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Effective biotransformation of benzaldehyde to a stable L-PAC using calcium alginate entrapped pyruvate decarboxylase of an auxotrophic *Saccharomyces cerevisiae*

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Abstract

The present study deals with the effective biotransformation of benzaldehyde to a stable L-PAC using calcium alginate entrapped pyruvate decarboxylase of an auxotrophic *Saccharomyces cerevisiae* via submerged fermentation. The wild-type of *Saccharomyces cerevisiae* ISL-7 was mutagenised using UV radiations. Significant increase in L-PAC production was found as compared to wild-type. Out of 29 mutants, UV-t6 was selected for maximum L-PAC production after primary and secondary screening. The auxotrophic mutant UV-t6 resulted in 1.25fold increase in L-PAC activity than wild-type ISL-7 when exposed to UV radiations. The effect of different benzaldehyde concentrations and incubation time on production of L-PAC was also studied. The maximum PDC activity 95.99±5.7 U/ml and L-PAC activity 22.46±1.34 g/l was observed after 4 h of incubation at 28ºC for 60 µl benzaldehyde concentration. The selected mutant strain UV-t6also showed maximum L-PAC production with better L-PAC fermentation kinetics as compared to wild-type ISL-7. The PDC of auxotrophic mutant strain of *Saccharomyces cerevisiae* UV-t6 was immobilized using calcium alginate beads. The effect of enzyme concentration, bead size and cell holding time on L-PAC activity was also studied. The immobilized enzyme exhibited maximum PDC and L-PAC activity i.e. 116.22±8.13 U/ml and 13.72±0.96g/l at enzyme concentration 1.5 ml as compared to free enzyme (PDC activity 115.20±8.06 U/ml and L-PAC activity 12.97±0.91 g/l). The maximum L-PAC activity was observed for bead size 3 mm i.e. 14.56±1.16 g/l along with cell holding time 10 min. This study revealed that auxotrophic mutant strain of *Saccharomyces cerevisiae* along with immobilized PDC exhibited 1.68fold increase in L-PAC production than wild-type.

Keywords *Saccharomyces cerevisiae*, L-phenylacetylcarbinol, Biotransformation, Benzaldehyde, Calcium alginate entrapment

Introduction

L-Phenylacetylcarbinol (L-PAC) is a key mediator for the production of L-ephedrine, norephedrine, pseudoephedrine, amphetamine or methamphetamine as well as adrenaline,

phenylpropanolamine and phenylamine (Suresh*et al*., 2015; Shukla and Kulkarni, 2000). L-Phenylacetylcarbinol (L-PAC) is also known as 1-hydroxy-1-phenylacetone, a-hydroxy benzyl methyl ketone, 1-hydroxy-1-phenyl-2-propanone or Neuberg'sketol (90-63-1). L-Phenylacetylcarbinol (L-PAC) has half-life of 240h, specific optical rotation (-375.8°) and melting point (172ºC) (Smallridge *et al*., 2001)**.** L-PAC do not evaluate as a market stock. It is manufactured by industries which utilize it as a synthon for several medicines or drugs. It has the properties of α, β adrenergic. These drugs include L-ephedrine, pseudoephedrine, norephedrine and pseudoephedrine (Shukla and Kulkarni, 2000). Neuberg and Hirsch published first introductory document on the biological output of a well-known α-hydroxy ketone almost a century ago in 1921 (Iranmanesh *et al*., 2019; Mahmoud *et al*., 1990; Adepoju*et al.,* 2013). Commercially its preparations are not available for use as standards in analysis (Nikolva*et al*., 1991).

L-Ephedrine is helpful for the treatment of the diseases such as asthma and congestion (Shukla *et al*., 2002; Park and Lee, 2001). Reports have also reflected its potential usage in the control of obesity (Shin and Rogers, 1995), for the medication of hypotension (Shukla and Kulkarni, 2000) and hay fever drugs (Kovacs and Mela, 2006). Dietary medications comprising ephedra have been widely facilitated in the US as a norm to lose weight and to stimulate energy levels in the body. Clinical surveys indicated that a mixture of ephedrine and caffeine with energy restrictive nutriment is a beneficial therapy for obesity (Kovacs and Mela, 2006; Astrup*et al*., 1995). In disparity, further researches have illustrated a substantial number of unfavorable effects related with ephedra and this included hypertension, palpitations, tachycardia (fast heartbeat), stroke and seizures (Haller and Benowitz, 2000). The action by the US Food and Drug Administration (FDA) to restrict ephedra-containing dietary complements in 2004 was regardless overruled due to the absence of evidence of its health hazards (Kovacs and Mela, 2006). In the sporting arenas, ephedrine could be utilized to empower rapid energy but its usage is prohibited by major organizations of sports (Calfee and Fadale, 2006). D-pseudoephedrine is consumed in the hay fever pills and in sinus (Kovacs and Mela, 2006) and as well as used as anti-asthmatic agent (Seifi*et al*., 2020; Miguez*et al*., 2012).

The cell immobilization is a process which precludes the problems of biotransformation processes. The most common and popular methods are adsorption and entrapment for immobilization of cell. A porous matrix is used to enclose the cells in entrapment method, in this way products diffuse away and substrates diffuse towards the cell (Seifi*et al*., 2020; Park and Lee, 2001). The cells of *Saccharomyces cerevisiae* which are immobilized in calcium alginate matrix are protected from toxic agents present in medium for L-PAC production (Seifi*et al*., 2020). The motive behind the immobilization direction was to formulate a diffusional restriction and a substrate gradient and solvent components between the catalyst and the majority of the substrate (Mahmoud *et al*., 1990; Seely*et al*., 1989). Immobilized yeast cells and ingredients were found to have outstanding benzaldehyde compassion and considerable L-PAC production than free cells (Shin and Rogers, 1995). Precise modelling and simulation benefited thorough kinetic examination offers key strategy for improving productivities and outputs of enzymatic and microbial cultivation methods. Prior kinetic equations formulated in surveys of the PAC biotransformation have either mentioned PDC deactivation by benzaldehyde or facilitated rate equations for PAC production implicating only one or two variables (Leksawasdi*et al*., 2004). L-PAC biotransformation is supposed as a

fascinating method as it comprises both end product inhibition and substrate (acetaldehyde and benzaldehyde) toxicity (Barberio*et al*., 2007). Though the usage of auxotrophic *Saccharomyces cerevisiae* and immobilization of PDC (pyruvate decarboxylase) has not been noted so far, so additional work and research is required on it for productive production of L-PAC.

Materials and methods

Necessary equipment such as hot plate line (D-MGST1-V2, Eisco Scientific, USA), weighing balance (NS-620A), microscope (CH3ORF200, Olympus Optical Co., Ltd., Japan), spectrophotometer (KT-40L, ALP Co., Ltd., Japan with 220 V), refractometer (ATAGO, Japan), autoclave (KT-40L, ALP Co., Ltd., Tokyo, Japan), laminar air flow cabinet, Incubator, centrifuge (LABCEN 60), and pH meter was used. These chemicals and others were of maximum purity and obtained from E-Merck (Germany), Oxyde (USA) and Sigma Aldrich (UK).

Microorganism

A wild-type strain of *S. cerevisiae* was obtained from IIB stock culture. The independent initial colonies were picked up aseptically and transfer to YPD-agar slants containing (g/L): yeast extract 10, peptone 10, dextrose 20 and agar 20 (pH 5). The slant cultures were incubated at 30°C for 1-2 days until maximum growth and stored at 4°C in a cold cabinet.

Inoculum preparation

Ten millilitres of sterile distilled water was added to the slant culture of *S. cerevisiae* having adequate growth. An inoculating wire-loop was used to disrupt the yeast cells under aseptic conditions. The tube was shaken to obtain a uniform homogenous suspension. A hemocytometer was used to count the yeast cells.

Analytical techniques

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 g yeast extract). The suspension was poured into a pre-weighed glass tube and recovered again. The cells were dried in an oven at 105°C for 24 h, cooled in a desiccator and reweighed.

Determination of protein content

Quantitative estimation of protein was made after (Bradford, 1976). Supernatant (half ml) and Bradford reagent (5 ml) were added in a glass test tube. Mixture was shaken well and incubated at 30°C for 20 min. Spectrophotometer was used to take absorbance at 575 nm. Control was also run parallel containing 0.5 ml of distilled water. The protein content was shown in mg/ml.

Estimation of L-PAC

The L-PAC concentration was determined by refractometer (ATAGO, Japan). Fermented broth was utilized for the estimation of L-PAC. Optical rotation (OR) was recorded and 1-hydroxy-1- phenyl-2-propanone (g/L) was calculated using the following formula:

g/L= 0R x 2 (dilution factor) x 1.11 (density of 1- hydroxy-1-phenyl-2-propanone)

PDC activity assay

Sodium citrate buffer (0.02 M, pH 6.0) 950 µl, $NADH^+$, Na salt (0.03 M, 10 mg/ml) 10 µl, sodium pyruvate (0.03M, 100 mg/ml), 03 µl of 10 mg/ml ammonium sulphate was pipetted out in a cuvette. It was incubated at 25°C for 5 min. The reaction was initiated by adding 5 µl of crude enzyme to the mixture. The absorbance was recorded at 410 nm. The decrease in absorbance was recorded for 5 min and compared to PDC standard.

One unit of enzyme activity was defined as that activity which converts 1 µmole of pyruvate to acetaldehyde per min at 25°C and pH 6.

Entrapment of PDC using calcium alginate

The crude extract of PDC of the selected auxotrophic yeast strain was mixed with equal volume of 5% sodium alginate solution. The mixture was squeezed through a hypodermic needle from the height of 5-10 cm in 3 M $CaCl_2$ solution. The beads were then allowed to cure in a $CaCl_2$ solution for approx. 30 min and stored at 4ºC.

Kinetic study

For determining the kinetic parameters of batch fermentation process, the procedures of Pirt (1975) and Lawford& Roseau (1993) were adopted. The values for specific growth rate i.e., µ (h^{-1}) were calculated from the plots of ln(X) vs. time of fermentation. The product yield coefficient$Y_{p/x}$ was determined by using the relationship $Y_{p/x}$ = dP/dX. The volumetric rates for product formation (Q_p) was determined from the maximum slopes in L-PAC produced vs. the time of fermentation. However, the volumetric rate for biomass formation (Q_x) was calculated from the maximum slope in plot of cell mass formation vs. the incubation time period.The specific rate constant for product formation (q_p) was determined by the equation$q_p = \mu \times Y_{p/x}$. The specific rate for cell mass formation (q_x) was, however, calculated by multiplying the specific growth rate (µ) with the growth yield coefficient ($Y_{x/s}$).

Statistical analysis

Treatment effects were compared by the protected least significant difference method and one-way ANOVA (Spss-9, version-4) after Snedecor and Cochran (1980). Significance difference among the replicates has been presented as Duncan's multiple ranges in the form of probability (<p>) values.

Results and discussion

Induced mutagenesis of *S. cerevisiae* for L-PAC production

The wild strain of *S. cerevisiae* ISL-7 was exposed to ultraviolet treatment at different distances (5, 10, 15, 20, 25, 30 cm) as shown in **Table 1**. Spore suspension was exposed to UV-light for 5 min at different distances. The fourteen mutants were selected for L-PAC activity assay. It was noted that after primary and secondary screening the increase in distance resulted in decline in L-PAC activity. Only one mutantUV-d9 selected from optimum distance 20 cm showed maximum OR 5.0 and L-PAC activity11.14 g/l. At distance 30 cm, lowest enzyme activity7.77-8.65 g/l was obtained. In a similar study, wild strain of *S. cerevisiae* was also exposed to ultraviolet treatment and suggested that mutant showed enhanced L-PAC production than wild-type (Doostmohammadi *et al*., 2016).

In the next step, yeast spore was exposed to UV treatment at different L-tryptophan concentrations (0.005, 0.01, 0.015, 0.02, 0.025, 0.03%). Out of 11 mutants, UV-tryp9 showed maximum enzyme activity11.32 g/land OR 5.1after primary and secondary screening at tryptophan concentration 0.025%. The further increase in tryptophan concentration showed lower enzyme activity. The mutagens inhibit growth and develop non-viability in microorganisms. Moustacchi and Enteric (1970) reported that at higher intensities of UV light, survival rate less than 10% might be due to the hypersensitivity in the cells for the mutagen.

Then spore suspension was also exposed to UV light at different time intervals (5, 10, 15, 20, 25, 30 min). The mutant UV-t6 showed maximum L-PAC activity10.87 g/l and OR 4.9 out of 9 auxotrophic strains after primary and secondary screening at time interval 15 min. With the increase in time interval, decline in L-PAC activity was observed. The lowest enzyme activity 7.77 g/l and OR 3.5wasobserved at 30 min. The enzyme activity of wild-type ISL-7 was 8.65 g/l and OR was 3.9. Brusick and Mayer (1973) suggested direct association between exposure time and cell lethality and reported maximum lethality at a higher exposure time. Hence auxotroph UV-t6was selected for biotransformation of benzaldehyde to L-PAC and calcium alginate entrapment of PDC of auxotrophic yeast for stability in L-PAC production. While wild-type ISL-7 showed lowest L-PAC activity than auxotrophic mutant strain of *S. cerevisiae*.

Table 1: Induced mutagenesis of *Saccharomyces cerevisiae* for effective biotransformation of benzaldehyde to L-PAC in batch culture*

Induced mutagenesis	Death rate (%)	No. of survivors	Strain coding	Primary screening	Secondary screening	
				OD_{575nm}	OR (Brix^o)	L-PAC (g/l)
Wild-type			ISL-7	0.092	3.9	8.65
Distance (cm)						
5	65	3	UV-d1 – UV-d3	0.043–0.068	3.2–3.6	7.14–7.99
10	70	3	UV-d4 – UV-d6	0.069–0.075	3.9–4.1	8.65–9.12
15	75	2	UV-d7 – UV-d8	0.077–0.124	4.2–4.4	9.32–9.76
20	80	1	UV-d9	0.181	5.0	11.14
25	70	2	UV-d10 – UV-d11	0.085–0.137	3.8–4.2	8.43–9.32
30	65	3	UV-d12 – UV-d14	0.068–0.084	3.5–3.9	7.77–8.65
Tryptophan level (%)						
0.005	70	3	UV-tryp1 – UV-tryp3	0.046–0.079	3.3–3.7	7.32–8.21
0.01	50	2	UV-tryp4 – UV-tryp5	0.064–0.088	3.5–4.1	7.77–9.12
0.015	80	2	UV-tryp6 – UV-tryp7	0.079–0.095	3.6–4.3	7.99–9.54
0.02	85	1	UV-tryp8	0.155	4.6	10.21
0.025	90	1	UV-tryp9	0.178	5.1	11.32
0.03	75	2	UV-tryp10 – UV-tryp11	0.075–0.085	3.7–4.4	8.21–9.76
Time exposure (min)						
5	75	3	UV-t1 – UV-t3	0.084–0.093	3.9–4.2	8.65–9.32
10	80	2	UV-t4 – UV-t5	0.087–0.124	4.2–4.5	9.32–9.98
15	95	1	UV-t6	0.182	4.9	10.87
20	90	1	UV-t7	0.139	4.4	9.76
25	85	1	UV-t8	0.101	4.1	9.12
30	80	1	UV-t9	0.068	3.5	7.77

*Time of incubation 6 h, temperature 28˚C, medium pH 6, inoculum size 2 ml (8%, v/v), wavelength~410 nm.

Absorption spectra of wild-type and auxotrophic mutant strain of *S. cerevisiae* for L-PAC production

The relative absorbance of wild-type and auxotrophic mutant strain of *S. cerevisiae* were compared as shown in Fig. 4.1. The absorbance for maximum L-PAC production found to be 1.98 nm at wavelength 535 nm. According to Lambert's law of absorption, equal parts in the same absorbing medium absorb equal fractions of the light that enters them. In contrast, Khan *et al*. (2012) reported highest level of L-PAC at 595 nm. Nikolva *et al*. (1991) suggested maximum L-PAC synthesis at 580 nm. They used Westerfeld method (1945). Mahmoud *et al*. (1990) determined enhanced L-PAC production at 420 nm and they used Groger and Erge method. The variation in results might be due to the different benzaldehyde concentration and toxic effects of benzaldehyde and by-products on L-PAC production.

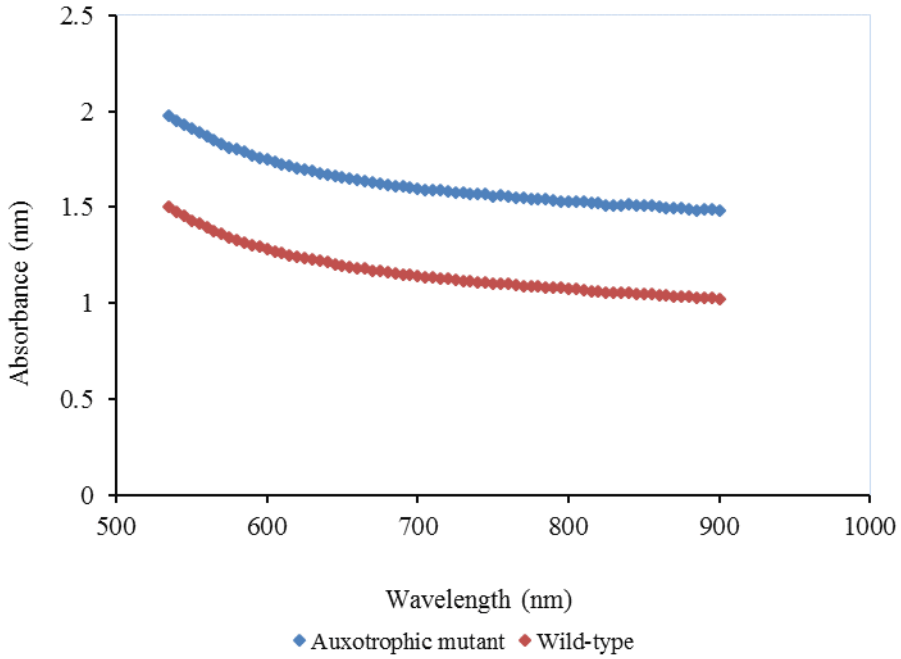

Fig. 4.1: Absorption spectra of wild-type and auxotrophic mutant strain of *S. cerevisiae* for L-PAC production

4.3. Effect of benzaldehyde on L-PAC production

The effect of benzaldehyde concentration as substrate on L-PAC production was studied as shown in Fig. 4.2. The different concentrations of benzaldehyde (10, 20, 30, 40, 50, 60, 90, 120, 150, 180 µl) were selected. The maximum PDC and L-PAC activity was observed for 60 µl benzaldehyde. The maximum PDC and L-PAC activity for auxotrophic strain UV-t6 was 88.88 U/ml and 11.76 g/l. The wild-type ISL-7 showed PDC and L-PAC activity as 14.13 U/ml and 9.12 g/l. With increase in benzaldehyde dosage, decline in PDC and L-PAC activity was observed. The OR of UV-t6 was 5.3, while of wild-type ISL-7 was 4.1. (Seifiet *al*. 2020) suggested benzaldehyde

concentration 2 g/l as optimal one for production of L-PAC. This was also shown by other studies (Doostmohammadi*et al*., 2016; Khan *et al*., 2012). In another study, maximum L-PAC production was also observed at 2 g/l benzaldehyde concentration using immobilized cells of *Candida utilis* (Shin and Rogers, 1995) but Mahmoud *et al*. (1989) suggested that 6 g/l benzaldehyde concentration resulted in maximum L-PAC production by *S. cerevisiae*. However, these workers used immobilized cells of different species. The viability of cells decreased with increase in concentration of benzaldehyde. The benzaldehyde concentration is very critical factor in determining benzaldehyde biotransformation to L-PAC (Long and Ward, 1989; Agarwal *et al*., 1987), but it also shows toxic effects on enzymatic functions and rate of cell growth (Long and Ward, 1989).

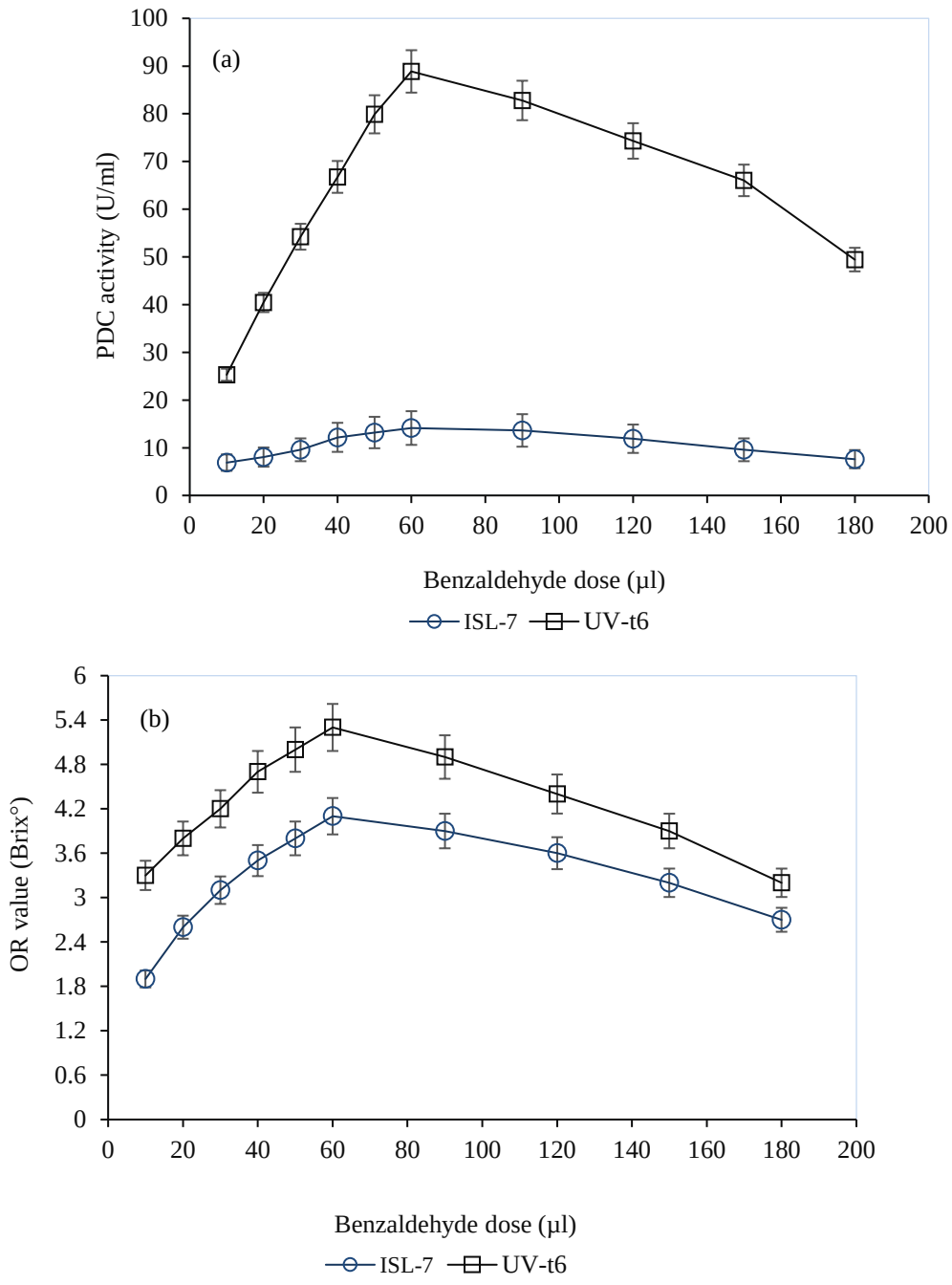

Fig. 4.2(a,b): Effect of benzaldehyde on PDC activity and OR value by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Time of incubation 6 h, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~535 nm.

The y-error bars indicate standard deviation (±sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

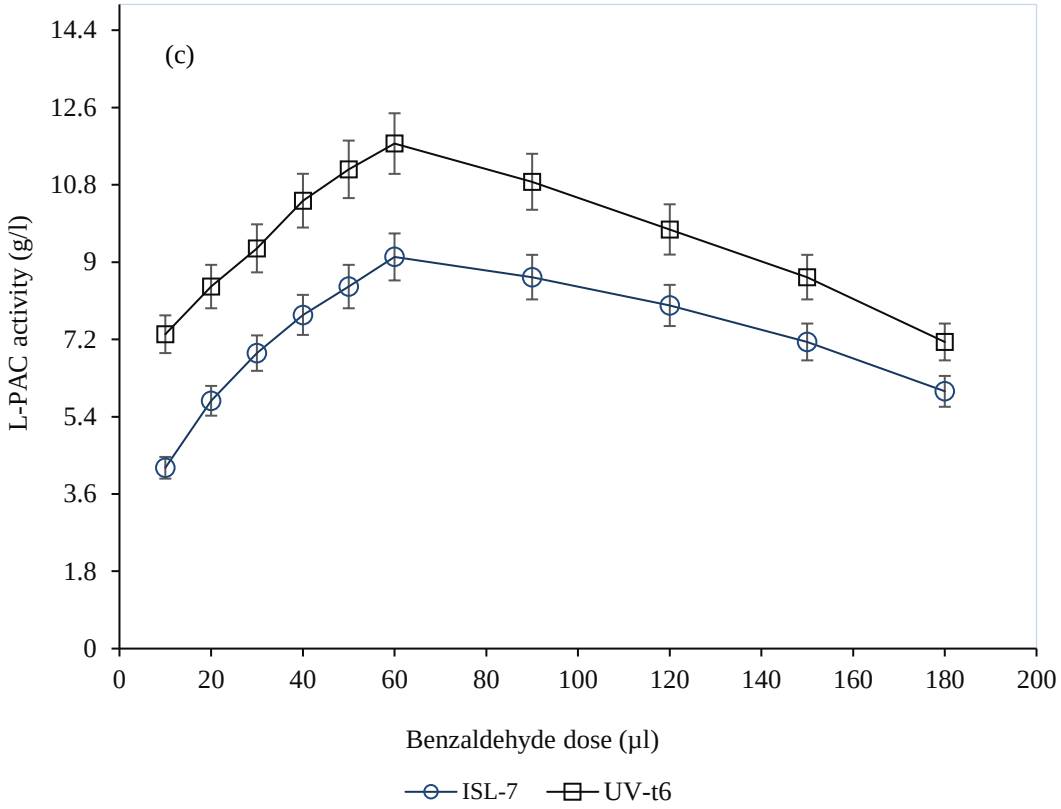

Fig. 4.2c: Effect of benzaldehyde on L-PAC activity by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Time of incubation 6 h, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~410 nm.

The y-error bars indicate standard deviation (±sd set at 5-10 %) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

4.4. Time of incubation for benzaldehyde biotransformation

The effect of incubation time was studied for wild-type and auxotrophic yeast strain of *S. cerevisiae* as shown in Fig. 4.3. The different time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9 h) were compared. The maximum PDC and L-PAC activity was observed after 4 h of incubation at 28ºC for 60 µl. The PDC and L-PAC activity was decreased with increase in incubation time. The maximum PDC and L-PAC activity observed for UV-t6 was 95.99U/ml and 22.46 g/l. While PDC and L-PAC activity of wild-type ISL-7 was 16.31U/ml and 15.89 g/l and it was low as compared to auxotrophic mutant strain. The supernatant OD_{575nm} of UV-t6 and ISL-7 was 0.242 and 0.121. The dry cell mass was also obtained for wild-type and auxotrophic mutant strain and it was 17 g/l for UV-t6

and 13 g/l for ISL-8. The optical rotation was also measured such as 5.8 for UV-t6 and 4.6 for ISL-7. While in a study conducted by Long and Ward (1989), maximum enzyme activity was observed after 6 h of incubation using 2 g/l benzaldehyde concentration. Mahmoud *et al*. (1990) suggested that benzaldehyde biotransformation rate increased during 2-3 h. The increase in biotransformation rate continued up to 5-9 h and it decreased after 10 h. The viability of cell is adversely affected by extending exposure to benzaldehyde (Mandwal *et al*., 2004).

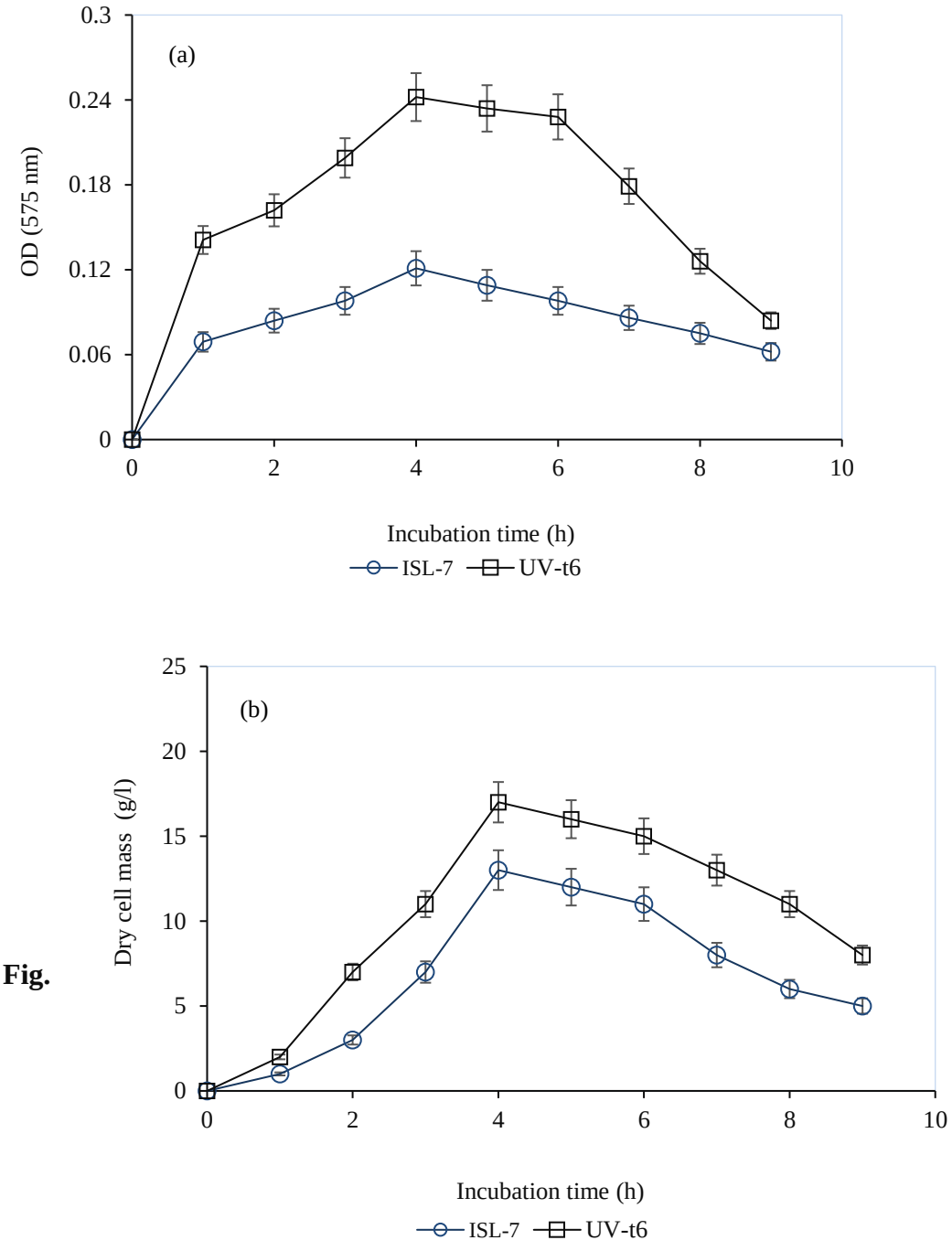

Fig.

4.3(a,b): Effect of incubation time on OD and dry cell mass by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Benzaldehyde 60 µl, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~535 nm.

The y-error bars indicate standard deviation (±sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

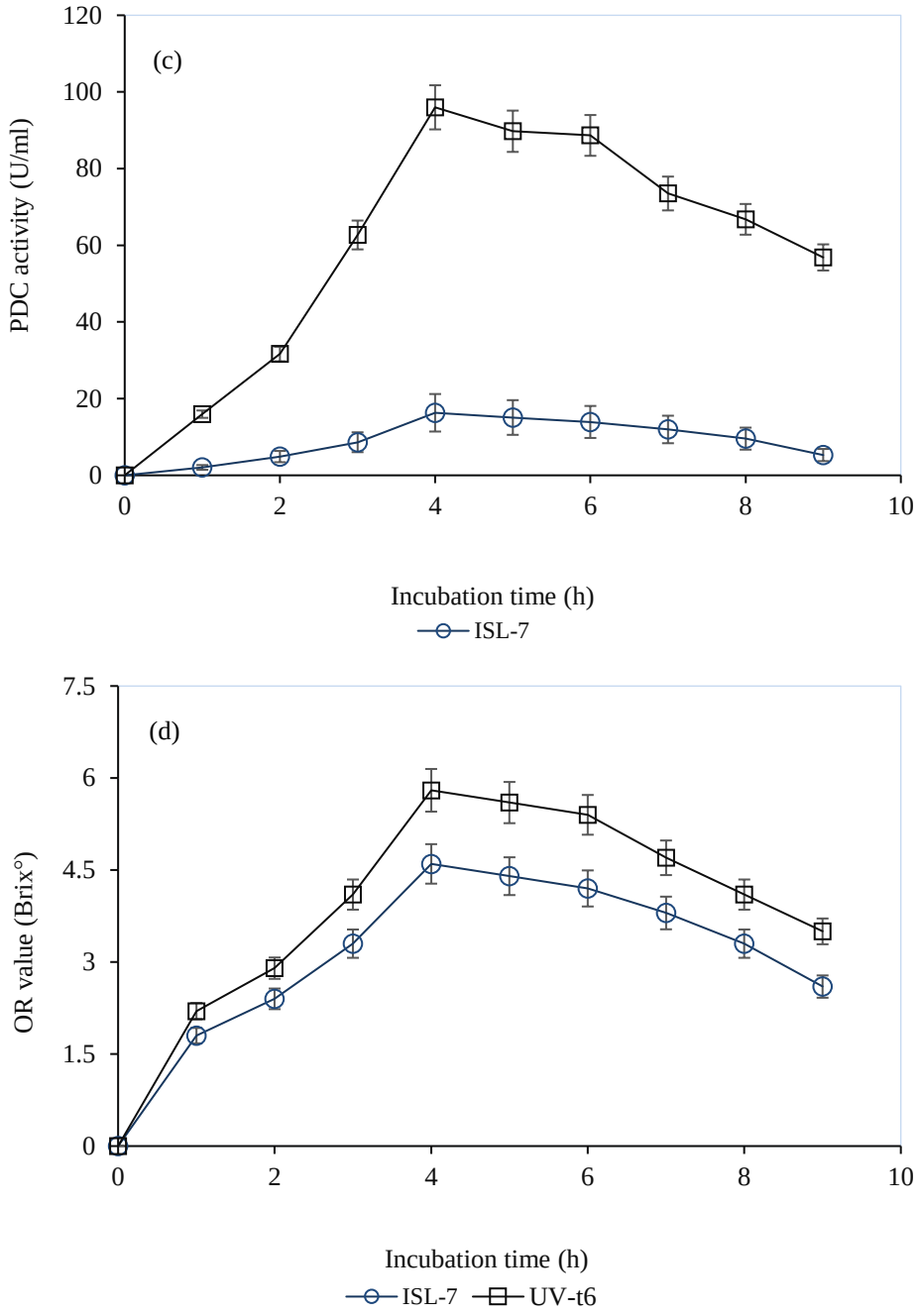

Fig. 4.3(c,d): Effect of incubation time on PDC activity and OR value by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Benzaldehyde 60 µl, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~410 nm.

The y-error bars indicate standard deviation (±sd set at 5-10 %) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

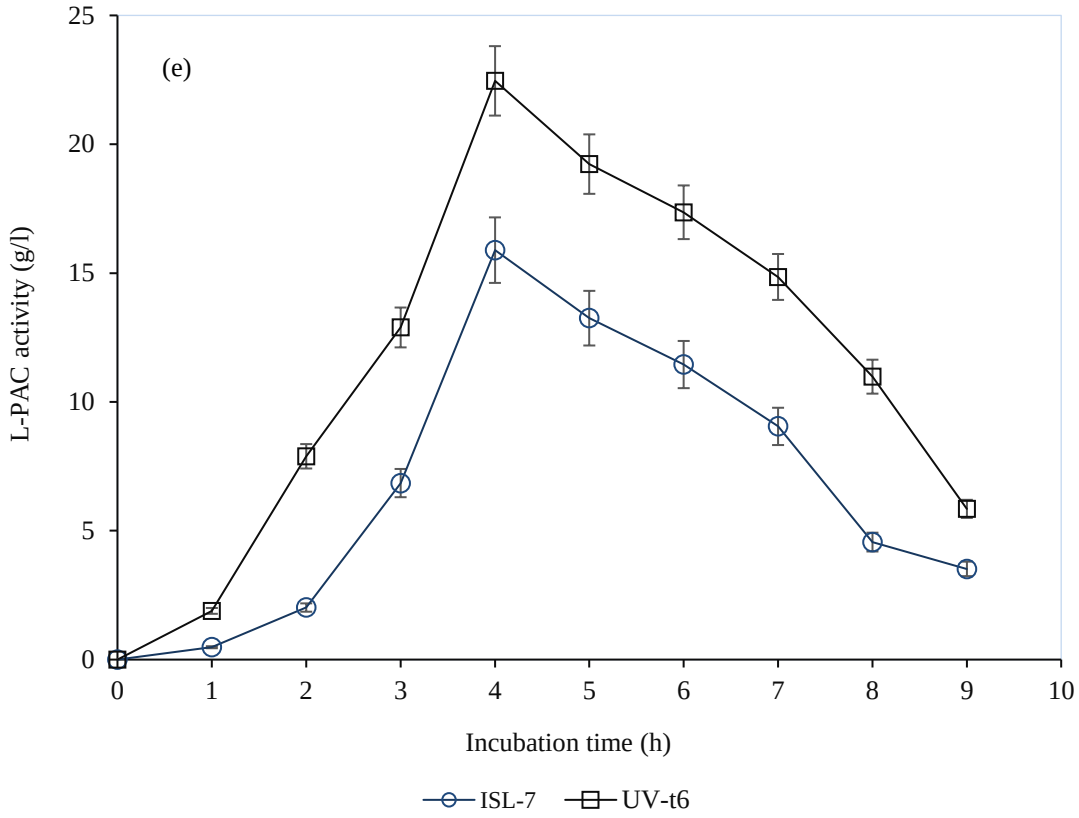

Fig. 4.3e: Effect of incubation time on L-PAC activity by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Benzaldehyde 60 µl, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~410 nm.

The y-error bars indicate standard deviation (±sd set at 5-10 %) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

4.5. Kinetic study for L-PAC production

The kinetic study was also performed for effective biotransformation of benzaldehyde to L-PAC. The different parameters such as specific growth rate µ(h^{-1}), product yield coefficient ($Y_{p/x}$), volumetric rate constant for product formation (Q_p), volumetric rate constant for cell mass production (Q_x), specific rate constant for product formation (q_p) and specific rate constant for cell mass production (q_x) were studied as shown in Fig. 4.4.The mutant exhibited higher value of L-PAC fermentative kinetics such as volumetric rate constant (Q_p) 5.61 g/g/h, (Q_x) 4.25 g/g/h and specific rate constant (q_p) 5.68 g/l/h, (q_x) 3.23 g/l/h as compared to wild-type. The mutant showed lower specific

growth rate 4.3 μ(h^{-1}) and lower product yield coefficient 1.32 g/g/l. Hence L-PAC productivity found to be more in auxotrophic mutant strain than wild-type. In a similar study, Seely*et al*. (1989) also reported that maximum specific rate ($Q_{p)}$ was exhibited by immobilized mutant *S. cerevisiae*. Hussain *et al*. (2012) also studied kinetic parameters and suggested that mutant showed enhanced production of L-PAC than wild-type.

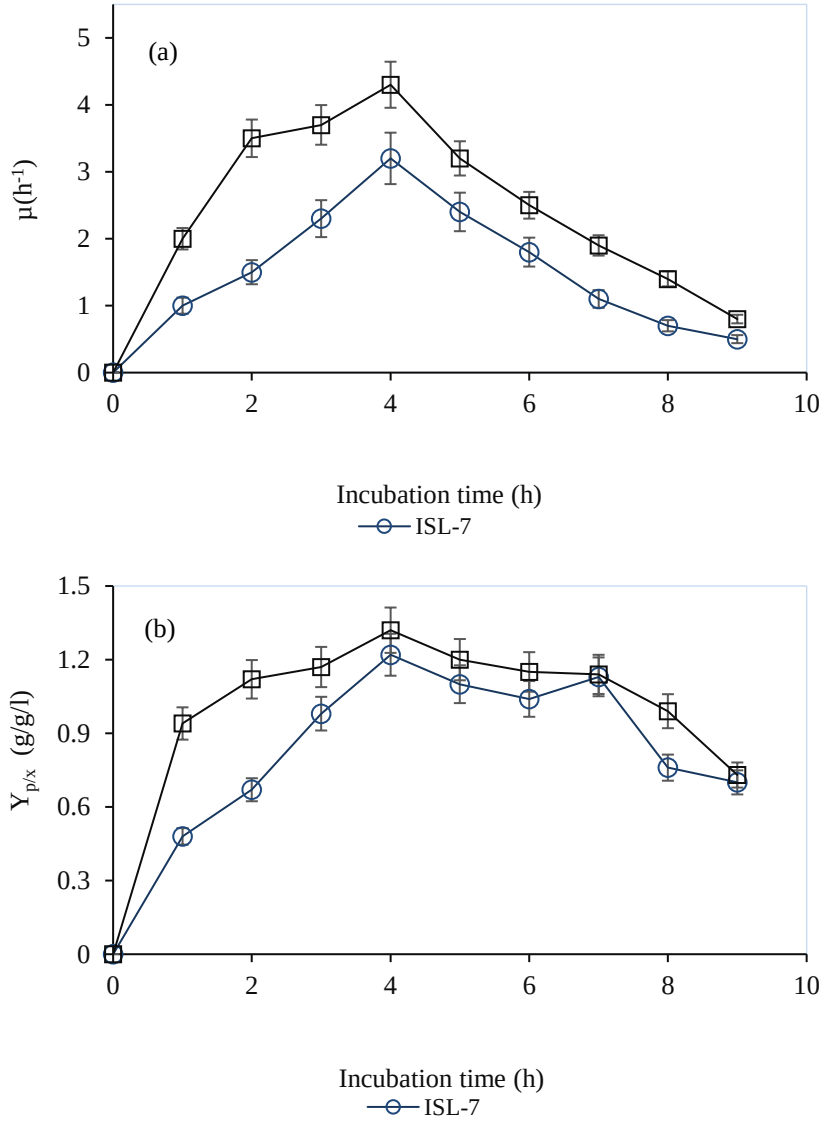

Fig. 4.4(a,b): Effect of specific growth rate μ(h^{-1}) and product yield coefficient ($Y_{p/x}$) on L-PAC production by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Fermentation conditions: Benzaldehyde 60 µl, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~535 nm

Kinetic variables: μ(h^{-1})= specific growth rate, $Y_{p/x}$(g/g/l)= product yield coefficient.

The y-error bars indicate standard deviation (±sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

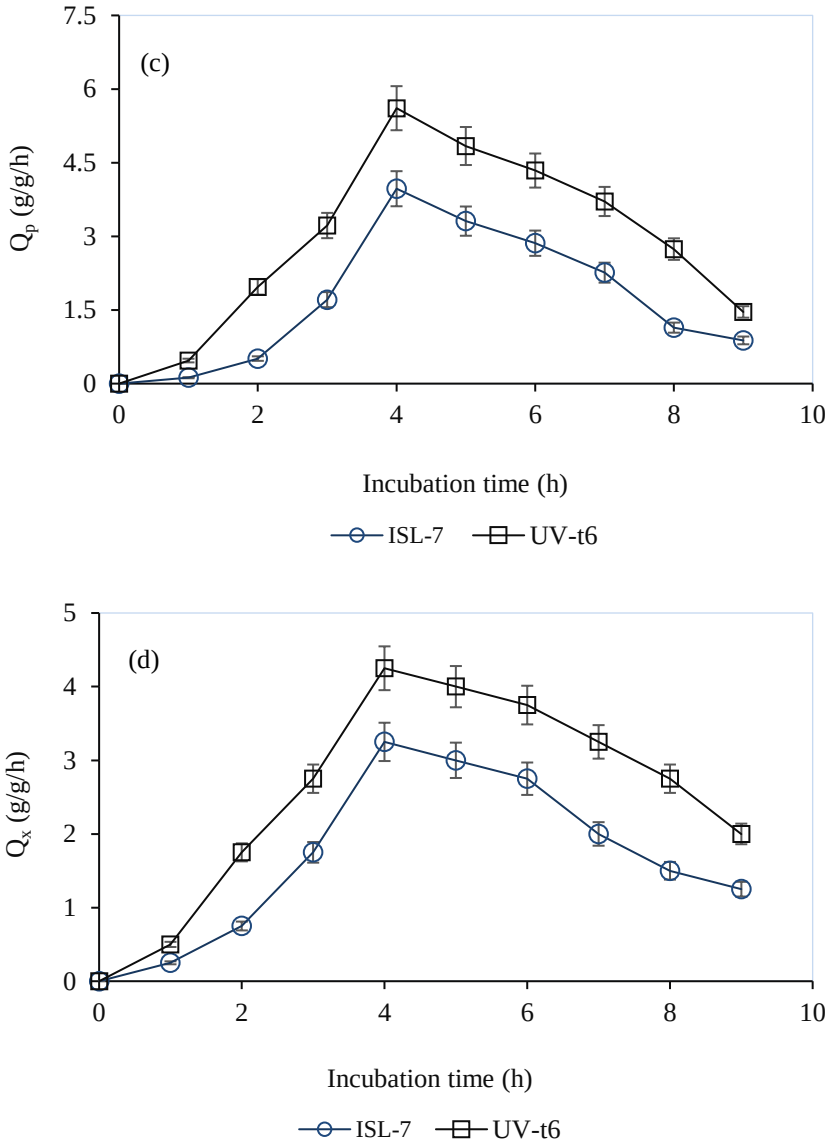

Fig. 4.4(c,d): Effect of volumetric rate constant for product (Q_p) and cell mass production (Q_x) on L-PAC production by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Fermentation conditions: Benzaldehyde 60 µl, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~410 nm

Kinetic variables: $\mu(h^{-1})$= Q_p(g/g/h)= volumetric rate constant for product formation, Q_x(g/g/h)= volumetric rate constant for cell mass production.

The y-error bars indicate standard deviation (±sd set at 5-10 %) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

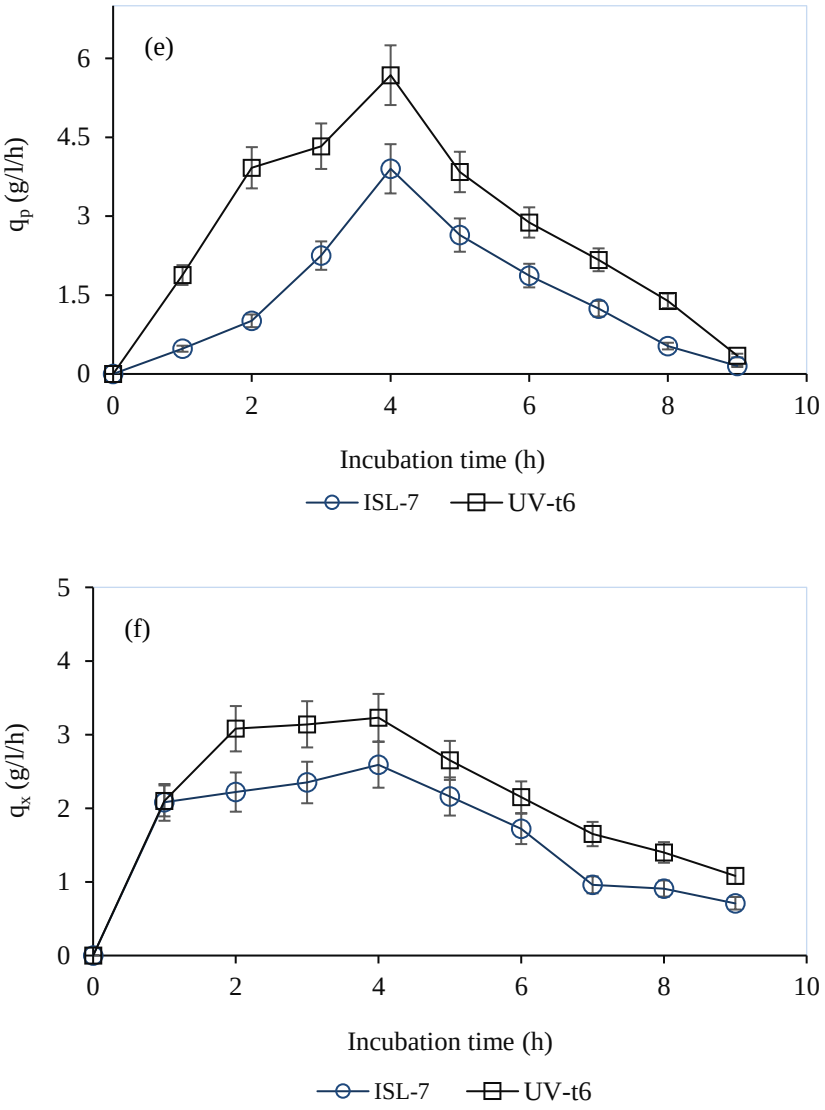

Fig. 4.4(e,f): Effect of specific rate constant for product (q_p) and mass formation(q_x) on L-PAC production by wild-type and auxotrophic mutant strain of *S. cerevisiae**

*Fermentation conditions: Benzaldehyde 60 µl, temperature 28˚C, medium pH 5, inoculum size 2 ml (8%, v/v), wavelength~535 nm

Kinetic variables: q_p(g/l/h)=specific rate constant for product formation, q_x(g/l/h)=specific rate constant for cell mass production.

The y-error bars indicate standard deviation (±sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

4.6. Comparative kinetic analysis for L-PAC production

The comparative kinetic analysis was also performed for effective biotransformation of benzaldehyde to L-PAC by wild-type and auxotrophic mutant strain of *S. cerevisiae* as

shown in table 4.2. The kinetic variables were studied at different time intervals (3, 4, 5 h). The auxotrophic mutant exhibited maximum L-PAC production than wild-type and maximum values were observed after 4 h of incubation. The auxotrophic mutant showed maximum Q_p and q_p values (5.61 g/g/h and 5.68 g/l/h) than wild-type (3.97 g/g/h and 3.90 g/l/h). The specific growth rate and product yield coefficient was also highest for auxotrophic mutant (4.3 $\mu(h^{-1})$ and 1.32 g/g/l) than wild-type (3.2 $\mu(h^{-1})$ and 1.22 g/g/l). In another study, Santianegara *et al*. (2006) also reported a higher volumetric rate constant. However, these workers used *C. utlilis* to produce R-PAC. While Rajoka *et al*. (2005) reported higher fermentative kinetic values and lower growth as compared to wild-type. The alterations observed in culture behavior by mutant might be due to mutation.

Table 4.2: Comparative analysis of kinetic variables for effective biotransformation of benzaldehyde to L-PAC by wild-type and auxotrophic mutant strain of *S. cerevisiae**

Kinetic variables	Kinetic quotients	Units	Kinetic models	Time of incubation (h)					
				3		4		5	
				ISL-7	UV-t6	ISL-7	UV-t6	ISL-7	UV-t6
Specific growth rate	μ	h^{-1}	Growth rate	2.3±0.23	3.7±0.29	3.2±0.2	4.3±0.34	2.4±0.2	3.2±0.2
Product yield coefficient	$Y_{p/x}$	g/g/l	Yield coefficient	0.9±0.06	1.2±0.08	1±0.08	1.3±0.09	1±0.07	1.2±0.08
Volumetric rate constant for product formation	Q_p	g/g/h	Volumetric rate	1.7±0.15	3.2±0.25	3.9±0.3	5.6±0.44	3.3±0.2	4.84±0.3
Volumetric rate constant for cell mass production	Q_x	g/g/h	Volumetric rate	1.75±0.1	2.7±0.19	3.2±0.2	4.2±0.29	3.0±0.2	4.0±0.28
Specific rate constant for product formation	q_p	g/l/h	Specific rate	2.25±0.2	4.33±0.4	3.9±0.4	5.68±0.5	2.6±0.3	3.8±0.38
Specific rate constant for cell mass formation	q_x	g/l/h	Specific rate constants	2.3±0.28	3.14±0.3	2.5±0.3	3.23±0.3	2.1±0.2	2.6±0.26

*Fermentation conditions: Benzaldehyde 60 µl, temperature 28˚C, medium pH 5, inoculums size 2 ml (8%, v/v), wavelength~535 nm.
Kinetic variables: $\mu(h^{-1})$=Specific growth rate, $Y_{p/x}$(g/g/l)=Product yield coefficient, Q_p(g/g/h)=Volumetric rate constant for product formation, Q_x(g/g/h)=Volumetric rate constant for cell mass production, q_p(g/l/h)=Specific rate constant for product formation, q_x(g/l/h)=Specific rate constant for cell mass production.

± indicate standard deviation (sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

4.7. Immobilization of PDC of auxotrophic mutant strain of *S. cerevisiae* using calcium alginate beads

4.7.1. Enzyme concentration

The PDC of auxotrophic mutant strain UV-t6 was immobilized by using calcium alginate beads. The different enzyme concentrations (0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3 ml) were compared. The maximum PDC and L-PAC activity (116.22 U/ml and 13.72 g/l) was observed for immobilized enzyme at enzyme concentration 1.5 ml as shown in Fig. 4.5 (a,b). While PDC and L-PAC activity for free enzyme was 115.20 U/ml and 12.97 g/l. The PDC and L-PAC activity started to decrease with increase in enzyme concentration. The lowest enzyme activity was observed for enzyme concentration 3 ml. In a similar study, Shin and Rogers (1995) also used calcium alginate beads to produce L-PAC and L-PAC activity reported was 15.2 g/l. However, these workers used *C. utilis* immobilized cells to produce L-PAC. In another study, PDC entrapment in polyacrylamide gel provided higher capacity of immobilization than to adsorption on cationic exchange resin surface (Shin and Rogers, 1995). Shukla and Kulkarni (2003) suggested barium alginate to be better immobilizing matrix than calcium alginate. The immobilized cells suggested enhanced production of L-PAC and increased resistance to benzaldehyde (Shin and Rogers, 1995; Mahmoud *et al*., 1990).

4.7.2. Effect of bead size

The effect of bead size was studied for effective biotransformation of benzaldehyde to a stable L-PAC by auxotrophic mutant strain of *S. cerevisiae* UV-t6. The calcium alginate beads of different sizes (0.5, 1, 1.5, 2, 2.5, 3, 3.5 mm) were formed. The maximum PDC and L-PAC activity was observed for bead size 3 mm i.e. 120.20 U/ml and 13.85 g/l. The lowest PDC and L-PAC activity was 67.64 U/ml and 10.16 g/l, which is observed for bead size 0.5 mm as shown in Fig. 4.5 (c). In contrary, Gilson and Thomas (1995) suggested that smaller size beads were less prone to as compared to beads of larger diameter.

4.7.3. Effect of cell holding time

The effect of cell holding time was observed for effective biotransformation of benzaldehyde to a stable L-PAC by auxotrophic mutant strain of *S. cerevisiae* UV-t6 by using free and immobilized enzyme as shown in Fig. 4.5 (d,e). The different time intervals (5, 10, 15, 20, 25, 30, 35 min) were compared. The maximum PDC and L-PAC activity was observed after 10 min for immobilized enzyme i.e. 136.15 U/ml and 14.56 g/l, while PDC and L-PAC activity was low for free enzyme i.e. 128.76 U/ml and 13.12 g/l. The lowest PDC and L-PAC activity was observed after 35 min that showed increase in time interval resulted in decline in PDC and L-PAC activity. Dorota *et al*. (2013) also suggested that immobilization is preferable than free cell system with respect to enzyme stability and productivity.

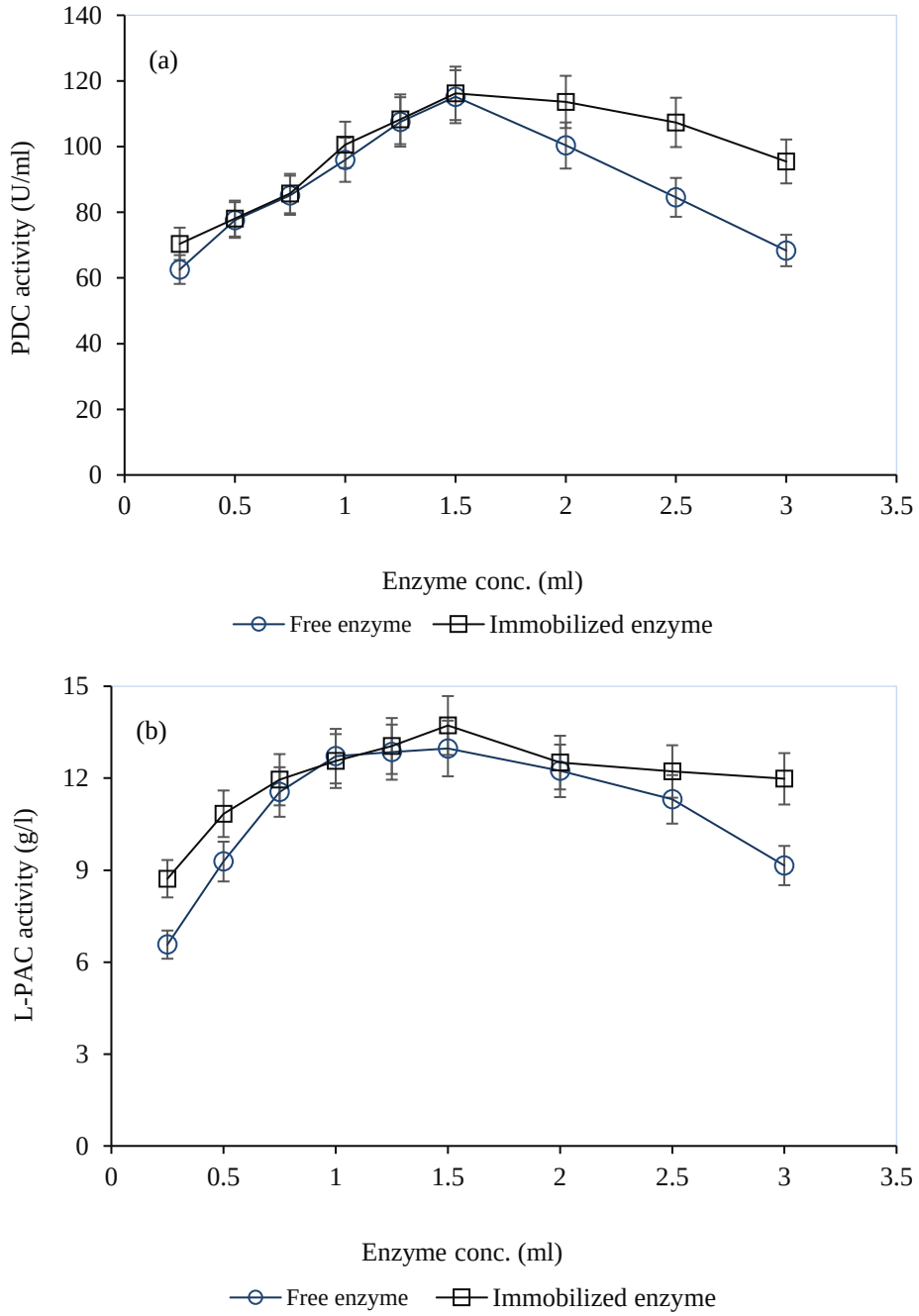

Fig. 4.5(a,b): Effect of enzyme conc. on PDC and L-PAC activity by auxotrophic mutant strain of *S. cerevisiae* using calcium alginate beads*

*Time of incubation 5 min, bead size 3 mm, temperature 28˚C, enzyme conc. 1.5 ml, wavelength~535 nm.

The y-error bars indicate standard deviation (±sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set under one way ANOVA.

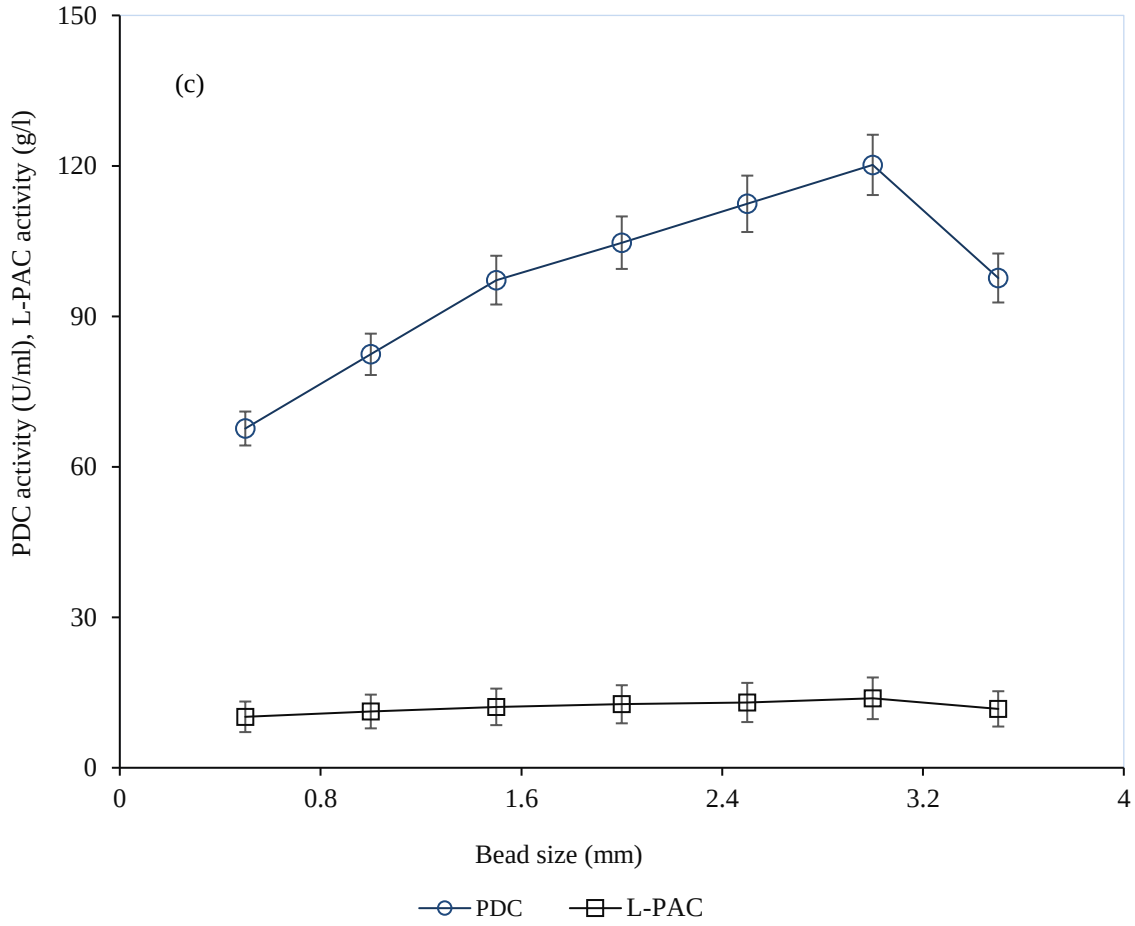

Fig. 4.5c: Effect of bead size on PDC and L-PAC activity by auxotrophic mutant strain of *S. cerevisiae* using calcium alginate beads*

*Time of incubation 5 min, temperature 28˚C, enzyme conc. 1.5 ml, wavelength~535 nm.

The y-error bars indicate standard deviation (±sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set under one way ANOVA.

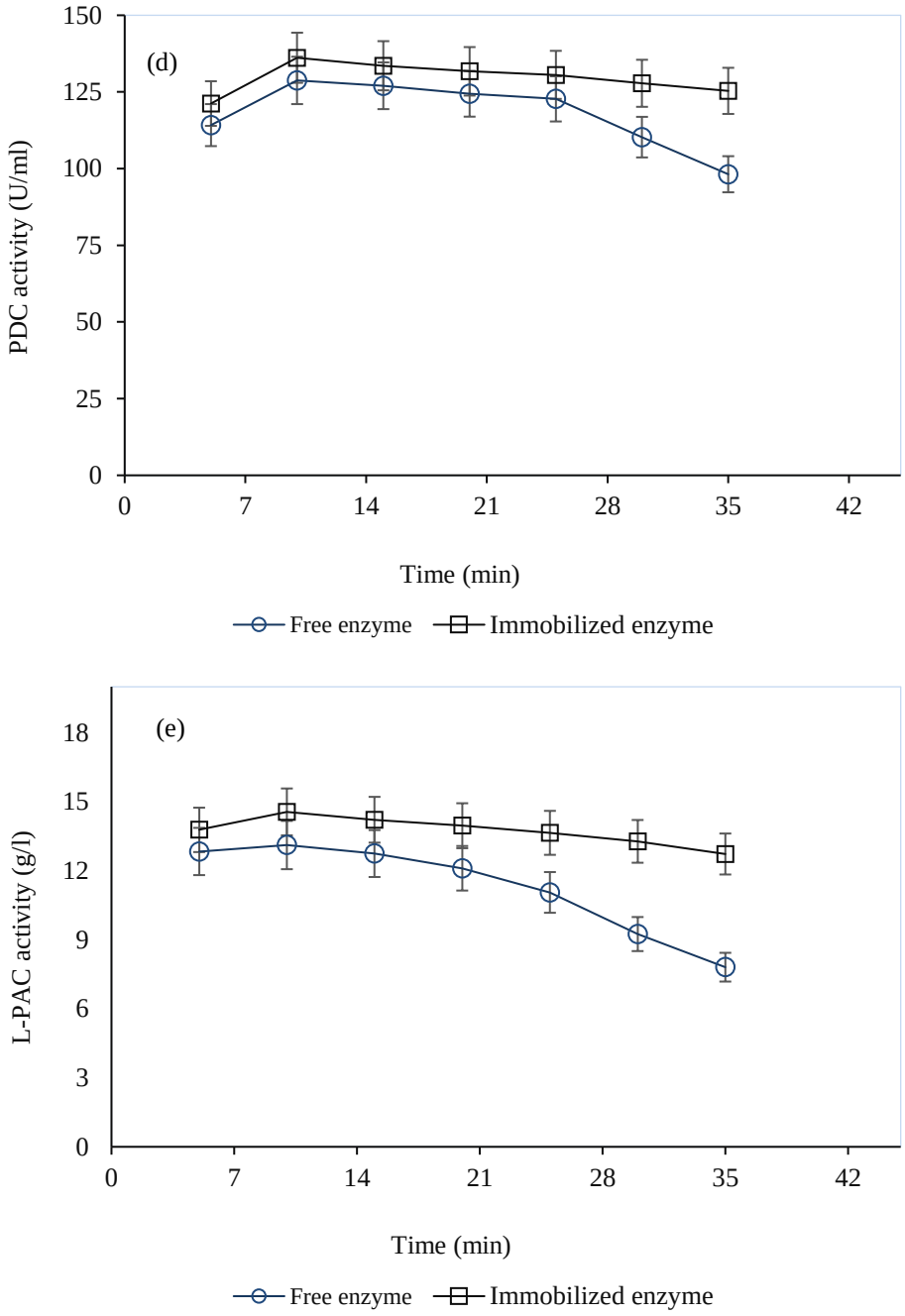

Fig. 4.5(d,e): Effect of time on PDC and L-PAC activity by auxotrophic mutant strain of *S. cerevisiae* using calcium alginate beads*

*Time of incubation 5 min, bead size 3 mm, temperature 28˚C, enzyme conc. 1.5 ml, wavelength~535 nm.

The y-error bars indicate standard deviation (±sd set at 5%) amongst the values of three parallel replicates. The sum means values differ significantly at $p \leq 0.05$ from each other in one set, under one way ANOVA.

Conclusion

The significant achievement of the present study lies in the development of an auxotrophic mutant strain and PDC immobilization of auxotrophic mutant by using calcium alginate beads for effective benzaldehyde biotransformation to a stable L-PAC. In the present study, the wild-type strain of *Saccharomyces cerevisiae* ISL-7 was exposed to UV treatment for mutagenesis. Among all auxotrophic mutant strains, UV-t6 was selected for better production of L-PAC. The auxotrophic mutant showed maximum L-PAC production than wild-type when exposed to UV radiations. The auxotrophic mutant exhibited 1.25-fold increase in L-PAC production than wild-type. The auxotrophic mutant also exhibited higher values of fermentative kinetics as compared to wild-type. The PDC of auxotrophic mutant was immobilized using calcium alginate beads and exhibited higher L-PAC activity than free enzyme. It also exhibited 1.68-fold increase in production of L-PAC than wild-type. The immobilization of PDC in calcium alginate is inexpensive and experimentally simple. The immobilization of PDC of auxotrophic mutant provided possible way of achieving long-term production of L-PAC.

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