

Process Optimization for Biosynthesis of Pyruvate Decarboxylase (PDC) and Neuberg's Ketol (PAC) from Novel *Pichia Cecembences* Through Response Surface Methodology using Industrial Waste as Substrate

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Research Article

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Abstract

Purpose

Phenyl acetyl carbinol (PAC) is an intermediate for the synthesis of active pharmaceutical ingredients (ephedrine, pseudoephedrine, norephedrine etc.) which are used for the production of antiasthmatics and decongestants. Chemical production of these APIs and extraction from plants is costly and cumbersome. Biosynthesis of PAC through condensation of benzaldehyde and acetaldehyde using PDC, a more effective method, is being used. These solvents can adversely inhibit PDC. Optimization of cointeraction of significant factors was done through Response surface methodology (RSM) in relatively short time.

Method

The effect of incubation time (8-18hrs), temperature (30-38°C), pH (4-10) and Inoculum size (4-10%, v/v) on PAC yield, sugar consumption and PDC activity was determined. PAC was quantified spectrophotometrically and HPLC. All results and models were statistically analysed. PDC, produced in 5L flask using molasses as substrate, was exposed to 40mM benzaldehyde as whole cells, crude extract and partially purified to determine its half life as residual activity.

Results

PDC activity and PAC yield were 56.27 U/ml and 8.44 g/L, respectively. The yield of PAC (2.22 to 8.44g/L) was increased by 71% after process optimization through RSM with time (13hrs), temperature (33°C) and total sugar conc. (18%, v/v) as significant factors (p-values, 0.902, 0.260 and 0.247, respectively). Process design had Adj R² 0.562, R-Squared 0.770, Adeq Precision 4.888 with a uniformly distributed standard error. PDC used in the form of *Pichia cecembences* cells revealed higher stability towards benzaldehyde and temperature as compared to partially purified PDC. Whole cells and partially purified PDC showed half-lives of 240 and 72hrs at 4°C whereas, 33 and 28.5hrs at 25°C. PAC purity through HPLC was 76.18%.

Conclusions

Time, temperature and sugar were significant factors as they increased the PAC biosynthesis. PDC from *Pichia cecembences* (crabtree negative; reported in other publication by same authors), as a whole cell and purified showed better half-lives at 4 and 25°C as compared to reported PDCs. Hence, it is a promising candidate for commercial production of PAC, as its PDC was stable at 4 and 25°C in presence of Benzaldehyde.

2.0 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 properties; Decarboxylation and Carboligation, through decarboxylation of pyruvate, it produces acetaldehyde and carbon dioxide (Nichols *et al.*, 2003) whereas incarboligation it adds carbon from Benzaldehyde into the acetaldehyde resulting in the production of a large number of products like phenyl acetyl carbinol (PAC), benzyl alcohol, benzoic acid, formic acid, acetaldehyde and other side products (Suresh *et al.* 2009, Meyer *et al.* 2010, Thitiprasert *et al.* 2014). Enzymes, involved in Decarboxylation (a critical reaction in fermentation process) including PDC are thymidine Phosphate (ThdP) dependent enzymes and belong 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, nor-ephedrine, nor-pseudoephedrine etc. Alternatively, Ephedrine being an important anti asthmatic drug is traditionally extracted from Ephedra (*Ephedra sinica*) which is not abundantly found in most of the 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 comprises of 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, co-solvent 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).

PDC can be produced from yeasts (*Saccharomyces cerevisiae*, *Candida utilis* etc.) and bacteria (*Zyomonas mobilis*) through fermentation but yeast PDC is preferable over bacterial PDC because bacterial PDC are more sensitive towards benzaldehyde and lose their productivity after a short time of exposure. Therefore, yeasts are the most suitable sources of PDC due to this and number of other factors (Raj KC *et al.* 2002). Fermentation process is affected by many physical and chemical parameters so the conventional optimization of fermentation (varying one parameter at a time) is a time consuming and hectic practice. 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). Therefore, in present study, a statistical method viz., Response Surface Methodology (RSM) is used to study influences of individual factors and their interactions. RSM is a statistical tool for experiment design, 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). RSM has been used by many researchers as it is an efficient statistical tool for process design and development of methods to produce many valuable biomolecules (Mushtaq *et al.* 2014, Othman *et al.* 2017).

In present study, initial experiments were designed through PBM via linear regression analysis to select significant factors from physical and chemical factors i.e., Time of fermentation (Hrs), incubation temperature (°C) for fermentation, inoculum size (% v/v), pH of medium, conc. of sugar (% v/v), urea (% w/v), MgSO₄ (% w/v) and TPP (% w/v). Central composite design was used for co-linear regression analysis. Stability of PDC as whole cells, crude extract and partially purified was evaluated.

3.0 Results And Discussion

Selection of correct carbon source is very critical in process optimization. Carbon sources were screened for the production of PDC and biosynthesis of PAC. It was observed that cane molasses was the best carbon source as it produced 2.22 g/L PAC whereas other sources produced lesser amounts. That is pure cheese whey (whey without suspended proteins) 1.11, whole cheese whey (whey with suspended proteins) produced 0.88, glucose 0.99, galactose 0.44, fructose 0.66, maltose 0.77 and Carbon sulphite liquor 0.33 (Fig. 1. Screening of carbon sources for PDC production.). Some other researchers stated that cane molasses as an excellent carbon source for growth of yeasts and production of different enzymes and other metabolites such as pectinases, proteases, xylanases and bioethanol etc. (Aguilar et al 2002, Sughra et al. 2013). As molasses is a rich source of sugars, vitamins, minerals and a number of other nutrients. Moreover this industrial waste and is easily available round the year (Darvishi and Moghaddami 2019).

3.1. Response Surface based optimization

The use of RSM for experiment design and statistical analysis of results is increasing day by day due to its robust response. 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 substrate (Asadi and Zilouei 2016).

3.2. Plackett Burman Burman Model (PBM)

By using the Design-Expert version 10.1.6 (Stat-Ease, Inc., Minneapolis, MN, USA) Sugar Conc. (% v/v), Incubation Time (Hrs) and Temperature of fermentation ($^{\circ}\text{C}$) were selected as significant factors through PBM. Other factors were non-significant as R^2 was greater than 1 and P value was out of standard limit. Linear regression analysis of eleven factors through four responses (Table 1A) was done using Plackett Burman Model. Standard error of design (Fig. 2) was smaller and similar within all types of co-efficients. VIF was 1.0 and hence satisfactory. R_i^2 for all factors was 0.00. All these aspects showed terms are not co-related and Model is significant.

As per ANOVA, P-values for initial sugar (% v/v), Time (Hrs) and Temperature ($^{\circ}\text{C}$) were less than 0.05 therefore these were significant factors. Central Composite Design was designed using selected factors to determine and optimize their co-relation for higher productions. Asif et al. 2012 designed experiments for proteases using the similar approaches.

3.3. Central Composite Design and evaluation

Sugar Conc. (% v/v), Incubation Time (Hrs) and Temperature of fermentation ($^{\circ}\text{C}$) were used as input factors for CCD (Table 1B.) and studied through four responses as PDC activity (mmol/L) final pH, sugar consumed (% v/v) and PAC (g/L) produced. The fitted models in terms of the coded values of Sugar Conc. (A), Time (B) and Temperature (C) are given below;

3.4. Evaluation of RSM Designe

Final Equation in Terms of Coded Factors:

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

Final Equation in Terms of Coded Factors:

$$Y_{\text{PAC(g/l)}} = +7.24 + 0.070 * A + 0.62 * B - 0.64 * C - 0.027 * A * B - 0.027 * A * C + 0.030 * B * C - 1.95 * A^2 - 1.21 * B^2 - 1.95 * C^2$$

where y is the PDC activity and PAC produced (g/L) positive sign in front of the terms indicates synergetic effect, whereas negative sign indicates antagonistic effect.

3.5. Analysis of variance (ANOVA)

ANOVA for Pyruvate decarboxylase activity showed that the F-values of Model (3.71, 18.4 and 6.08), P-Values less than 0.005 for PDC activity, Sugar consumed and PAC produced implies that the models for all these responses were significant. Whereas P-Value for lack of fit was greater than 0.005 so the lack of fit was non-significant for all responses, which made them more significant (Table 2). There are only 2.65, 2.2 and 2.9% chances that the "Model F-Value" could occur due to noise.

In this case A^2 , B^2 , C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 1.72 implied that the Lack of Fit was not significant relative to the pure error. There was a 28.30% chance that a "Lack of Fit F-value" could occur due to noise. Moreover,

Std. Dev. 12.7

R-Squared 0.770

Mean 24.9

Adj R-Squared 0.562

C.V. % 51.1

PRESS 8.62E+003

Adeq Precision 4.888

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 4.888 indicated an adequate signal. This model can be used to navigate the design space.

Optimizations (Figs. 3A, 3B) goals for sugar consumed, PDC activity and PAC were maximized. All possibilities of interaction and their co-relations were studied. Importance for all was of high importance i.e., +++. 3D graphs to study the co-relation of Time, sugar concentration and temperature were plotted.

Effects of these interactions over PDC activity and PAC produced (g/L) were studied. As shown in Fig. 4A and 5A.

Linear plots (Fig.3A.) 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 PDC activity, PAC and sugar Conc. showed that Lambda for PDC activity and PAC produced were near to the ideal values that is 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.

According to ANOVA and statistical analysis temperature is significant. As per analysis of Ramps (3C. I,II,III), temperature significantly effected activity of PDC and hence yield of PAC. Whereas sugar conc. and time were not significant. Smooth ramps showed that these factors have no significant effect on production of PDC and its products. Positive impact of temperature have been reported by many researchers (Shukla and kulkarni 2002, Andrews and McLeish 2012). The 38°C (Fig. 3C.I) was optimum temperature for PDC production from *Pichia cecembencesthis* goes with Shukla and kulkarni 2002.

Interaction of Temperature & sugar and Temperature & Time positively effect (4A) the PDC activity which rose to 42.8 U/ml. In simple words it could 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. Co-relation of Time and Sugar was not effective for higher PDC activity (14 U/ml). Standard error for PDC for all co-relations was almost similar as shown in 3D plots in Fig. 4B.

Interactions of Temperature with sugar Conc. and Time, enhanced PAC produced but interactions of Time and Sugar conc. could not produce good yields of PAC (g/L). Standard error of interactions was uniform for all interactions (Fig.5A, 5B). Hence, through CCD design it was found that Temperature was the factor which can produce higher activity of PDC 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 %, v/v, incubation temperature °C, mixing rate rpm, and incubation period (Hrs)). In present study eleven factors through PBM and three factors through CCD were optimized to enhance the yield of PDC and PAC. Design Expert 6.0.7 software (Stat-Ease Inc., Minneapolis, USA) was reported for optimization of alcoholic fermentation. We used Design-Expert version 10.1.6 (Stat-Ease, Inc., Minneapolis, MN, USA) for optimization. According to the RSM optimization process, the response for each fermentation parameter was defined within the studied levels range to get the highest performance.

RSM combines the individual desirability, into a single number and searched to optimize the function, based on the response. Arroyo-López et al. (2009) calculated bioethanol production 30.7 g/L with bioethanol yield of 42% under fermentation conditions; pH 5, 25% initial molasses concentration, 35°C, 116 rpm, and 60 hrs. The experimental result of these conditions was found to be 32 g/L with 43.57

%bioethanol yield. In present model maximum PDC activity was 56.27 U/ml producing 8.44 g/L PAC. The yield was increased by 71% under optimized fermentation conditions, initial pH 5.0, total sugar concentration 18% (v /v), Incubation temperature 33°C and 13hrs of incubation. Retention times (Table 3A) for PAC, Benzoic acid and Benzyl alcohol were 5.5-6.0, 17.5 and 1.5-2.0 min through HPLC purification.

Whole cells of *Pichia cecembences* (Table 3B) have better half-life at 4°C with and without 40mM benzaldehyde (240hrs and 336hrs, respectively) as compared to *candida utilis* reported by Satianegara et al. 2006. Who reported half-life of 228hrs in presence of benzaldehyde at same temperature. Whereas crude extract exhibits extended half-lives at 25°C with and without Benzaldehyed (24 and 32.5 Hrs) rather than crude extracts of *Candida utilis* 12.9 and 26.3Hrs respectively it goes in agreement with Leksawasdi 2004. Partially purified PDC in current research work have better half life time (72Hrs) in presence of benzaldehyde as compared to partially purified PDC of *Candida utilis* which losses its activity by one half in presence of benzaldehyde at 6°C within 60.5Hrs.

4.0 Materials And Methods

4.1. Microorganism and maintenance

Pichia cecembensis used in the present study was locally isolated from *Prunus persica* (peach). It was revived and maintained on Malt agar slants containing (g/L) yeast extract 7.5, malt Extract 10, Glucose 7.5, peptone 7.5 and agar 15 for 72hrs at 30°C. The slants after sufficient growth of the yeast were stored at 4°C.

4.2. Plackett Burman Model for selection of significant factors

PBM can only be used for linear regression analysis of different factors but not for their interaction (Plackett & Burman, 1946) so it was used for screening of eleven factors effecting production of PDC during present study. These factors were sugar conc. (A) incubation time (B), incubation temperature °C (C), pH (D), Inoculum size %, v/v (E), Urea %, w/v (F), MgSO₄.7H₂O (G), TPP (H), K₂HPO₄ (I), Na₂HPO₄ (J) (NH₄)₂ SO₄ (K) and H₃PO₄ (L). These factors were tested for low, medium and high values. All possible combinations of these factors were investigated in duplicates. Linear regression analysis was studied using the approach:-

$$Y = \beta_0 + \sum \beta_i x_i \quad (i=1-k)$$

In this equation, Y is the target function, β_0 and β_i are the intercept and regression coefficient, respectively. Effect of every variable was tested by the following equation:-

$$E(X_i) = 2 (\sum M_i^- - M_i^+) / N$$

In this equation, $E(X_i)$ = the effect of the tested variables M_i^- and M_i^+ = the total production from the trials where the variable X_i 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.

4.3. Experimental design and process optimization by Response Surface Methodology

Significant factors selected by PBM were optimized by Response Surface Methodology (RSM) using central composite design (CCD).

4.4. Statistical analysis

Coded equation for significant factors was:-

$$Z = (X - X^0) / \Delta X$$

Where Z = coded value of independent variable, X = the corresponding real value; X^0 = 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:-

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

Where, Y = predicted response, β_0 = 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.

5.0 Fermentation Technique For Pdc Production

Submerge fermentation (SmF) in shake flasks was carried out for the biosynthesis of PDC. Molasses, pure cheese whey (whey without suspended proteins), whole cheese whey (whey with suspended proteins), carbon sulphite liquor, glucose, galactose, maltose and fructose were screened as carbon sources. Seed inoculum was prepared in a medium containing (g/L): treated molasses 80, Urea 1.0, $MgSO_4$ 1.0 (pH was adjusted at 6.0) final volume was made upto 1L with distilled water. Inoculum media was inoculated with 72Hrs old *Pichia cecembensis* and incubated at 34°C and 150RPM for

24Hrs.Fermentation medium containing constituents (Treated Molasses, Urea, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, Thiamine Pyrophosphate, K_2HPO_4 , Na_2HPO_4 , $(\text{NH}_3)_2\text{HPO}_4$) and phosphoric acid) in amounts according to 1st (Plackett Burman Model) and 2nd level (RSM) factorial designs was sterilized and inoculated with 24Hrs old inoculum having viable cell count of 120×10^6 cells/ml. Fermentation flasks were incubated under the fermentation conditions (time, temperature and pH) according to both the factorial designs. All experiments were performed in triplicates and results were average values of actual results. Cell count was determined through standard lab practices using Haemocytometer.

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, 1 tablet Complete Protease Inhibitor Cocktail EDTA-free) by vortexing with glass beads. Cell debris was removed by centrifugation at 6000RPM and clear supernatant was used for enzyme assay.

6.0 Analytical Techniques

6.1. Carboligase Assay

PDC assay was carried out as Carboligase activity and estimated through the quantitative estimation of PAC produced at 30°C for 30min. 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 mM TPP, 1.5M 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 15min. PAC was quantified through spectrophotometric analysis at 570nm. 500 μl Carboligase assay mixture was mixed with colorless tetrazolium 500 μl (0.1%, w/v) in presence of 1ml 3M NaOH. PAC and other products of PDC reduced colorless tetrazolium salt into red salt. Absorbance at 570 was carried out to quantify 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.

6.2. Partial purification of PDC

A scaled up batch for PDC production using the optimized constituents through RSM was carried out in 5L flask containing 1500ml fermentation medium. The flasks were inoculated with 8% (v/v) of 24hrs old vegetative inoculum and incubated at 33°C for 13hrs. 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 sulphate precipitation.

6.3. Determination of residual PDC activity

PDC as partially purified enzyme, crude extract and whole cells were mixed with 40mM 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).

6.4. 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 20µl aliquot was chromatographed. Lameda max for PAC, benzyl alcohol and benzoic acid were 283, 263 and 250, respectively. Mobile phase consisted of a degassed filtered mixture of acetonitrile and water (70:30 % v/v).

7.0 Declarations

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Authors' contributions

The main idea of the study, supervision of project, rational analysis of data and experiment designs were conducted by Hamid Mukhtar. Manuscript was finally approved by Hamid Mukhtar for publication. Write up, experiment performance, data collection and arrangements were conducted by Zareena Mushtaq.

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Availability of data and materials

All data used in paper is original.

Ethics approval and consent to participate

Both authors are 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

N/A

8.0 References

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Tables

Table 1A: Plackett Burman Model for linear regression analysis of all factors

Std	Run	Block	Factor 1 A: Sugar Conc. %	Factor 2 B: Time Hrs	Factor 3 C: Temperature oC	Factor 4 D: pH	Factor 5 E: Inoculum Size %, (v/v)	Factor 6 F: Urea %, (w/v)	Factor 7 G: MgSO4 %, (w/v)	Factor 8 H: TTP %, (w/v)	Factor 9 I: Di. Ammonia g/L	Factor 10 K: Di. Sodium g/L	Factor 11 L: Di. Pot g/L	Response 1 PDC activity U/ml	Response 2 Final pH	Response 3 sugar consumed %, v/v	Response 4 PAC g/l
12	1	Block 1	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 1	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 1	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 1	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 1	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 1	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 1	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 1	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 1	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 1	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 1	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 1	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 1	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 1	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

Table 1B: 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

Table 2: Analysis of Variance (ANOVA) for the fitted models of CCD for Responses

ANOVA for PDC activity (U/ml)

Response	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 (%age, v/v)

Response	sugar consumed					
ANOVA for Response Surface Mean 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	0.000	0				
Residual	6.84	19	0.360			
Lack of Fit	6.71	14	0.479	18.4	0.00229	significant
Pure Error	0.130	5	0.0260			
Cor Total	6.84	19				

ANOVA for PAC produced (g/L)

Response	PAC					
Transform:	Square root	Constant:	0.000			
ANOVA for selected factorial 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	0.000	0				
Curvature	1.45	1	1.45	6.08	0.0297	significant
Residual	2.87	12	0.239			
Lack of Fit	2.38	11	0.216	0.439	0.840	not significant
Pure Error	0.492	1	0.492			
Cor Total	4.32	13				

Table 3A: Determination of PAC, Benzoic acid and Benzyl Alcohol through HPLC

Products of PDC	Retention Time (min)	Purity %
Phenyl acetyl carbinol (PAC)	5.5-6.0	76.18
Benzoic acid	17.5	0.12
Benzyl alcohol	1.5-2.0	7.1

Table 3B: The calculated half-lives of different preparations of *P. cecembances* PDC in 40 mM benzaldehyde at 4 and 25°C

Different preparations	Half-life (hrs) at 4°C		Half-life(hrs) 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

Figures

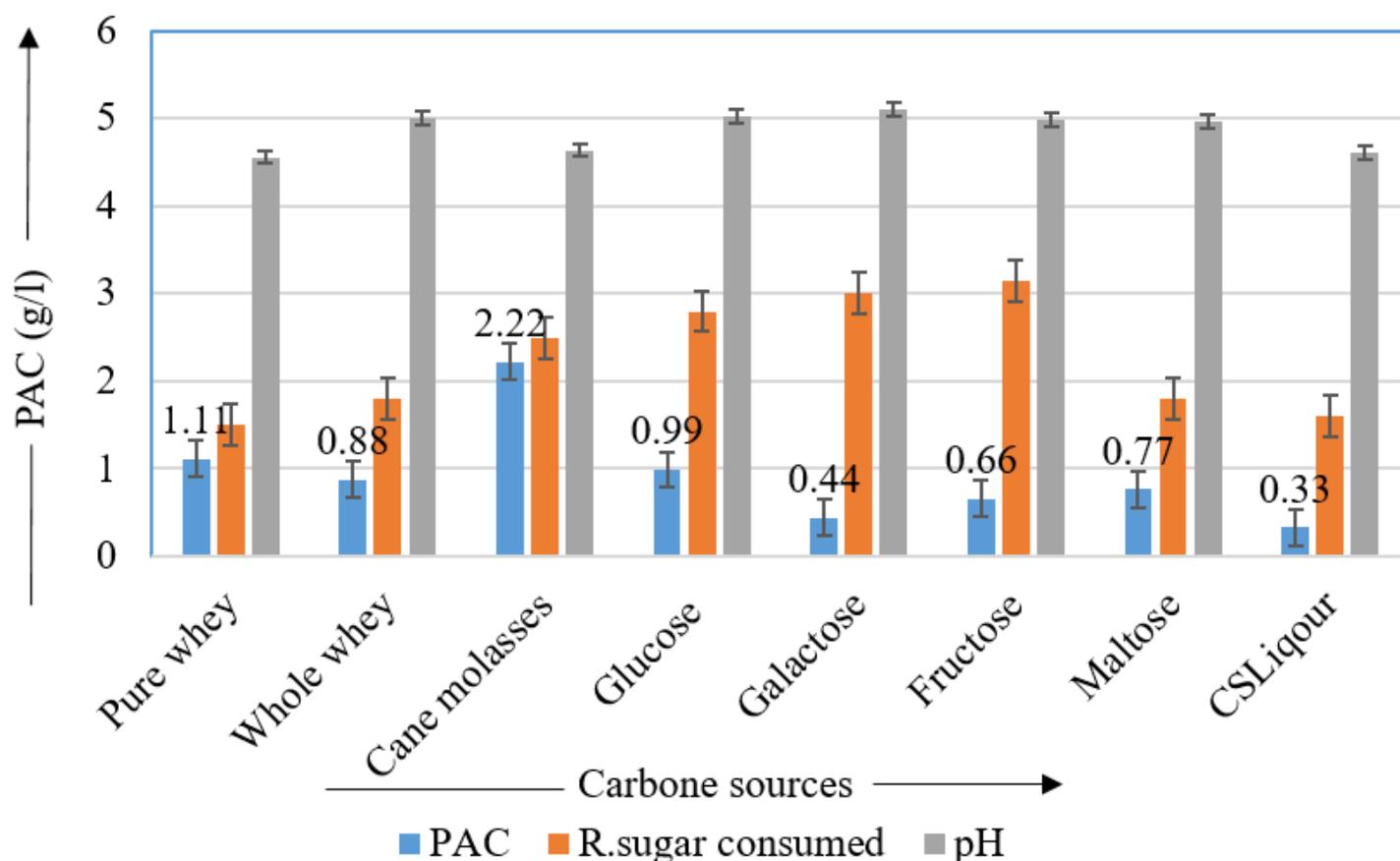


Figure 1

Screening of Carbon sources for Pyruvate Decarboxylase production

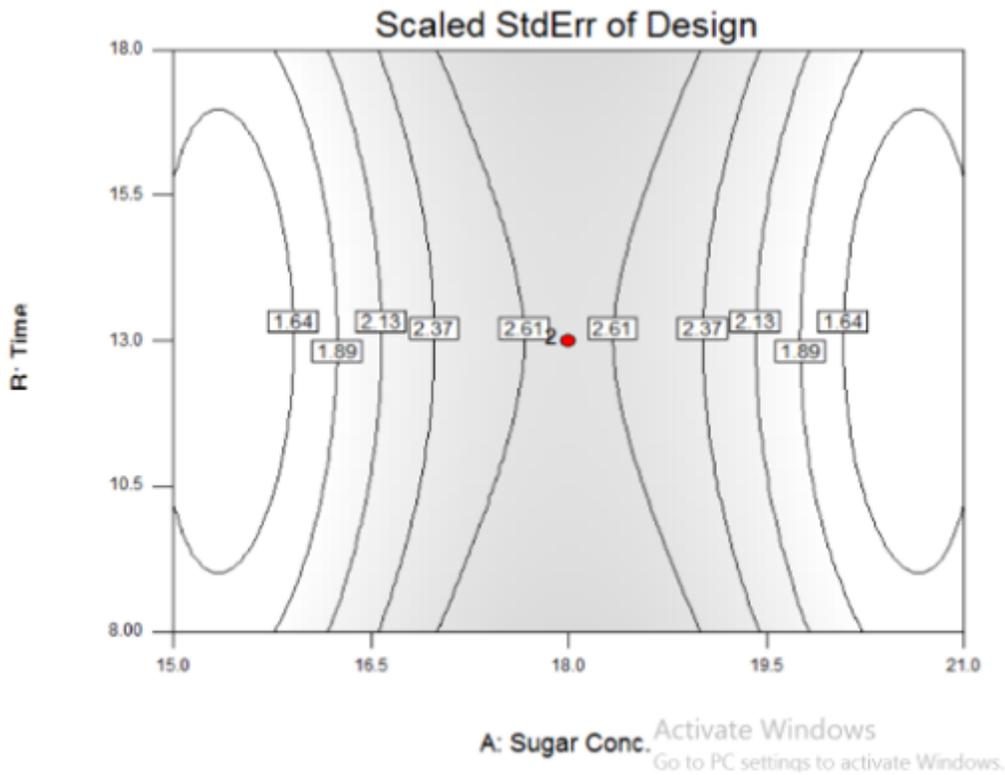


Figure 2

Scaled Standard Error of design

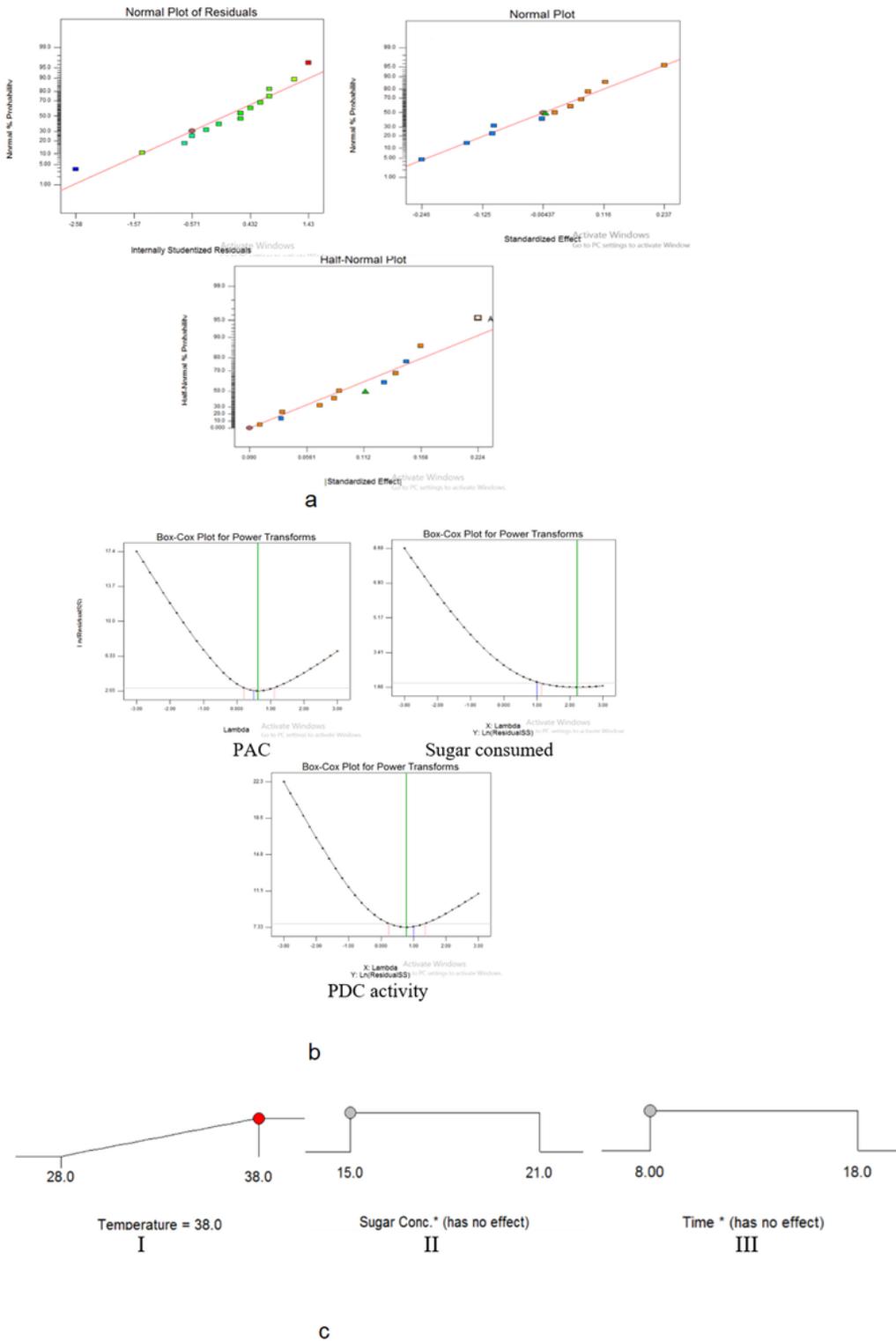
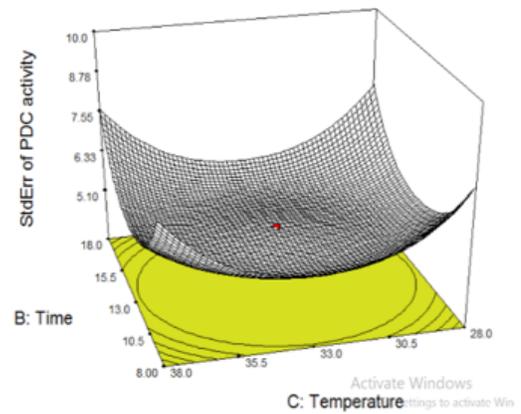
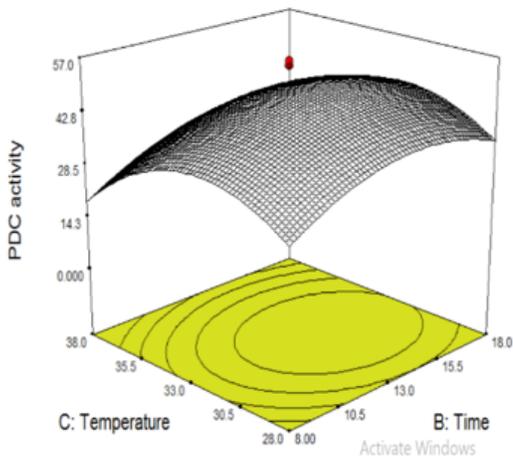
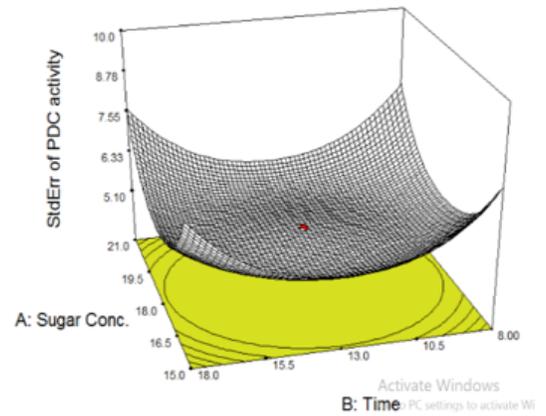
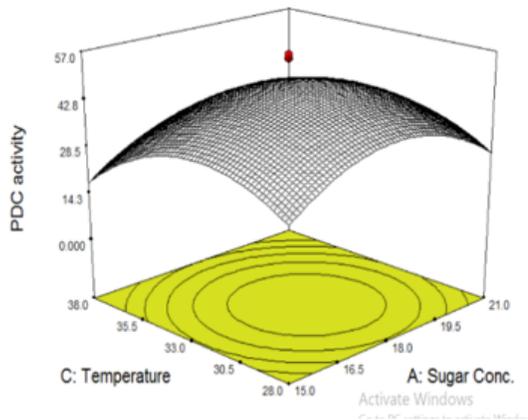
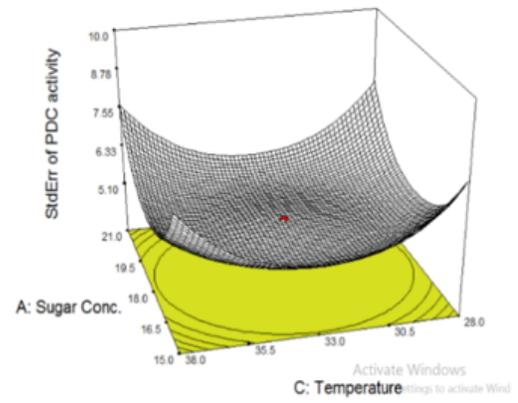
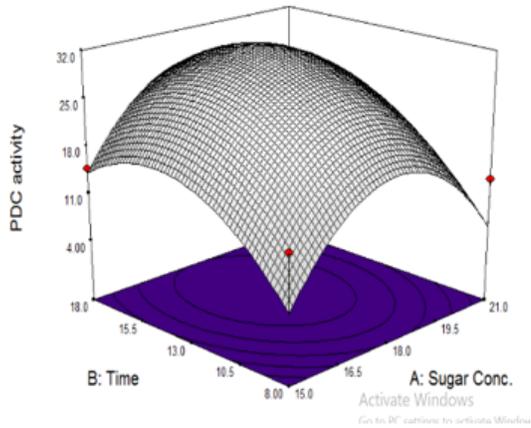


Figure 3

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.

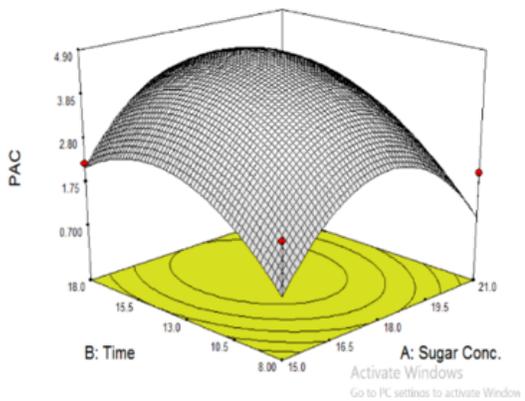
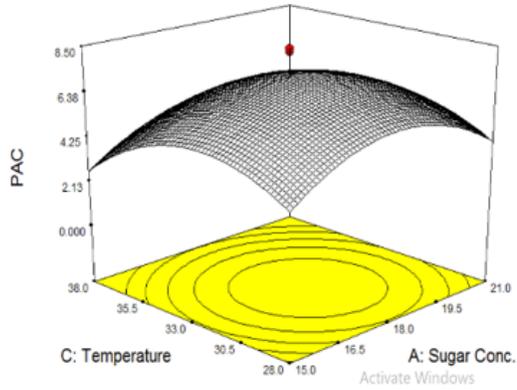
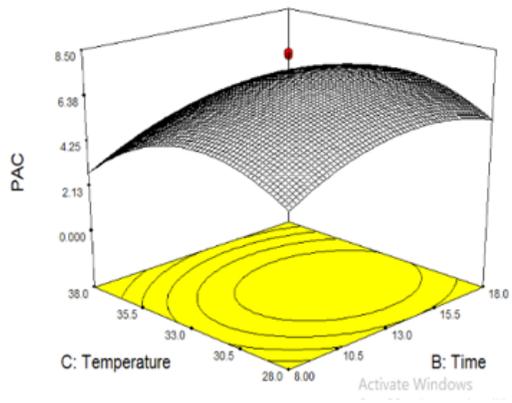


4A

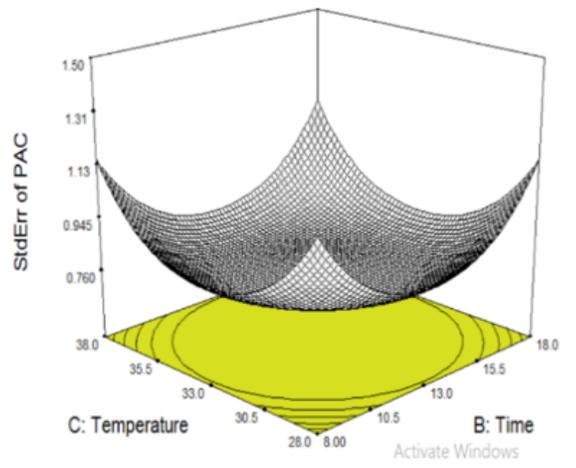
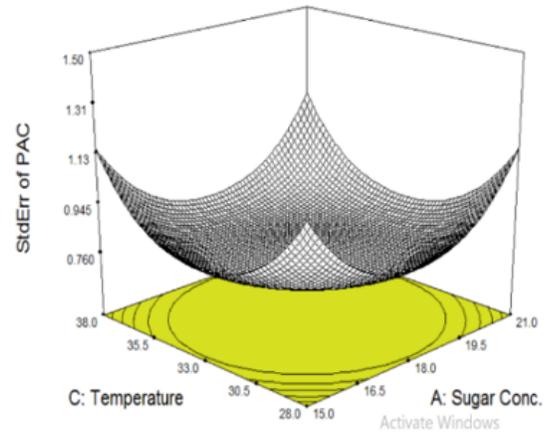
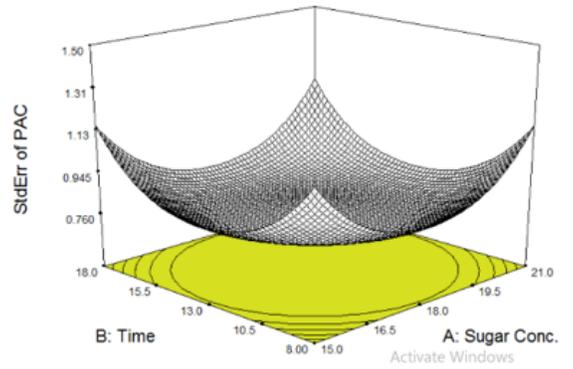
4B

Figure 4

A: Effect of Co-relations of Temperature (oC), Time (Hrs) and Sugar Conc. (% age, v/v) on PDC activity. B: Standard errors for co-relation of factors (Temperature (oC), Time (Hrs) and Sugar Conc. (% age, v/v)).



5A



5B

Figure 5

A: Effects of Co-relation of Time & sugar Conc., Temperature & sugar Conc., and Temperature & Time on PAC produced (g/L). B: Standard error of co-interaction of Time & sugar Conc., Temperature & sugar Conc., and Temperature & Time.