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Process optimization for biosynthesis of pyruvate decarboxylase (PDC) and Neuberg's ketol (PAC) from a novel *Pichia cecembensis* through response surface methodology

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Abstract

Purpose: Phenylacetylcarbinol (PAC) is an intermediate for the synthesis of several active pharmaceutical ingredients (ephedrine, pseudoephedrine, norephedrine, etc.) used for the production of antiasthematics and decongestants. An efficient biosynthesis of PAC through condensation of benzaldehyde and acetaldehyde catalyzed by a solvent tolerant pyruvate decarboxylase (PDC) is being reported. A process for the biosynthesis of PAC was designed and optimized through response surface methodology (RSM) in the present study.

Methods: The effects of incubation time (8–18 h), incubation temperature (30–38 °C), medium pH (4–10), and inoculum size (4–10%) on PAC yield, sugar consumption, and PDC activity were determined through submerged fermentation using a newly isolated potent yeast strain of *Pichia cecembensis*. PAC was quantified spectrophotometerically and through HPLC. PDC produced was exposed to 40 mM benzaldehyde as whole cells, crude extract, and partialy purified preparation to check its stability against the said solvent.

Results: The highest PDC activity and PAC yield during present study were found to be 56.27 U/ml and 8.44 g/l, respectively. The yield of PAC was increased by 71% (2.22 to 8.44 g/l) after process optimization through RSM with incubation time of 13 h, incubation temperature of 33 °C, and 18% total sugar as significant factors (*P*-values, 0.902, 0.260, and 0.247, respectively). R-squared value of 0.770 and Adeq Precision value of 4.888 show the goodness of fit of the process design. PDC is used in the form of *Pichia cecembensis* whole cells revealed higher stability towards benzaldehyde and elevated temperature as compared to partially purified PDC. Whole cells and partially purified PDC showed half-lives of 240 and 72 h at 4 °C, whereas 33 and 28.5 h at 25 °C. PAC was purified though HPLC with a purity level of 76.18%.

Conclusion: Incubation time, temperature, and sugar concentration were found to be significant factors for the biosynthesis of PAC. A newly isolated *Pichia cecembensis* produced a highly active, solvent, and temperature-tolerant pyruvate decarboxylase (PDC) which is superior to its counterpart being presently used in the industry. Hence, this novel yeast species is a promising candidate for commercial production of PAC and other related APIs owing to its highly stable PDC.

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Keywords: Response surface methodology, Statistical methodology, Phenylacetylcarbinol, Pyruvate decarboxylase, Biotransformation, *Pichia cecembensis*

Background

Pyruvate decarboxylase (PDC) is a highly important enzyme in the pharmaceutical and chemical industries but unfortunately has been ignored for centuries (Strommer and Garabagi 2009). PDC exhibits two types of activities, namely decarboxylation and carboligation. In the former, it produces acetaldehyde and carbon dioxide from pyruvate (Nichols et al., 2003), whereas in the latter, it adds carbon from benzaldehyde into the acetaldehyde resulting in the production of a large number of products like phenylacetylcarbinol (PAC), benzyl alcohol, benzoic acid, formic acid, and acetaldehyde (Suresh et al. 2009, Meyer et al. 2010; Thitipraser et al. 2014). Enzymes, involved in decarboxylation (a critical reaction in fermentation process) including PDC, is thymidine phosphate (ThdP)-dependent enzymes and belongs to ligases (Nichols et al. 2003; Andrews and McLeish 2012).

PAC is an intermediate compound in the production of active pharmaceutical ingredients (API), i.e., norephedrine, L-ephedrine, pseudoephedrine, norephedrine, and norpseudoephedrine. Alternatively, ephedrine being an important anti-asthmatic drug is traditionally extracted from ephedra (*Ephedra sinica*), a gymnosperm, not abundantly found in most parts of the world. In addition, they are not easy to cultivate, and the extraction procedures are cumbersome (Abourashed et al. 2003). Ephedrine and pseudoephedrine comprise a large group of molecules which participate in important physiological functions and pharmaceutically valuable bioactivities (Moris et al. 2018). Other products of PDC are important bacteriostatic preservatives (benzoic acid and its salts), paint emulsifiers, bacteriostatic agents, and cosolvent in a variety of liquid pharmaceutical preparations (benzyl alcohols) and food preservatives (salts of benzoic acids) (Roshe et al. 2002a; Sudareva and Chubarova 2006; Suresh et al. 2009).

Pyruvate decarboxylase can be produced from yeasts (*Saccharomyces cerevisiae*, *Candida utilis*, *Cyberlindnera jadinii*, etc.) and bacteria (*Zymomonas mobilis*) through fermentation. Yeast pyruvate decarboxylase is preferable over bacterial pyruvate decarboxylase because bacterial pyruvate decarboxylases are more sensitive towards benzaldehyde and lose their productivity after a short time of exposure to benzaldehyde. Therefore, yeasts are the most suitable sources of PDC production on industrial scale (Raj et al. 2002). Fermentation process is affected by many physical and chemical parameters, so the conventional optimization of fermentation by varying one parameter at a time is time-consuming and difficult to manage. Moreover, interaction among variables is not considered in such practices. If interaction among the factors is ignored, it is difficult to reach optimum factors with best interactions (Mason et al. 2003). Response surface methodology (RSM) is a statistical tool for experimental design and screening of factors to select significant factor (through linear and co-relation regression analysis) and selection of the best influence of the significant factors over product formation (Mason et al. 2003; Mushtaq et al. 2014).

In present study, initial experiments were designed through Plackett-Burman model (PBM) via linear regression analysis to select significant factors from among different physical and chemical factors, i.e., time of fermentation (h), incubation temperature (°C), inoculum size (%, v/v), pH of medium, conc. of sugar (%, v/v), conc. of urea (%, w/v), conc. of $MgSO_4$ (%, w/v), and conc. of TPP (%, w/v). Central composite design (CCD) was used for colinear regression analysis. Stability of PDC as whole cells, crude extract, and partially purified enzyme was also evaluated.

Results and discussion

Different carbon sources were screened for the production of PDC and biosynthesis of PAC. Cane molasses were the best carbon source as it produced highest amounts of PAC, 2.22 ± 0.02 (g/l) as compared to other sources like pure cheese whey (whey without suspended proteins), whole cheese whey (whey with suspended proteins), glucose, maltose, fructose, and carbon sulfite liquor (Fig. 1). Researchers have reported cane molasses as a rich source of sugars, vitamins, minerals, and a number of other nutrients and an excellent carbon source for growth of yeasts for the production of different enzymes and other metabolites (Aguilar et al 2002; Sughra et al. 2013) besides its easy availability round the year.

Response surface optimization

Response surface methodology was used in the present study for the optimization of important fermentation parameters. Several researchers have used it for many novel research projects like synthesis of nanoparticles (Othman et al., 2017), bioethanol (Darvishi and Moghaddami, 2019), and pretreatment of certain substrates (Asadi and Zilouei, 2016) due to its robust action and sound statistical studies.

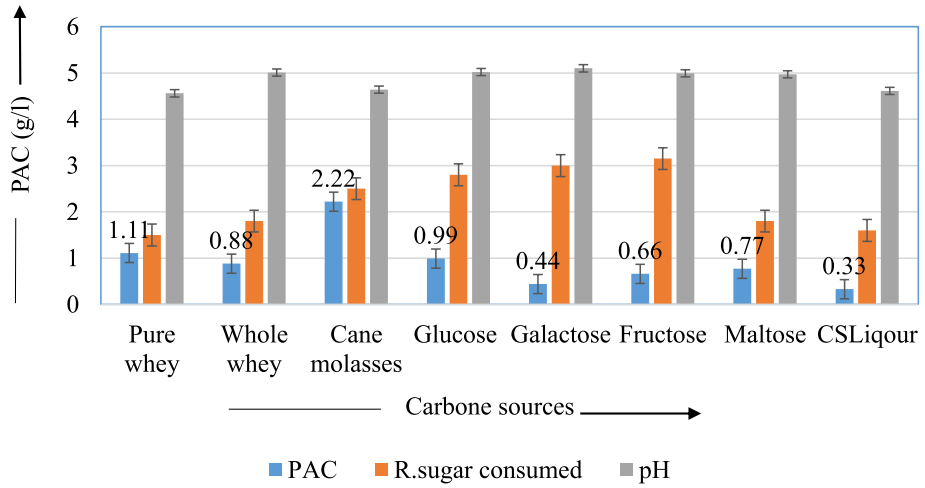

Fig. 1 Screening of carbon sources for pyruvate decarboxylase production

Plackett-Burman model (PBM)

Plackett-Burman model (PBM) is used for linear regression analysis of factors. Four responses (PDC activity, final pH, sugar consumed, and PAC) were used for analysis of eleven factors (Table 1). The model was significant as *P*-value for design was less than 0.05. Standard error of design (Fig. 2) was smaller and similar within all types of coefficients. Variance inflation factor (VIF) was found to be 1.0, hence satisfactory. Ri^2 for all factors was 0.00 which reveals that the individual factors have no interference with the others. Sugar conc. (%), incubation time (h), and temperature of fermentation (°C) were selected as significant factors. The factors are significant if they have *P*-values less than 0.05, R^2 less than 1, and factors are not correlated with one another.

As per ANOVA, *P*-values for initial sugar (%), time (h), and temperature (°C) were less than 0.05; therefore, these were significant factors. Central composite design (CCD) was designed using selected factors to determine their corelation and to optimize for higher production. Asif et al. (2012) has also designed experiments for the optimization of protease production using central composite design.

Central composite design and evaluation

Sugar conc. (%), incubation time (h), and temperature of fermentation (°C) were used as input factors for CCD (Table 2) and studied through four responses as PDC activity (mmol/l) final pH, sugar consumed (%), and PAC (g/l) produced. The fitted models in terms of

Table 1 Plackett-Burman model for linear regression analysis of all factors

	Std	Run	Block	Factor 1 A:Sugar Co %	Factor 2 B:Time Hrs	Factor 3 C:Temperature oC	Factor 4 D:pH	Factor 5 E:Inoculum Siz %, (v/v)	Factor 6 F:Urea %, (w/v)	Factor 7 G:MgSO4 %, (w/v)	Factor 8 H:TPP %, (w/v)	Factor 9 J:Di.Ammoniu g/L	Factor 10 K:DI. Sodium g/L	Factor 11 L:Di.Pot g/L	Response 1 PDC activity Unit	Response 2 Final pH	Response 3 sugar consume %, v/v	Response 4 PAC g/l
	12	1	Block	15.0	8.00	30.0	4.00	4.00	0.0100	0.00100	0.0200	0.100	0.100	0.100	5.80	4.01	3.00	0.880
	3	2	Block	21.0	8.00	38.0	10.0	4.00	0.500	0.100	0.0800	0.100	0.100	0.100	14.8	9.17	2.80	2.22
	14	3	Block	18.0	13.0	34.0	7.00	7.00	0.255	0.0505	0.0500	1.55	1.55	1.55	45.8	6.46	2.50	6.88
	1	4	Block	21.0	18.0	30.0	10.0	10.0	0.500	0.00100	0.0200	0.100	3.00	0.100	7.40	9.86	3.40	1.11
	7	5	Block	21.0	8.00	30.0	4.00	10.0	0.0100	0.100	0.0800	0.100	3.00	3.00	13.2	3.91	1.90	1.99
	13	6	Block	18.0	13.0	34.0	7.00	7.00	0.255	0.0505	0.0500	1.55	1.55	1.55	17.7	6.67	2.00	2.66
	9	7	Block	21.0	18.0	38.0	4.00	4.00	0.0100	0.100	0.0200	3.00	3.00	0.100	11.8	3.89	2.18	1.77
	5	8	Block	15.0	8.00	38.0	4.00	10.0	0.500	0.00100	0.0800	3.00	3.00	0.100	11.8	3.87	2.18	1.77
	11	9	Block	21.0	8.00	38.0	10.0	10.0	0.0100	0.00100	0.0200	3.00	0.100	3.00	16.2	9.45	2.80	2.44
	4	10	Block	15.0	18.0	30.0	10.0	10.0	0.0100	0.100	0.0800	3.00	0.100	0.100	5.10	10.0	3.75	0.770
	2	11	Block	15.0	18.0	38.0	4.00	10.0	0.500	0.100	0.0200	0.100	0.100	3.00	5.13	4.02	5.00	0.000
	10	12	Block	15.0	18.0	38.0	10.0	4.00	0.0100	0.00100	0.0800	0.100	3.00	3.00	8.86	9.98	3.00	1.33
	6	13	Block	15.0	8.00	30.0	10.0	4.00	0.500	0.100	0.0200	3.00	3.00	3.00	16.3	9.79	2.70	2.44
	8	14	Block	21.0	18.0	30.0	4.00	4.00	0.500	0.00100	0.0800	3.00	0.100	3.00	20.7	4.17	3.15	3.11

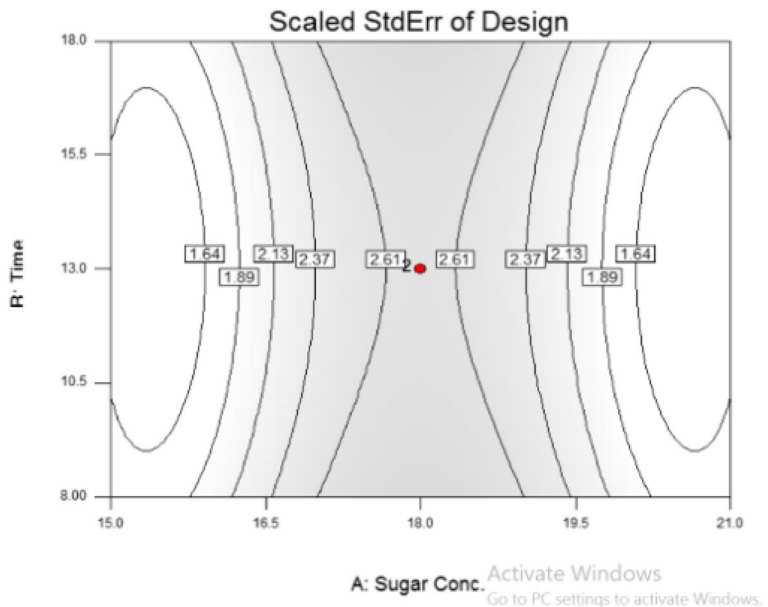

Fig. 2 Scaled standard error of design

the coded values of sugar conc. (A), time (B), and temperature (C) are given below:

$$Y_{\text{PDC activity(mM/ml)}} = +48.25 + 0.44 * A + 4.12 * B - 4.24 * C - 0.27 * A * B - 0.12 * A * C + 0.26 * B * C - 13.05 * A^2 - 8.08 * B^2 - 13.05 * C^2$$

$$\begin{aligned} Y_{\text{pH}} = {} & 2.23 + 0.008 * A + -0.045 * B + 0.010 * C \\ & + -0.017 * AB + -0.0169 * AC + -0.0 \\ & * BC + 0.02 * A^2 + 0.0129 * B^2 + 0.034 \\ & * C^2 + -0.04 * ABC + 0.04 * A^2B \\ & + -0.027 * A^2C + -0.0199 * AB^2 \\ & + 0 * AC^2 + 0 * B^2C + 0 * BC^2 + 0 * A^3 \\ & + 0 * B^3 + 0 * C^3 + -0.03 * A^2B^2 \\ & + 0 * A^2BC + 0 * A^2C^2 + 0 * AB^2C \\ & + 0 * ABC^2 + 0 * B^2C^2 + 0 * A^3B \\ & + 0 * A^3C + 0 * AB^3 + 0 * AC^3 \\ & + 0 * B^3C + 0 * BC^3 + 0 * A^4 + 0 * B^4 \\ & + 0 * C^4 + 0 * A^2B^2C + 0 * A^2BC^2 \\ & + 0 * AB^2C^2 + 0 * A^3B^2 + 0 * A^3BC + 0 * A^3C^2 \\ & + 0 * A^2B^3 + 0 * A^2C^3 + 0 * AB^3C + 0 * ABC^3 \\ & + 0 * B^3C^2 + 0 * B^2C^3 + 0 * A^4B + 0 * A^4C \\ & + 0 * AB^4 + 0 * AC^4 + 0 * B^4C + 0 * BC^4 + 0 * A^5 \\ & + 0 * B^5 + 0 * C^5 \end{aligned}$$

$$Y_{\text{sugar consumed (\%, v/v)}} = +1.53254 + 0.029824 \times A + 0.0998773 \times B + -0.0382367 \times C + 0.0994692 \times AB + 0.0381083 \times AC + 0.00524738 \times BC + 0.0684663 \times A^2 + -0.0860421 \times B^2 + 0.0465525 \times C^2$$

$$Y_{\text{PAC(g/l)}} = +7.24 + 0.070 \times A + 0.62 \times B + -0.64 \times C + -0.027 \times AB + -0.027 \times AC + 0.03 \times BC + -1.95 \times A^2 + -1.20 \times B^2 + -1.95 \times C^2$$

where Y is the PDC activity (mM/ml) and PAC produced (g/l), positive sign in front of the terms indicates synergetic effect, whereas negative sign indicates antagonistic effect. RSM models for sugar consumed (%) and final pH were not significant.

Analysis of variance (ANOVA)

F-values of model for PDC activity and PAC produced were greater than 1.0 (3.71 and 3.69), *P*-values were less than 0.05 (0.0265 and 0.0269), and *P*-value for lack of fit was greater than 0.05 (0.283 and 0.2808), so the models for these responses were significant and lack of fit non-significantly. There are only 2.65 and 2.69% chances of PDC activity and PAC produced, respectively, which the *F*-value of model could occur due to noise (Table 3),

Table 2 Central composite design for regression analysis of significant factors

Std	Run	Block	Factor 1 A:Sugar Conc. %	Factor 2 B:Time Hrs	Factor 3 C:Temperature oC	Response 1 PDC activity U/ml	Response 2 Final pH	Response 3 sugar consumed %, v/v	Response 4 PAC g/l
1	1	Block 1	15.00	8.00	28.00	25	5.3	3	3.77
4	2	Block 1	21.00	18.00	28.00	26	5.4	2.7	3.99
12	3	Block 1	18.00	21.41	33.00	32.5	4.8	2.8	4.88
8	4	Block 1	21.00	18.00	38.00	14.8	4.6	3.3	2.22
13	5	Block 1	18.00	13.00	24.59	5.86	5.34	3.2	0.88
20	6	Block 1	18.00	13.00	33.00	56.27	5.11	2.2	8.44
16	7	Block 1	18.00	13.00	33.00	54.8	5.09	2.3	8.22
17	8	Block 1	18.00	13.00	33.00	55.53	4.99	2.21	8.33
15	9	Block 1	18.00	13.00	33.00	56.27	5.1	2.5	8.44
6	10	Block 1	21.00	8.00	38.00	13.27	5.3	2.7	1.99
11	11	Block 1	18.00	4.59	33.00	1.47	5.5	0.5	0.22
18	12	Block 1	18.00	13.00	33.00	37	4.8	2.4	5.55
14	13	Block 1	18.00	13.00	41.41	0	5.5	1.8	0
19	14	Block 1	18.00	13.00	33.00	32.53	4.8	2.6	4.88
3	15	Block 1	15.00	18.00	28.00	26.6	5.1	2.21	3.99
5	16	Block 1	15.00	8.00	38.00	13.27	5	3	1.99
7	17	Block 1	15.00	18.00	38.00	14.8	5.4	2.1	2.22
2	18	Block 1	21.00	8.00	28.00	26.6	5.1	2.4	3.99
10	19	Block 1	23.05	13.00	33.00	4.4	5.3	2.8	0.66
9	20	Block 1	12.95	13.00	33.00	1.46	5.17	2.5	0.22

whereas for sugar consumed and final pH, lack of fit was significant, and model was non-significant.

In case of PDC activity (Table 3) and PAC produced, coefficients of quadratic terms (A^2, B^2, and C^2) were significant model terms. *F*- and *P*-value of lack of fit (0.9529 and 0.283) implied that the lack of fit was not significant relative to the pure error. There was a little chance (28.30 and 28.0% for PDC activity and PAC models) that a "lack of fit" could occur due to noise. Morover, "Adeq Precision" measures the signal to noise ratio. Adeq Precision for PDC activity (4.888) and PAC (4.882) indicated an adequate signal. So, these models can be used to navigate the design. Models for sugar and final pH were not significant. Model for final pH considered in the fit summary was aliased; in this case, *B*, C^2, and *ABC* were significant model terms. So, the model for final pH cannot be accurately fit with design and therefore was not considered for analysis.

All possible interactions and corelations of time, sugar concentration, and temperature were very important and plotted as 3D surface graphs. Effects of these interactions over PDC activity (U/ml) and PAC produced (g/l) were studied as shown in Figs. 4A and 5A. Linear plots (Fig. 4) between standardized effects and normal %age probability for PDC activity, PAC, and sugar consumed during fermentation showed that the resultant values were uniformly distributed along a linear trend line. Box-Cox plots (Fig. 3B) for pyruvate decarboxylase activity, PAC, and sugar conc. showed that lambda for PDC activity and PAC produced was close to ideal values that are 1 for PAC and 0.048 for PDC, whereas for sugar consumed, it was beyond the limit for good models (shown by blue lines in Fig. 3B).

Moreover, as per analysis of ramps (Fig. 3C I, II, III), temperature significantly affected the activity of PDC and yield of PAC, whereas smooth ramps for sugar conc. and time showed that these factors have no significancant effect on production of PDC and its products. Positive impact of temperature has been reported by many researchers (Shukla and Kulkarni, 2002; Andrews and McLeish, 2012). Optimum temperature

Table 3 Analysis of variance (ANOVA) for the fitted models of CCD for responses

ANOVA for PDC activity (U/ml)

Response 1 PDC activity

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	5.42E+003	9	603.	3.71	0.0265	significant
A-Sugar Conc.	*2.59*	*1*	*2.59*	*0.0159*	*0.902*	
B-Time	*232.*	*1*	*232.*	*1.43*	*0.260*	
C-Temperature	*246.*	*1*	*246.*	*1.51*	*0.247*	
AB	*0.605*	*1*	*0.605*	*0.00373*	*0.953*	
AC	*0.125*	*1*	*0.125*	*0.000770*	*0.978*	
BC	*0.530*	*1*	*0.530*	*0.00327*	*0.956*	
A^2	*2.45E+003*	*1*	*2.45E+003*	*15.1*	*0.00302*	
B^2	*940.*	*1*	*940.*	*5.79*	*0.0369*	
C^2	*2.45E+003*	*1*	*2.45E+003*	*15.1*	*0.00302*	
Residual	1.62E+003	10	162.			
Lack of Fit	*1.03E+003*	*5*	*205.*	*1.72*	*0.283*	*not significant*
Pure Error	*597.*	*5*	*119.*			
Cor Total	7.05E+003	19				

ANOVA for Sugar consumed (%)

Response 3: sugar consumed
ANOVA for Quadratic Model
Analysis of variance [Sum of squares is **Type III – Partial**]
Transform: Square Root

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.4864	9	0.0540	1.00	0.4941	not significant
A-Sugar Conc.	0.0121	1	0.0121	0.2253	0.6452	
B-Time	0.1362	1	0.1362	2.53	0.1430	
C-Temperature	0.0200	1	0.0200	0.3703	0.5564	
AB	0.0792	1	0.0792	1.47	0.2535	
AC	0.0116	1	0.0116	0.2155	0.6525	
BC	0.0002	1	0.0002	0.0041	0.9503	
A^2	0.0676	1	0.0676	1.25	0.2892	
B^2	0.1067	1	0.1067	1.98	0.1898	
C^2	0.0312	1	0.0312	0.5792	0.4642	
Residual	0.5392	10	0.0539			
Lack of Fit	0.5256	5	0.1051	38.57	0.0005	significant
Pure Error	0.0136	5	0.0027			
Cor Total	1.03	19				

ANOVA for PAC produced (g/L)

Response 4. PAC produced(g/L)
ANOVA for Quadratic Model
Analysis of variance [sum of squares Type III-partial]

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	121.90	9	13.54	3.69	0.0269	significant
A-Sugar Conc.	0.0675	1	0.0675	0.0184	0.8948	
B-Time	5.31	1	5.31	1.45	0.2564	
C-Temperature	5.67	1	5.67	1.55	0.2420	
AB	0.0061	1	0.0061	0.0017	0.9684	
AC	0.0061	1	0.0061	0.0017	0.9684	
BC	0.0072	1	0.0072	0.0020	0.9655	
A^2	55.02	1	55.02	15.01	0.0031	
B^2	21.03	1	21.03	5.74	0.0376	
C^2	55.02	1	55.02	15.01	0.0031	
Residual	36.66	10	3.67			
Lack of Fit	23.24	5	4.65	1.73	0.2808	not significant
Pure Error	13.42	5	2.68			
Cor Total	158.56	19				

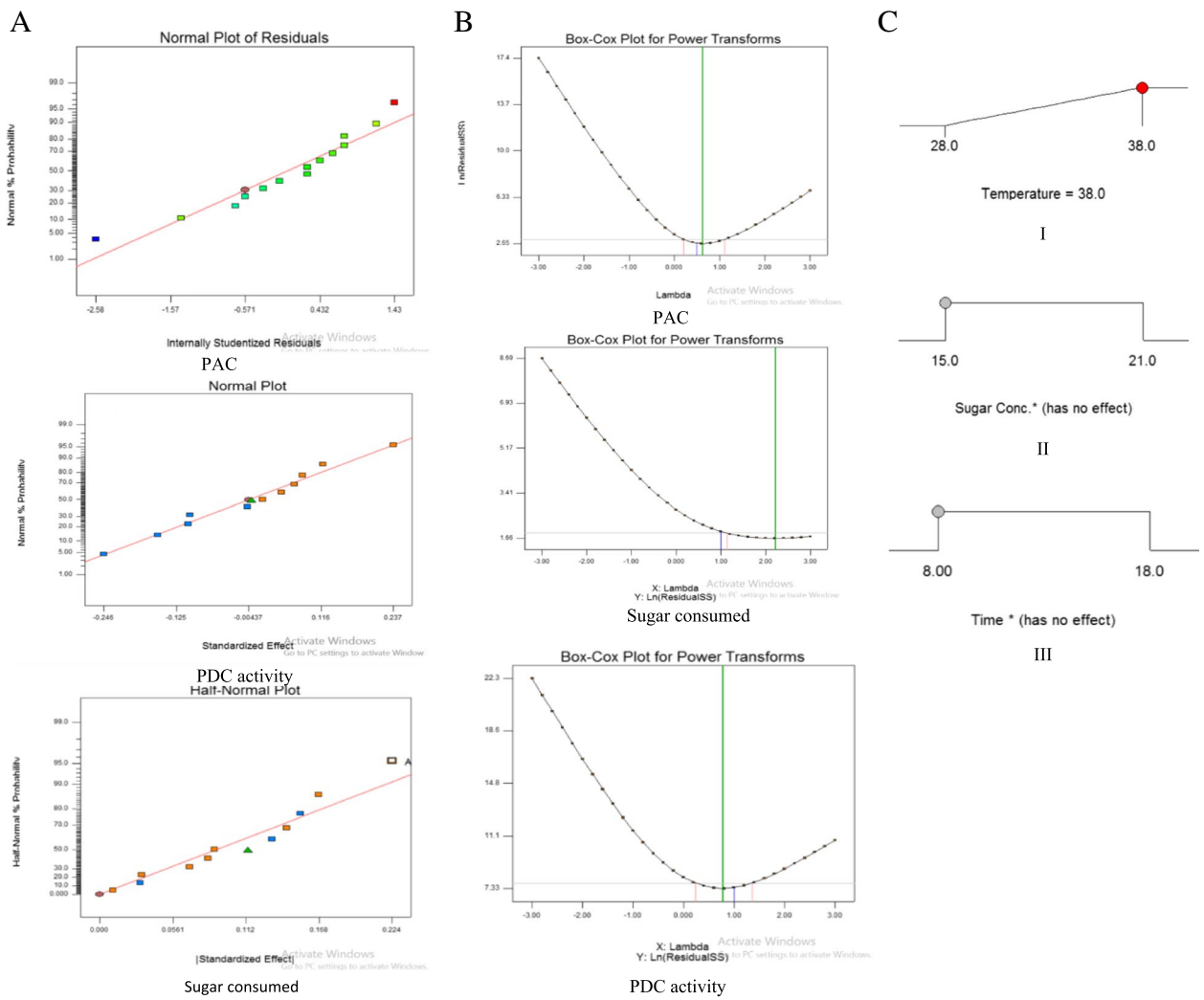

Fig. 3 **A** Linear plot for PAC, PDC activity, and sugar consumed. **B** *Box-Cox graphs for PAC (g/l), sugar consumed (% age, v/v), and PDC activity (U/ml).* **C** Ramps depicting the impact of factors on pyruvate decarboxylase activity. Sugar conc. and incubation time have no effect. Temperature is significant and have positive impact

for PDC production from *Pichia cecembensis* was 38 °C (Fig. 3C.I) which goes with Shukla and Kulkarni (2002).

Interaction of temperature and sugar and temperature and time positively affected (4A) the PDC activity which rose to 42.8 U/ml (indicated by concentric red data points). In simple words, it can be suggested that temperature was a key factor to enhance the activity of PDC. Interaction of temperature with sugar concentration and incubation time produced significant response surface models and 3D surface graphs. However, corelation of time and sugar was not effective for higher carboligase activity (14 U/ml, indicated by red data spots). Standard error for PDC for all corelations was almost similar as shown in 3D plots in Fig. 4B.

Interactions of temperature with sugar conc. and time enhanced the pyruvate decarboxylase activity (6.38 U/ml), but interactions of time and sugar conc. produced lower pyruvate decarboxylase (approximately 2.0). Standard error of interactions was uniform for all interactions (Fig. 5 A and B). Hence, temperature was the factor which can produce higher activity of pyruvate decarboxylase and its products. Arroyo-López et al. (2009) reported temperature and sugar as significant parameters affecting the microbial growth and product formation using CCD of RSM. They maximize the yield by process optimization for five factors (initial pH, initial molasses concentration %, incubation temperature °C, mixing rate rpm, and incubation period h). In present study, eleven factors through

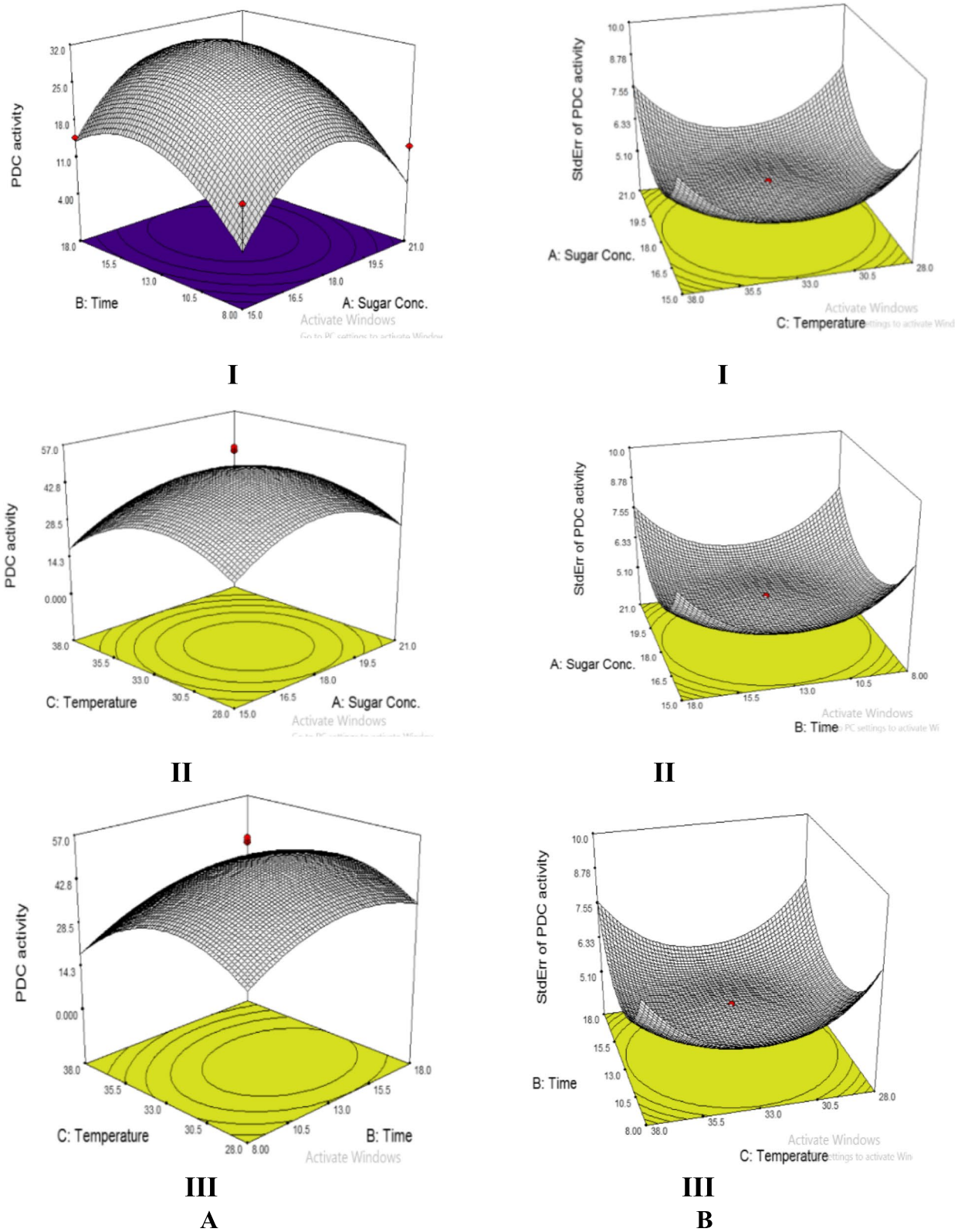

Fig. 4 **A** Effect of corelations of temperature (°C), time (h), and sugar conc. (% age) on PDC activity. **B** Standard errors for corelation of factors (temperature (°C), time (h), and sugar conc. (%)

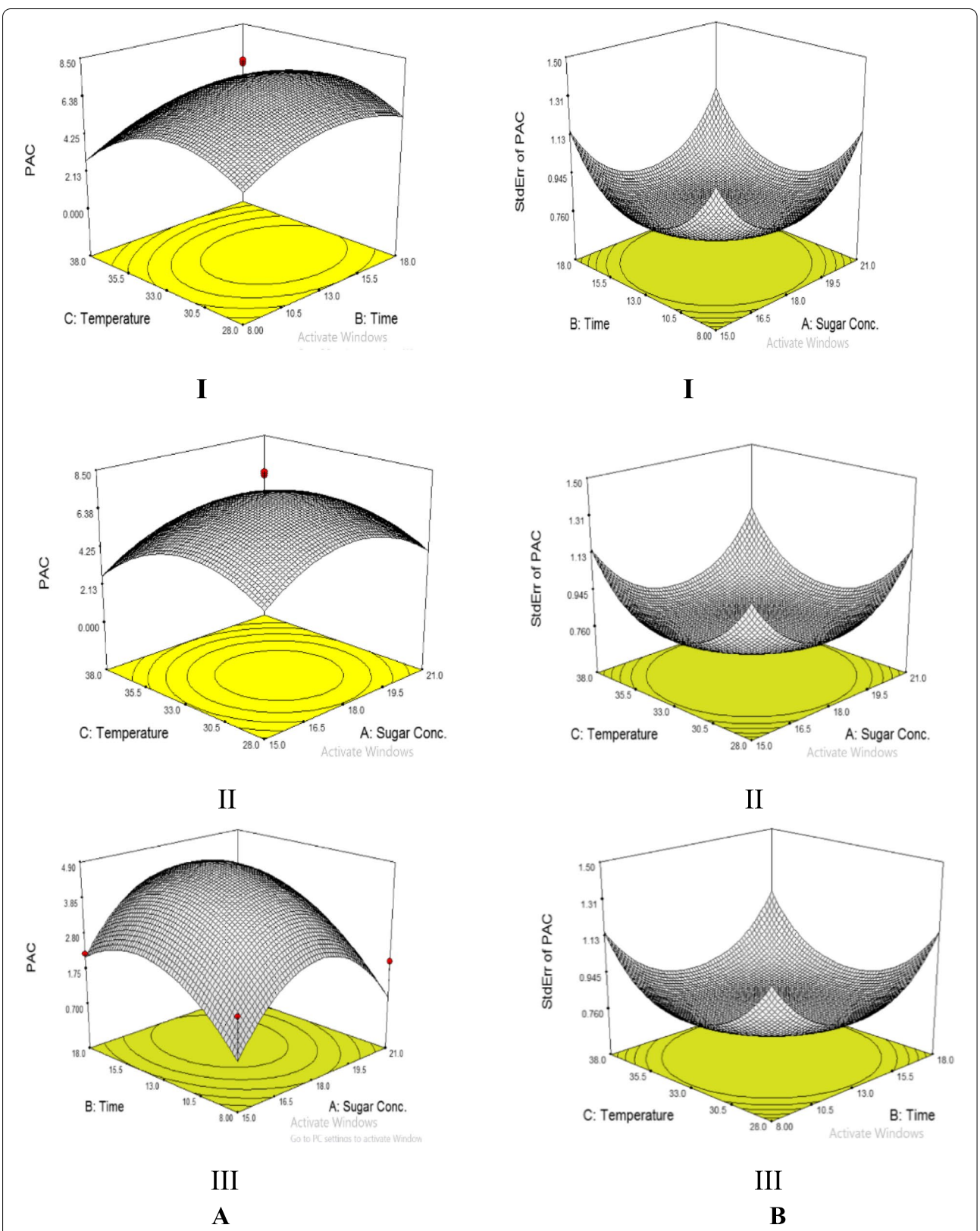

Fig. 5 **A** Effects of corelation of time and sugar conc., temperature and sugar conc., and temperature and time on PAC produced (g/l). **B** Standard error of co-interaction of time and sugar conc., temperature and sugar conc., and temperature and time

PBM and three factors through CCD were optimized to enhance the yield of pyruvate decarboxylase and PAC. According to the response surface optimization process, the response for each fermentation parameter was defined within the studied levels range to get the highest performance.

In present model, maximum PDC activity was 56.27 U/ml resulting in the production of 8.44 g/l PAC as compared to another study which has reported a maximum yield of 2.4 g/l by *Saccharomyces cerevisiae* using cane molasses (Doostmohammadi et al., 2016). In present study, the yield was increased by 71% under optimized fermentation conditions, initial pH of 5.0, total sugar concentration at 18%, incubation temperature of 33 °C, and 13 h of incubation time. Hussain et al. (2012) reported different strains of *Saccharomyces cerevisiae* producing 2.58 g/l maximum PAC. Retention times (Table 4) for PAC, benzoic acid, and benzyl alcohol were found to be 5.5–6.0, 17.5, and 1.5–2.0 min through HPLC purification.

Whole cells of *Pichia cecembensis* (Table 5) have better half-lives of 240 h and 336 h when incubated with and without 40 mM benzaldehyde, respectively at 4 °C as compared to a half-life of 228 h of the whole cells of *Candida utilis* (Satianegara et al., 2006). Crude extract of PDC from *Pichia cecembensis* exhibited extended half-life of 24 h with benzaldehyde and 32.5 h without benzaldehyde at 25 °C, as compared to the crude extracts of PDC from *Candida utilis* showing half-lives of 12.9 and 26.3 h under same conditions (Leksawasdi, 2004). Partially purified PDC in current research work has better half-life (72 h) in the presence of benzaldehyde as compared to partially purified PDC of *Candida utilis* which lost its activity within 60.5 h by one-half in the presence of benzaldehyde at 6 °C *utilis* (Satianegara et al., 2006).

Table 4 Determination of PAC, benzoic acid, and benzyl alcohol through HPLC

Products of PDC	Retention time (min)	Purity %
Phenylacetylcarbinol (PAC)	5.5–6.0	76.18
Benzoic acid	17.5	0.12
Benzyl alcohol	1.5–2.0	7.1

Table 5 The calculated half-lives of different preparations of *P. cecembances* PDC in 40 mM benzaldehyde at 4 and 25 °C

Different preparations	Half-life (h) at 4 °C		Half-life(h) at 25 °C	
	Control	40mM	Control	40mM
Partially purified	168	72	48	33
Crude extract	90	30	32.5	24
Whole cells	336	240	50	28.5

Materials and methods

Microorganism and maintenance

Pichia cecembensis used in the present study was locally isolated from *Prunus persica* (peach) fruit. It was selected on the basis of temperature tolerance, solvent resistance, and least crab tree effect (data is reported by same authors in another publication). It was maintained by weekly transferring to malt agar slants containing (g/l) yeast extract 7.5, malt extract 10, glucose 7.5, peptone 7.5, and agar 15. The slants after sufficient growth of the yeast were stored at 4 °C.

Plackett-Burman model for selection of significant factors

PBM can be used for linear regression analysis of different factors but not for their interaction (Plackettt & Burman, 1946), so it was used for screening of eleven factors affecting production of PDC during present study. These factors were sugar conc. (A), incubation time (B), incubation temperature (C), pH (D), inoculum size (E), urea (F), $MgSO_4.7H_2O$ (G), TPP (H), K_2HPO_4 (I), Na_2HPO_4 (J), $(NH4)_2\ SO_4$ (K), and H_3PO_4 (L). These factors were tested for low, medium, and high values. All possible combinations of these factors were investigated in triplicates. Linear regression analysis was studied using the approach as follows:

$$Y = \beta_o + \sum \beta_i x_i (i = l - k) \quad \text{(Eq. 1)}$$

In Eq. 1, Y is the target function, and β_o and β_i are the intercept and regression coefficient, respectively. Effect of every variable was tested by the following equation:

$$E(Xi) = 2(\sum Mi^- - Mi^+)/N \quad \text{(Eq. 2)}$$

In Eq. 2, $E\ (X_i)$ = the effect of the tested variables Mi^- and Mi^+ = the total production from the trials where the variable Xi measured at low and high levels, respectively, and N = the number of experiments. Responses were studied as PDC activity, sugar consumed, and final pH. Theoretical yields were compared with actual yield to select significant factors.

Experimental design and process optimization by response surface methodology

Significant factors were selected by PBM and optimized by response surface methodology (RSM) using central composite design (CCD).

Statistical analysis

Coded equation for significant factors was as follows:

$$Z = (X - X^0)\Delta X \quad \text{(Eq. 3)}$$

where Z = coded value of independent variable, X = the corresponding real value, X^o = real value of an independent variable at the center point, and ΔX = step change of real value at the variable for Z the value.

The relationship between the response and the independent variables was explained by using second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} {x_i}^2 + \sum \beta_{ij} x_i x_j \quad \text{(Eq. 4)}$$

where Y = predicted response, β_o = the interception coefficient, β_i = linear coefficient, β_{ii} = quadratic coefficient, and β_{ij} = interception coefficients. Software package Design-Expert version 10.1.6 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for multiple regression analysis and construction of response surface models and their studies. The significance of regression equation was studied by *F*-test and lack of fit and explained by coefficient of determination R^2 that is adjusted R^2, predicted R^2, and coefficient of variance. The 2nd-order fitted polynomial equation was explained through three dimensional graphs to show the relationship among the response and experimental factors. The maximum response of each variable was optimized through point optimization method. This method was validated through optimized variables producing maximum response.

Fermentation experiments

Submerged fermentation (SmF) in shake flasks was carried out for the biosynthesis of PDC. Inoculum was prepared in the medium containing (g/l) the following: treated molasses 80, urea 1.0, and $MgSO_4$ 0.5 (pH was adjusted at 6.0). Twenty five milliliter of the same medium was used in 250 ml flasks which was inoculated with 72-h-old *Pichia cecembensis* culture and incubated at 34 °C and 150 RPM for 24 h.

Fermentation medium containing constituents (treated molasses, urea, $MgSO_4.7H_2O$, thiamine pyrophosphate, K_2HPO_4, Na_2HPO_4, $(NH_3)_2HPO_4$), and phosphoric acid) in amounts according to the 1st (Plackett-Burman model as given in Table 1) and 2nd level (RSM as given in Table 2) factorial designs were prepared and sterilized. All fermentation media (25 ml each in 250 ml flask) were inoculated with 2 ml of 24-h-old inoculum having viable cell count of 120×10^6 cells/ml. Cell count was determined through standard lab practices using hemocytometer. The flasks (factorial designs) were incubated under the fermentation conditions (time, temperature, and pH) according to both the factorial designs in Tables 1 and 2. All experiments were performed in triplicates, and results were average values of actual results.

At the completion of fermentation, yeast cells were harvested after centrifugation and washed. Yeast cells were disrupted in breakage buffer (50 mM MES/KOH, pH 7.0, 20 mM $MgSO_4$, 1 mM thiamine pyrophosphate, protease inhibitor cocktail) by vortexing with glass beads. Cell debris was removed by centrifugation at 10,000 RPM, and clear supernatant was used for enzyme assay.

Analytical techniques

Carboligase assay

PDC assay was carried out as carboligase activity and estimated through the quantitative estimation of PAC produced at 30 °C for 30 min. Enzyme extract was incubated at 30 °C with 40 mM benzaldehyde and 100 mM pyruvate in carboligase buffer (50 mM citrate/KOH, 20 mM $MgSO_4$, 1 mMTPP, 1.5 M ethanol, pH 6.0) for 30 min in water bath. The reaction was stopped by adding trichloroacetic acid (10%, w/v), and precipitated proteins were removed by centrifugation at 6000 RPM for 15 min. PAC was quantified through spectrophotometric analysis at 570 nm. A total of 500 µl carboligase assay mixture was mixed with colorless tetrazolium 500 µl (0.1%, w/v) in the presence of 1 ml 3M NaOH. PAC and other products of PDC reduced the colorless tetrazolium salt into red salt. Absorbance was read at 570 nm to quantify the products.

One unit of carboligase activity was defined as the amount of PDC required to produce 1.0 mM PAC from pyruvate and benzaldehyde per min at pH 6.0 and 30 °C. Calibration curves of pyruvate, benzaldehyde, PAC, benzoic acid, and benzyl alcohol were used.

Partial purification of PDC

A scaled-up batch for PDC production using the optimized constituents through RSM was carried out in 5 l flask containing 1500 ml fermentation medium. The flasks were inoculated with 8% (v/v) of 24-h-old vegetative inoculum and incubated at 33 °C for 13 h. Yeast cells were harvested after centrifugation and washed with deionized water. Enzyme extract was prepared as described earlier and was used for the partial purification of the enzyme through ammonium sulfate precipitation.

Determination of residual PDC activity

PDC as partially purified enzyme, crude extract, and whole cells were mixed with 40 mM benzaldehyde in Teflon screw cap glass vials (2.0 ml reaction volume) at 4 °C and 25 °C. Residual carboligase activities for each condition and their controls (without benzaldehyde) were

determined over time by withdrawing and processing the samples for subsequent analysis. From these data, the half-life values of various PDC preparations were calculated (Satianegara et al. 2006).

Quantification of metabolites of PDC

An isocratic high-performance liquid chromatography system (Shimadzu, Tokyo, Japan), comprising a LC-6A pump, LC real-time analysis system, a diode array detector SPD-M20A, a communication bus module CBM-20A, degasser (prominence) DGU-20A, and column oven CTO-20A with oven temperature 40 °C was used. HPLC was equipped with reverse phase C18 column, detection wavelength range 100–200, and flow rate 1.5 mL/min, whereas a 20 µl aliquot was chromatographed. Lambda max for PAC, benzyl alcohol, and benzoic acid was 283, 263, and 250, respectively. Mobile phase consisted of a degassed filtered mixture of acetonitrile and water (70:30% v/v).

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NA.

Authors' contributions
The main idea of the study, supervision of project, rational analysis of data, and experiment designs were conducted by HM. Manuscript was finally approved by HM for publication. Write-up, experiment performance, data collection, and arrangements were conducted by ZM. The authors read and approved the final manuscript.

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All data used in paper is original and available in the data repository of the institute.

Declarations

Ethics approval and consent to participate
Both authors agreed and have consent for publication and participation. This article does not contain any materials that violate any personal or proprietary rights of any other person or entity.

Consent for publication
I have informed the co-author, and she is fully agreed and authorized me to submit.

Competing interests
The authors declare that they have no competing interests.

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