75	1.60	1.60	1.50	1.60	1.75	1.50	1.50	1.50	1.40	
	(ii) 0h	0.58				0.45														0.20	
	48h	0.85				1.50														0.54	
(b) Difference in turbidity	(i)	1.12	0.55	1.00	0.40	0.90	0.50	1.05	0.60	0.50	1.00	0.60	0.92	0.82	1.00	0.65	1.10	1.00	0.96	0.65	0.03
	(ii)	0.27				0.60														0.34	0.02

reduction in specific gravity and the mean difference in turbidity in the sucrose medium was positive and non-significant. This suggests that there was a tendency for the yeasts with a high difference in turbidity to have greater percent reduction in specific gravity. Carlson and Bolstein (1983) showed that yeasts contain up to six mutant sucrase genes, and Stewart et al., (1983) showed that the fermentation of sucrose becomes faster or slower depending on which of these genes are carried in the yeast.

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