IMPROVEMENT OF *SACCHAROMYCES CEREVISIAE* GCU-36 THROUGH INDUCED MUTAGENESIS FOR L-PHENYLACETYLCARBINOL PRODUCTION

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ABSTRACT: The present study deals with the improvement of a *Saccharomyces cerevisiae* strain GCU-36 for L-phenylacetylcarbinol (L-PAC) production through randomely induced mutagenesis. The wild culture gave 2.58 g/l L-PAC production. An enhancement in the production was, however observed when yeast cells were exposed to mutagenic agents viz. ultraviolet (UV) radiations, nitrous acid (HNO_2) and ethylmethane sulphonate (EMS). The mutant culture gave a maximal of 5.61 g/l L-PAC production which was over 2.17 fold improvement in terms of product formation. The outcome of an inferior mutant with declined L-PAC production level marked the termination of further mutagenesis.

Key words: Saccharomyces cerevisiae, random mutagenesis, shake flask culture, benzaldehyde, gas chromatography, L-phenylacetylcarbinol

INTRODUCTION

In phamaceutical industries, L-phenylacetylcarbinol (L-PAC) has been used as an intermediate in the production of drugs such as ephedrine and pseudoephedrine (Rosche *et al*., 2007). L-ephedrine is useful as a topical nasal decongestant and bronchodilator where as D-pseudoephedrine mainly works as an antiasthmatic agent. Commercialy, L-PAC is produced from *Saccharomyces cerevisiae* pyruvate decarboxylase (PDC)-mediated condensation of added benzaldehyde with acetaldehyde (Arasaratnam *et al*., 2001). Being a GRAS (generally regarded as safe) organism, *S. cerevisiae* has extensively been used for the purpose of random mutagenesis. Effective mutagenesis accompanied by successful mutant screening and selection results in the achievement of a potent strain (Snow 1967; Tuite and Cox, 1980). The rate of decarboxylation and carboligation processes of L-PAC mechanism increases when mutations impart positive effect on active site of PDC (Culic *et al*., 1984; Anonymous, 2007).

In the present paper, a yeast culture *Saccharomyces cerevisiae* GCU-36 (isolated from cane-molasses and preserved at the Culture Collection of IIB) was improved through random mutagenesis for L-PAC production. The cells were exosed to different mutagenic agents i.e. ultraviolet (UV) radiations, nitrous acid (HNO_2) and ethylmethane sulphonate (EMS) applying protocols of single, reciprocal or alternate mutagen (s).

MATERIALS AND METHODS

Organism: *Saccharomyces cerevisiae* strain GCU-36 was maintained on YPD-agar slopes containing (g/l): yeast extract 10, peptone 10, dextrose 20, agar 15 at pH 5.0 and stored at 4ºC in a cold cabinet (MPR-1410, Sanyo, Japan).

Inoculum Preparation: The cell suspension of *S. cerevisiae* GCU-36 (2 ml) was inoculated to 18 ml of YPD-medium containing (g/l): yeast extract 10, peptone 20, glucose 30 at pH 5.0 using a 100 ml Erlenmeyer flask. The flask was incubated in a rotary shaker (200 rpm) at 30°C for 8 h. The broth was re-inoculated to 180 ml of the same medium in a1000 ml Erlenmeyer flask and allowed to grow till the cell count reached to 105×10^6 cells/ml (cal. 3 %, w/v) after Shukla *et al*. (2001). The cells were recovered by centrifugation (9500×*g* for 15 min) and water washed twice.

Fermentation Procedure: Two hundred millilitre of GP-medium containing **(**g/l): glucose 50, peptone 6 at pH 5.0 was taken in a 1000 ml capacity Erlenmeyer flask and cotton plugged. It was autoclaved at 15 lb/in^2 pressure (121ºC) for 20 min. After cooling at an ambient temperature (20ºC), the medium was aseptically inoculated with 3 % (w/v) inoculum. The cells were allowed to adapt for approximately 1 h in a rotary shaking (200 rpm) incubator at 30°C. Five doses (each of 1.2 ml/l acetaldehyde + 1.20 ml/l benzaldehyde) were added at 1 h interval. Broth sample was collected for analysis after the last dose.

Analytical Techniques: Yeast cells were recovered from the broth by centrifugation (9500×*g* for 15 min) and supernatant was used to prepare analytical sample. The biotransformation sample (1.0 ml) was dispensed in 5.0 ml of dichloromethane using a screw-capped test tube and vortexed for 2-3 min till the appearance of two layers (organic and aquous). Two microlitres of the sample from organic layer was injected into a gas chromatograph (GC-B14, Shimadzo, Kyoto, Japan), equipped with Class-LC 10 Integrator. L-PAC showed its peaks at retention time of 5.57-6.02 min. GC (%) result was computed using normalization method on GC peak areas. The result was then applied in material balance of benzaldehyde used to estimate L-PAC.

Induced Mutagenesis and Selection of Hyper-Producer L-PAC Mutants: UV treatments were carried out using modified procedure reported by Zouari et al. (2004) while the technique of randomly induced mutagenesis is depicted in Fig. 1. Twenty five millilitre of cell suspension (1×10^7 cells/ml) was poured into a glass petriplate and stirred continuously. The cells were irradiated for 100 min by UV light (Fluence rate, 1.2×10^2 J/m^2/s; Mineral light, UVS. 12, California, USA), placed at 6-12 cm. Sample (1.0 ml) was collected every 10 min interval. Five hundred microlitres of the cell dilution (cell density 500-600 cells/ml) was spreaded on YPG plate containing (%) yeast extract 1.0, peptone 2.0, glycerol 3.0, pH 5.0. The plates were incubated at 30ºC for 4 days. The developed colonies were replica plated with velveteen on to YPD-agar plate and incubated (30ºC) for another 2 days.

Nitrous acid treatment was followed by the procedure of Snow (1967). One millilitre of freshly prepared nitrous acid (1.0-2.5 M by dissolving sodium nitrite in 0.2 M sodium acetate buffer, pH 4.5) was added to 9 ml of cell suspension (2×10^8 cells/ml in the same buffer) using a screw-capped glass tube. The mixture was thoroughly shaken for 25 min. Samples (0.5 ml) were withdrawn every 5 min interval and added to 4.5 ml of 0.05 M tris (hydroxymethyl) aminomethane buffer (pH 8.0) to terminate the reaction. Colonies were developed on YPD plates at 30ºC for 2 days.

Ethylmethane sulphonate (EMS) treatment was used after Lindegren *et al*. (1965). To 5 ml of cell suspension (2×10^8 cells/ml in 0.1 M phosphate buffer, pH 7.0), EMS (3-6 %, v/v) was added. The assay mixture was shaken vigorously and incubated at 25ºC for 90-120 min. Samples (0.2 ml) were withdrawn at regular intervals of 10 min and added to 8.0 ml of sodium thiosulphate solution (5 %, w/v) to quench the

reaction. The colonies were developed on YPD plates. The survival frequency of the cells was calculated as: No. of colonies on YPD plate after mutagenic treatment/No. of untreated cells in the start of mutagenesis.

The procedures developed by Prakash and Prakash (1977) and Agustin and Marcel (2004) were adopted for the selection of hyper-producer L-PAC mutants. The colonies grown on YPD plates were replica plated with velveteen on 0.11 % (w/v) acetaldehyde (minimum growth inhibitory concentration) added YPD plates and incubated (30°C) for 4 days. The mutant frequency was calculated as: No. of colonies on acetaldehyde-supplemented YPD plate/No. of untreated cells in the beginning of mutagenesis. Acetaldehyde resistant mutants were screened for L-PAC production using shake flask technique.

Statistical Analysis: Treatment effects were analysed through analysis of variance technique (Snedecor and Cochran, 1980). Duncan's multiple range test (DMRT) was applied to compare the means (Duncan, 1955) at $P<0.05$.

RESULTS AND DISCUSSION

Cell Survival Rate: In the present study, the cells of *S. cerevisiae* strain GCU-36 were exposed to ultraviolet (UV) irradiations, nitrous acid (HNO_2) and ethylmethane sulphonate (EMS) for improvement in terms of L-PAC production before colony development on YPD-agar plates. The survival frequency decreased with the increase in concentration of chemical mutagens (or decrease in distance between UV source and yeast cells) or increase in exposure time. The results are given in Fig. 2a, 3a and 4a. The cell mortality was possibly due to a defective repair system or damage to cellular or sub-cellular components (Kozmin *et al*., 2005). Different survival frequencies were noticed when mutants derived after single mutagen treatment were subjected to second mutagen during the scheme of reciprocal mutagens. The values were found to be 3.13, 1.82 and 4.26 % after EMS, UV and again UV exposure to mutants GCUV-3, GCUN-4 and GCUE-9, respectively. The values were lower than those obtained after single treatment of the second mutagen. The findings showed a good agreement with the Arasaratnam *et al*. (2001) who exposed UV or nitrous acid sensitive cells to EMS. Sensitivity to series of mutagens was also reflected from the frequencies of 7.14 and 5 % obtained after exposing double mutants GCUV/E-3 and GCUN/V-2 to alternate mutagens i.e. UV radiations and nitrous acid, respectively.

Frequency of Mutant Strains: The survivors were replica plated on YPD-plates containing 0.11 % (w/v) acetaldehyde (minimal growth inhibitory concentration, MIC) to observe the mutant frequency**.** The results are depicted in Fig. 2b, 3b and 4b. Mutant frequency showed no correlation with survivors suggesting independent occurrence of the frequencies. However, incidence of higher mutant frequency (per survivors) is a sign of effective mutagenic action. In some cases of double mutagenesis, the frequency was a simple additive product of the values obtained after applying each of the 2 mutagen singly. The finding is in accordance with Ager and Haynes (1990) focusing on additive outcomes of mutant frequency after exposing the cells by two consecutive mutagens.

L-PAC Production: The quantitave screening of the selected mutants was carried out using shake flask technique. Three derived, better mutants GCUV-3, GCUN-4 and GCUE-9 (using single mutagen) gave significantly ($p\leq0.05$) higher L-PAC production i.e. 4.15, 4.55 and 4.61 (g/l), respectively compared to the wild strain *S. cerevisiae* GCU-36 (2.58 g/l) as shown in Tables 1a, 1b and 1c. The outcome of the better mutants reflected a positive impact of mutation on benzaldehyde biotransformation to L-PAC. In the previously reported studies, Culic *et al*. (1984) investigated mutation-based beneficial alterations in the active sites of PDC enzyme. Similarly, a higher effectivity of EMS is also evident from the studies of Lesilie *et al*. (2006) who applied the same mutagen for

Table 1a. L-PAC production by UV-irradiated mutants of *S. cerevisiae* GCU-36

Mutant GCUV.[1/]	PAC (g/l)	Mutant GCUV..	PAC (g/l)	Mutant GCUV	PAC (g/l)
1	1.63 ±0.10 ^{hi}	13	1.76 ±0.16^{h}	25	2.38±0.13^{defg}
2	2.23 ±0.11^{efg}	14	1.0±0.10^{j}	26	2.15±0.16^{g}
3	4.15 ±0.15^{a}	15	2.4±0.13^{def}	27	2.43±0.12^{de}
4	2.12 ±0.13^{g}	16	2.26±0.13^{efg}	28	2.45±0.14^{de}
5	2.79±0.15^{bc}	17	1.57±0.15^{hi}	29	1.46±0.10^{i}
6	2.83±0.14^{b}	18	2.43±0.23^{def}	30	2.89±0.12^{b}
7	2.32±0.13^{efg}	19	1.52±0.13^{hi}	31	1.65±0.1^{hi}
8	2.31±0.15^{efg}	20	1.56 ±0.13^{hi}	32	1.22±0.12^{j}
9	1.22±0.12^{j}	21	1.77 ±0.12^{h}	33	2.38±0.22^{defg}
10	1.60 ± 0.10^{hi}	22	2.37±0.13^{defg}	34	2.32±0.13^{efg}
11	1.60±0.15^{h}	23	2.17±0.14^{fg}	Parent strain	2.58±0.15^{cd}
12	2.3±0.12^{defg}	24	2.33±0.08^{efg}		

Five sequential doses (each benzaldehyde 1.20 ml/l + acetaldehyde 1.20 ml/l); dose interval 1 h. Means followed by the same letter (s) are not significantly different at 5% level of significance, DMRT.
[1/] alphabet (V) following general code, GCU represents UV sensitive mutant while dots show the number of the mutant (written in the coloumn).

Table 1b. L-PAC production by HNO_2 treated mutants of *S. cerevisiae* GCU-36

Mutant GCUN..[1/]	PAC (g/l)	Mutant GCUN..	PAC (g/l)	Mutant GCUN..	PAC (g/l)
1	1.94±0.17^{fgh}	9	1.37±0.15^{h}	17	2.56±0.18^{gh}
2	2.19±0.20^{cdefg}	10	1.85±0.15^{gh}	18	2.27±0.16^{bcdef}
3	2.42±0.23^{bcde}	11	2.44±0.22^{bcde}	19	2.41±0.20^{bcde}
4	4.55±0.17^{a}	12	2.12±0.17^{efg}	20	1.86±0.15^{h}
5	2.18±0.25^{defg}	13	2.53±0.14^{bcd}	21	2.26±0.16^{bcdef}
6	1.85±0.38^{gh}	14	2.14±0.21^{efg}	Parent strain	2.58±0.15^{b}
7	1.38±0.18^{h}	15	2.38±0.18^{bcde}		
8	2.16±0.15^{efg}	16	1.64±0.18^{gh}		

[1/] alphabet (N) following general code, GCU represents nitrous acid sensitive mutant while dots show the number of the mutant (written in the coloumn).

Table 1c. L-PAC production by EMS treated mutants of *S. cerevisiae* GCU-36

Mutant GCUE..[1/]	PAC (g/l)	Mutant GCUE..	PAC (g/l)	Mutant GCUE..	PAC (g/l)
1	2.58±0.15^{bc}	6	2.51±0.21^{bcd}	11	2.57±0.15^{bc}
2	2.50±0.20^{bcd}	7	1.21±0.20^{f}	12	2.26±0.16^{cde}
3	2.19±0.17^{de}	8	2.56±0.17^{bc}	13	2.63±0.18^{b}
4	2.46±0.18^{bcd}	9	4.61±0.20^{a}	Parent strain	2.58±0.15^{bc}
5	2.34±0.16^{bcde}	10	2.11±0.15^{e}		

[1/] alphabet (E) following general code, GCU represents EMS sensitive mutant while dots show the number of the mutant (written in the coloumn).

Table 2. L-PAC production by mutants derived after reciprocal treatments

Mutants derived after reciprocal treatments on GCUV-3		Mutants derived after reciprocal treatments on GCUN- 4		Mutants derived after reciprocal treatments on GCUE-9	
Mutant	PAC (g/l)	Mutant	PAC (g/l)	Mutant	PAC (g/l)
Parent mutant	4.15± 0.1^{bc}	Parent mutant	4.55±0.17^{bc}	Parent mutant	4.61±0.20^{a}
GCUV/E-1	4.18±0.18^{bc}	GCUN/V-1	4.0±0.20^{d}	GCUE/V-1	4.19±0.20^{b}
GCUV/E-2	3.94±0.16^{cd}	GCUN/V-2	5.06±0.15^{a}	GCUE/V-2	3.61±0.20^{c}
GCUV/E-3	5.17±0.20^{a}	GCUN/E -1	4.28±0.17^{cd}	GCUE/N-1	4.35±0.17^{ab}
GCUV/N-1	2.60±0.20^{e}	GCUN/E -2	4.85^{ab}±0.19^{ab}	GCUE/N-2	3.73±0.18^{c}
GCUV/N-2	4.26±0.16 ^{bc}	GCUN/E -3	4.23±0.17^{d}		
GCUV/N-3	4.31±0.17^{b}	GCUN/E -4	4.65±0.16^{b}		
GCUV/N-4	4.20±0.21^{bc}				
GCUV/N-5	4.15 ± 0.15^{bc}				

Alphabet (V, N or E)) following general code, GCU represents the subsequent mutagens (UV, nitrous acid or EMS) to which mutant has

conferred sensitivity.

Table 3. L-PAC production by mutants derived after alternate treatments

Alternate treatment	Code of mutant	PAC (g/l)	Code of mutant	PAC (g/l)
		1st step		
	GCUV/E-3 (parent)	5.17±0.20[a]	GCUN/V-2 (parent)	5.06±0.15[b]
	GCUV/E/V- 1	4.57±0.18[b]	GCUN/V/N-1	5.45±0.20[a]
	GCUV/E/V- 2	4.64±0.20[b]	GCUN/V/N-2	4.85±0.25[b]
	GCUV/E/V- 3	4.62±0.19[b]	GCUN/V/N-3	4.94±0.18[b]
	–	–	GCUN/V/N-4	4.49±0.17[c]
	–	–	GCUN/V/N-5	5.0±0.16[b]
2nd step				
	GCUN/V/N-1 (parent)	5.45±0.20[a]		
	GCUN/V/N/V-1	4.70±0.24[b]		
	GCUN/V/N/V-2	4.80±0.37[b]		

improvement in an industrially important yeast strain. In the present study, some mutants gave L-PAC significantly lower than parent. The decreased production was probably due to substantial DNA damage.

Two mutants GCUV/E-3 (derived after EMS exposure to mutant GCUV-3) and GCUN/V-2 (derived after UV irradiations to mutant GCUN-4) gave significantly higher L-PAC i.e. 5.17 and 5.06 (g/l), respectively when compared to their respective parent mutants (Table 2). The enhancement in the production seems to be a synergistic effect of the mutagens of double mutagenesis. However outcome of inferior mutants might be the antagonistic actions of the two successive mutagens. A mutant GCUN/V/N-1 (derived from nitrous acid exposure to GCUN/V-2 during 1st step of alternate treatments) gave significantly higher L-PAC production (5.45 g/l) than that of parent mutant (Table 3). However, L-PAC production decreased to 4.8 (g/l) by a mutant GCUN/V/NV-2 (derived after UV exposure to GCUN/V/N-1 during 2nd step of alternate treatment). The incidence of inferior mutant was possibly due to Plateau effect (cessation of mutation by the same mutagen due to saturation value of positive mutation) as reported by Markhanen and Suihko (1974).

Fig.1 Flow sheet of culture improvement.→ Denotes mutagenic stages; 1st (using single mutagen), 2nd (using reciprocal mutagens), 3rd (using alternate mutagen; 1st step) and 4th (using alternate mutagens; 2nd step). Treatment: UV = 30 min from 6 cm, HNO_2 = 15 min in 0.15 M HNO_2 and EMS = 90 min in 5% (v/v) EMS. 1/Finally selected mutant.

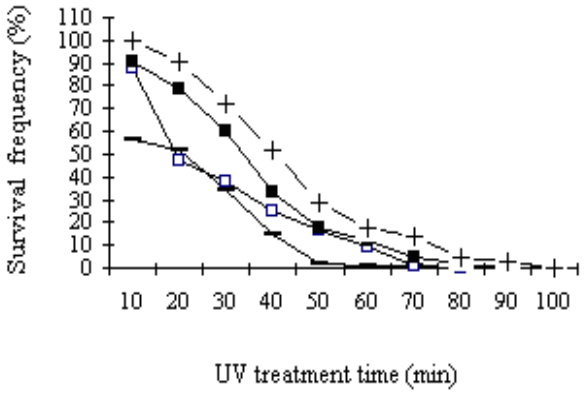

Fig.2a Survival frequencies (%) after UV treatments on *S. cerevisiae* GCU-36. (-×- at 6 cm distance, -□- 8 cm distance, -■- 10 cm distance, -12 -cm distance)

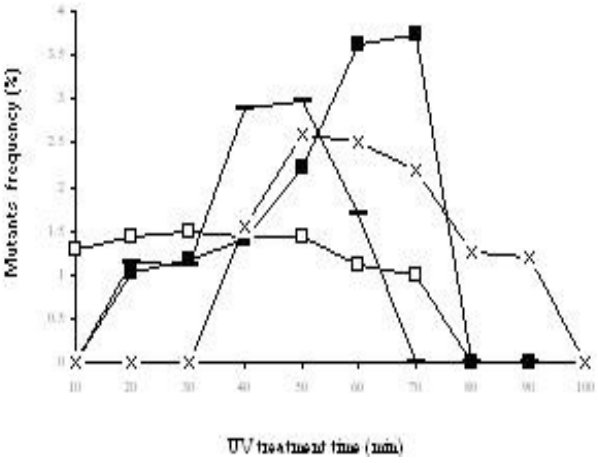

Fig.2b Mutants frequencies (%) after UV treatments on *S. cerevisiae* GCU-36. (-□- at 6 cm distance, -■- 8 cm distance, -10 -cm distance, -×- 12 cm distance)

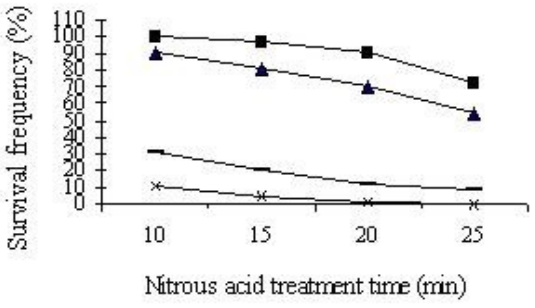

Fig.3a Survival frequencies (%) after nitrous acid treatments on *S. cerevisiae* GCU-36. (-■- 0.1 M, -▲- 0.15 M, - 0.2 -M, -×- 0.25 M)

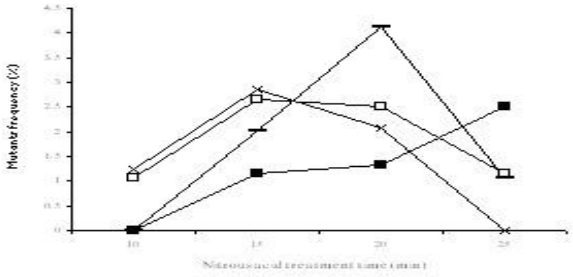

Fig.3b Mutants frequencies (%) after nitrous acid treatments on *S. cerevisiae* GCU-36. (-■- 0.1 M, -□- 0.15 M, - 0.2 -M, -×- 0.25 M)

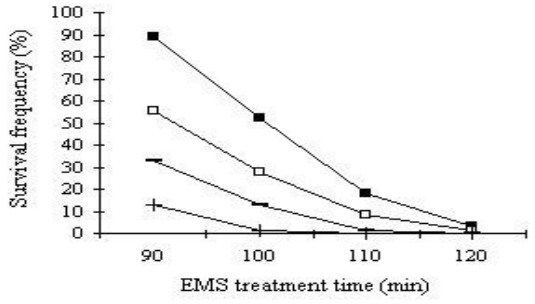

Fig.4a Survival frequencies (%) after ethylmethane sulphonate treatments on *S. cerevisiae* GCU-36 (-■- 3 % v/v, -□- 4 % v/v, - 5 -% v/v, -+- 6 % v/v)

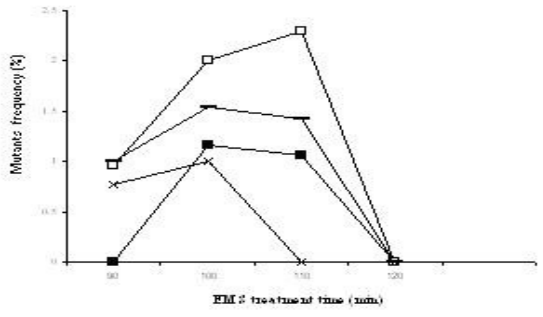

Fig.4b Mutants frequencies (%) after ethylmethane sulphonate treatments on *S. cerevisiae* GCU-36 (-■- 3 % v/v, -□- 4 % v/v, - 5 -% v/v, -+- 6 % v/v)

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