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BIOSYNTHESIS OF L-PHENYLACETYLCARBINOL FROM LOCALLY ISOLATED YEASTS

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Abstract

In the present study, 250 yeast strains were isolated from samples of different natural sources as cane-molasses, decaying vegetables and bagasse using glucose enriched medium. Among these, 106 strains showed no growth in acetaldehyde (1 g/l) supplemented yeast extract-peptone dextrose plates during qualitative screening. In the course of quantitative screening, 64 acetaldehyde tolerants gave almost negligible L-PAC production (≤0.5 g/l) using glucose-peptone medium in shake flasks. A comparatively better L-PAC production was observed with the rest of strains. The isolate *Saccharomyces cerevisiae* GCU-36 exhibited higher L-PAC production (2.58 g/l). However, lower sugar consumption and subsequent biomass formation was noted. Therefore, yeast GCU-36 was selected as a hyperproducer of L-PAC in batch culture.

Introduction

L-Ephedrine and its derivative D-pseudoephedrine are widely used in pharmaceutical formulations as anti-asthmatic and decongestant, respectively. L-Phenylacetylcarbinol (L-PAC) is a non-marketed intermediate of L-ephedrine. Commercially, it is obtained through yeast pyruvate decarboxylase (PDC)-mediated condensation of acetaldehyde with the supplementation of benzaldehyde (Smallridge *et al*., 2009). Other microbial sources such as fungi and bacteria have also been reported for this purpose. However, yeast source remained predominant with potential industrial applications (Cindy *et al*., 2007). Amongst yeasts, *Saccaromyces cerevisiae* is an organism of choice on account of non-pathogenicity, faster growth rate and easy handling. Culture isolation followed by extensive screening usually results in the selection of a hyperproducer strain. A suitable yeast strain is one of the determinants for economic feasibility of a fermentation process. PDC deficient strains show impaired growth in glucose enriched medium as repoted by Hohmann (1991). Therefore, the use of such media for yeast isolation screened out the non L-PAC producing strains in terms of net PDC quantity. The qualitative screening involves acetaldehyde tolerance as it intracellularly contributes in the biotransformation of benzaldehyde into L-PAC (Allen 2006). During quantitative screening, better L-PAC producing strains with low level of sugar consumption and biomass formation have been preferred. In the present paper, we report on the isolation of yeasts prior to intensive screening for L-PAC biosynthesis in batch culture.

Materials and Methods

Isolation of yeast strains: Samples from different sources such as cane-molasses, decaying vegetables and bagasse were collected in sterilized polythene bags and diluted following serial dilution method (Clark *et al*., 1958). Five hundred microlitres of each dilution was transferred to a Petri plate (15 cm dia.) containing 25 ml of Wickreham's MYPD-agar medium (g/l): malt extract 3, yeast extract 3, peptone 5, glucose 10, agar 20 (pH 5) after modified method of Arasaratnam *et al*., (2001). The plate was rotated clock- and counter-clock wise to spread the inoculum uniformly and placed in an incubator at 30°C for 2-3 days.

The independent initial colonies were picked up aseptically and transferred to YPD-agar slants containing (g/l): yeast extract 10, peptone 10, dextrose 20, agar 20 (pH 5). The slant cultures were incubated at 30°C for 2-3 days until maximum growth and stored at 4°C in a cold cabinet (MPR-1410, SANYO, Japan).

Screening of isolates for L-PAC biosynthesis

i. Qualitative method: The yeast strains were screened for acetaldehyde tolerance after Agustin and Marcel (2004). Three hundred and eighteen microlitres of acetaldehyde (10% v/v in water) was spreaded on a plate containing 25 ml of solidified YPD agar medium (pH 5) and allowed to diffuse overnight at 4°C. Half millilitre of cell suspension (~500-600 cells/ml) was spreaded on the YPD agar plate (with or without acetaldehyde). The plates were incubated at 30°C until colonies appeared (2 days for control and 4 days for the test). The colonies were counted using a digital colony counter (560, Suntex, Switzerland).

ii. Quantitative method

Inoculum preparation: Two millilitres of cell suspension 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 a 1000 ml Erlenmeyer flask and allowed to grow till yeast cell count reached to 105×10^6 cells/ml (cal. 3%, w/v) after Shukla & Kulkurni (2001). The cells were recovered by centrifugation (9500×*g*) for 15 min and water washed twice.

Fermentation procedure: Two hundred millilitre of sterilized medium containing **(**g/l): glucose 50, peptone 6 at pH 5 was taken in a 1000 ml capacity Erlenmeyer flask and cotton plugged. The medium was aseptically inoculated with 3% (w/v) inoculum. The cells were allowed to adapt for approximately 1 h in a rotary shaking incubator (200 rpm) at 30±2°C. Five doses (each of 1.2 m l/l acetaldehyde +1.2 ml/l benzaldehyde) were added at 1 h interval.

Identification of isolate: The isolate GCU-36 was characterized using the procedures of Lodder & Rij (1952).

Analytical techniques

Estimation of L-PAC: Yeast cells were recovered from the broth by centrifugation (9500×*g*) for 15 min. One millilitre of the supernatant was dispensed in 5 ml of dichloromethane in a screw-capped test tube and vortexed 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-LC10 Integrator. GC (%) result was computed using the normalization method on peak areas of the chromatograph. Mean of three replicates was taken. The identities of the components were confirmed according to the retention time with those of L-PAC standard (from ICI). The concentration of L-PAC was calculated on the basis of benzaldehyde utilized.

Estimation of dry cell mass: Ten milliliter of the culture broth was taken in a glass tube and centrifuged at 8500×*g* for 10 min. The biomass was water-washed twice and suspended in 0.9% (w/v) saline solution (0.085% NaCl, 0.5 yeast extract). The suspension was poured into a pre-weighed glass tube and recovered again. The cells were dried in an oven (UM-400, MEMMERT, Germany) at 105°C for 24 h, cooled in a desiccator and reweighed.

Estimation of residual glucose: It was estimated by the method of Miller (1959).

Statistical analysis: Treatment effects were compared after Snedecor & Cohran (1980). Duncan's multiple range test (DMRT) was applied under one-way analysis of variance (Duncan, 1955). Significance has been presented in the form of probability ($p \leq 0.05$) values.

Results and Discussion

Two hundred and fifty yeast strains were isolated from samples of different sugar-rich sources using malt extract, yeast extract-peptone dextrose-agar medium. The isolates developed hyper-osmotic tolerance through adaptation. This adaptability is just like thermotolerance in yeasts of warm habitats (Sandrasegarampillai *et al*., 2001) or lipases productivity in fungae of lipid-rich habitats (Tehreema *et al*., 2011a). According to Hohmann (1991), glucose of the media discourages the growth of pyruvate decarboxylase deficient yeast cultures. Therefore, culture screening followed isolation in the present studies. Amongst the isolates, 106 could not grow on acetaldehyde (1 g/l) added yeast extract-peptone dextrose (YPD) plates during the course of primary screening (Table 1). In the analysis of the data, maximum number (25) was found against each of cane molasses and sugar mill effluents. The acetaldehyde lacking tolerance may be the consequence of acetaldehyde-mediated repression. In another study, Pamment *et al*., (1997) exploited acetaldehyde-based poisoning of the medium which was of prime importance to study cell viability.

Table 1. Isolation sources and acetaldehyde non-tolerant or low L-PAC producing yeast isolates.

Solation source	Code of yeast isolate (GCU…)[1/]	
	Lacking acetaldehyde tolerance	**Producing L-PAC ≤ 0.5 (g/l)**
Decaying fruit	4, 6, 22 and 26 (4)[2/]	9, 13 and 14 (3)
Cane molasses	32, 34, 37, 38, 41, 47, 50-52, 70, 72, 73, 82-88, 90, 94-97 and 99 (25)	30, 31, 35, 43-45, 61, 75, 76, 79, 80, 81, 89, 91-93 and 98 (17)
Decaying rice hulls	107-114, 116-118, 127, 128, 131, 132 and 141 (16)	101-106, 120, 121-125, 129, 134-137, 139 and 140 (19)
Decaying vegetables	143, 144, 147, 148, 150-154, 157, 159, 161, 163 and 164 (14)	156 (1)
Distilleries'soil	168-170 (3)	172, 173 and 176 (3)
Wet bagasse	180-189, 191-195, 202-205 (19)	178, 179, 196-200 (7)
Sugar Mills'effluents	207-221, 228-230, 235, 237, 238, 241, 242, 246 and 247 (25)	223-227, 231, 233, 234, 239, 240, 243, 244, 249 and 250 (14)

[1/]Dots following GCU denote a number given below in the table. [2/]Denotes total numbers of isolate(s)

The acetaldehyde tolerant strains gave L-PAC at different levels when subjected to secondary screening using glucose-peptone medium in shake flasks. As shown in the Table 1, a total of 64 strains gave almost negligible amounts of L-PAC (≤0.5 g/l). The findings were in disparity to Long & Ward (1989) who reported a direct relationship between cell viability in acetaldehyde and subsequent L-PAC production. Insignificant L-PAC production by highly viable cells was due to the failure of cell divergence towards L-PAC. Eighty isolates gave notable production. The statistics of Table 2 shows comparative account of some better strains. The better L-PAC productivity was the result of possible synergistic activity of favourable cytogenetics and optimal physiological status of the strain as reported by Kulkurni & Shukla (2000). A significantly higher L-PAC (2.58 g/l) accompanied by lower sugar consumption and subsequent biomass formation was noticed when GCU-36 was used as the organism of choice in batch culture. The lower values of the subsidiary parameters agreed with the findings of

Iwan *et al*., (2001) suggesting better L-PAC production at low profiles of the parameters. The isolate GCU-36 was designated as *S. cerevisiae* GCU-36 after characterization. The identification is necessary for onward studies of a strain as reported by Tehreema *et al*., (2011b) using *Penicillium chrysogenum* (MBL 22) for biosynthesis of lipases. Most importantly, the yeast was frequently applied for the production of L-PAC at an industrial scale. GC trace of reaction mixture using *S. cerevisiae* GCU-36 for the bioconversion of benzaldehyde to L-phenylacetylcarbinol showed peaks of different components (Fig. 1). L-PAC peak at retention time of 6.212 min accompanied by peak area (9522879 µV. sec) led to 0.96 g of L-PAC/l on the basis of benzaldehyde utilized.

From the present results, it was concluded that yeast isolation supported the screening factor during the selection of a suitable strain in terms of L-PAC production. The selected strain may contribute towards economic feasibility of the bioconversion.

Table 2. Screening of wild yeast strains in glucose medium using shake flasks.

Isolation source	Code of yeast isolate	Glucose consumed (g/l)	Dry biomass formed (g/l)	L-PAC produced (g/l)
Decaying fruit	GCU-2	$2.81^{a} \pm 0.09$	$0.82^{a} \pm 0.08$	$2.35^{bc} \pm 0.12$
-	GCU-7	$1.95^{b} \pm 0.15$	$0.34^{c} \pm 0.03$	$2.20^{b} \pm 0.10$
-	GCU-16	$1.78^{bc} \pm 0.2$	$0.38^{c} \pm 0.12$	$2.16^{bc} \pm 0.08$
Bagasse	GCU-25	$1.60^{c} \pm 0.12$	$0.27^{cd} \pm 0.06$	$2.10^{c} \pm 0.07$
-	GCU-28	$1.94^{b} \pm 0.15$	$0.2^{d} \pm 0.07$	$2.16^{bc} \pm 0.11$
Cane molasses	GCU-36	$1.72^{bc} \pm 0.07$	$0.60^{b} \pm 0.09$	$2.58^{a} \pm 0.15$
-	GCU-60	$2.71^{a} \pm 0.18$	$0.34^{c} \pm 0.04$	$2.16^{bc} \pm 0.12$
-	GCU-66	$1.87^{bc} \pm 0.23$	$0.29^{cd} \pm 0.03$	$2.09^{c} \pm 0.08$

Means followed by the same letter(s) are not significantly different at 5% level of significance, DMRT

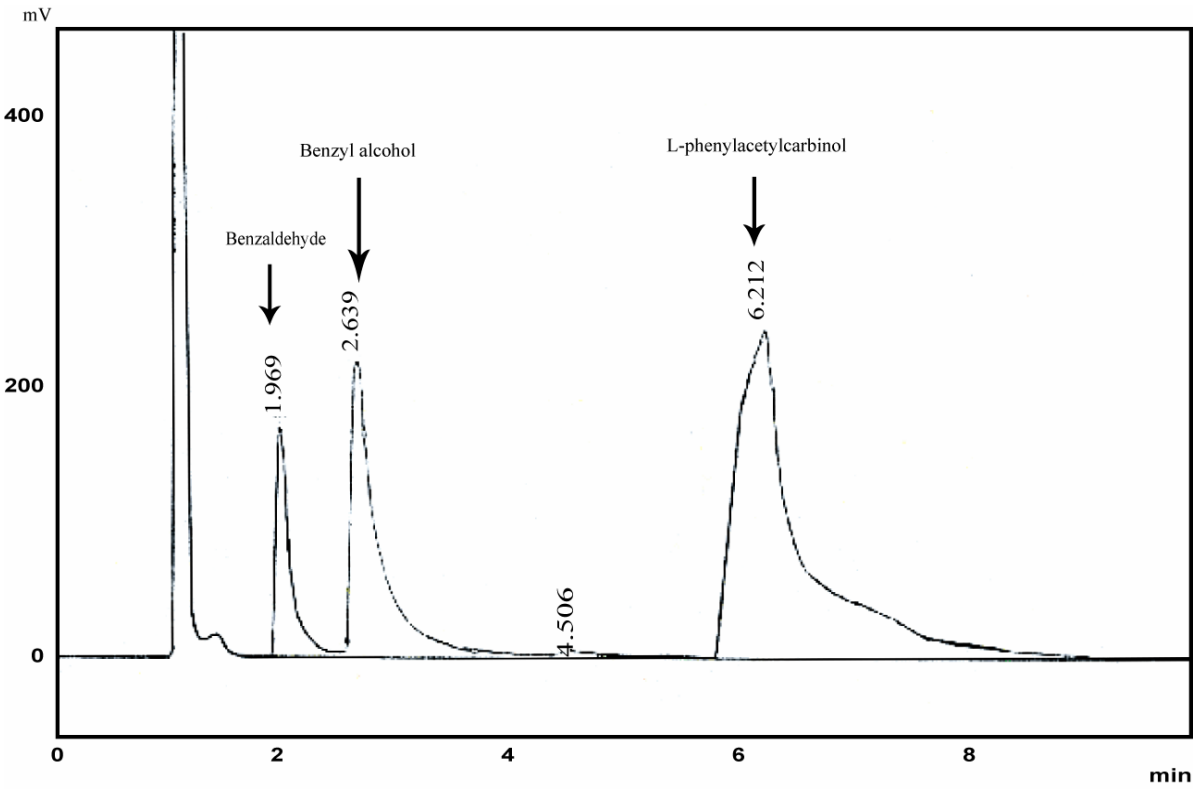

Fig. 1. GC traces of reaction mixture using *S. cerevisiae* GCU-36 for the bioconversion of benzaldehyde to L-phenylacetylcarbinol. Biotransformation broth was withdrawn before 2nd dose of acetaldehyde-benzaldehyde mixture.

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