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Screening and formulation of culture conditions for pyruvate production by candida glabrata MCC 1800 from corn steep liquor

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Abstract
Eleven culture parameters were screened by the Plackett–Burman design for pyruvate production to develop a standard protocol for media formulation. In an incubator shaker, the screened optimal conditions for pyruvate production were: carbon source 24.642g/L, nitrogen source 11.89 g/L, KH_2PO_4 1.185 g/L, thiamine 13.302 µg/L, biotin 13.927 µg/L, $CaCO_3$ 43.432g/L, $MgSO_4 \cdot 7H_2O$ 0.346 g/l, inoculum age 3 days, inoculum volume 6% (v/v), agitation 220 rpm, incubation time 46.412 h and incubation temperature 34.582℃. Except $CaCO_3$ all factors were significant and agitation was considered a dummy factor in the experiment.

Keywords: Corn steep liquor, Plackett-burman design, *candida glabrata*, Optimisation

1. Introduction
Microbial fermentation is considered as the preferred production method for products because it is a low cost and environmentally friendly process that provides high quality products (Liu *et al*. 2002) [6]. It is rich in amino acids and polypeptides, which act as an excellent source of nitrogen for almost all micro-organisms (Liggett and Koffler, 1948) [5]. Some researchers have advocated the use of corn steep liquor as an intermediate component in biological processes. In the laboratory, corn-soaked water can be used in place of extracts or as the main source of nitrogen and carbon for many microorganisms.
Pyruvic acid is an intermediate in hexose catabolism and is widely used in the pharmaceutical, cosmetics, agricultural chemistry, chemistry and food industries (Li *et al*. 2001a; Yonehara and Miyata 1994) [6, 13]. According to known methods, it is mainly used industrially as a raw material for the biosynthesis of drugs such as L-tryptophan, L-tyrosine, alanine and L-DOPA (Uchio *et al*. 1976) [12]. It is also used in the manufacture of phytosanitary products, polymers, cosmetics and food additives. Calcium pyruvate can also promote fatty acid metabolism in the human body, so it also has a strong fat-reducing effect (Roufs 1996) [10].
On an industrial scale, pyruvate can be produced by chemical and direct fermentative methods. Compared with the chemical method, direct fermentative production of pyruvate from a carbon source (such as glucose) has merits in terms of both cost-effectiveness and high purity of the product. The main objective of this work is to screen, optimise and formulate a standard media composition with varied nutrient sources. The Plackett-Burman design is being employed in this study as a classical approach in the screening tests. This study would act as a benchmark in replacement of the several alternate nutrient sources in the organic form *viz*. Agro-Industrial waste. This would further propagate the ideology to reduce the media costs and overall pyruvate production costs.

2. Materials and Methods
2.1 Micro-organism and materials
Candida glabrata MCC 1800 was procured from NCMR, NCCS, Pune and was used in this study because the micro-organism shows high pyruvate production in agitated cultures (Li *et al*., 2016). Pure corn steep liquor (Roquette Ridhhi Sidhhi Pvt. Ltd, Rudrapur, Udham Singh Nagar, Uttarakhand) was used as the nitrogen source in the culture medium and has a nitrogen

content of 8.2% w/w. Chemicals and glasswares were purchased from standard suppliers and were of laboratory grade, and were used without further purification.

2.2 Culture conditions
The culture media formulation has following levels of composition parameters

Table 1: Previously used media formulations

Sr No.	Factor	Miyata & Yonehara, 2000 [8]	Hua *et al*. 1999[2]	Li *et al*. 2001[6]	Luo *et al*. 2018[7]
1	Carbon source	10g	10g	20g	30g
2	Nitrogen source	6g	3g	5g	10g
3	Time	2-3days	40-50h	48h	
4	Temperature	30℃	30℃	30℃	30℃
5	pH	5.5	5.5	5.0	5.5
6	Agitation	200-225rpm		500-700rpm	220rpm
7	KH_2PO_4	0.5g	0.1g	1g	1g
8	Thiamine	30µg	40µg	0.1mg	
9	Biotin	30µg	30µg	0.06mg	
10	$MgSO_4.7H_2O$	0.04g	0.05g	0.5g	0.5g
11	$CaCO_3$		40g	40g	

2.3 Response analysis
Dilute each sample 100-fold by adding 10 µL filtrate to 0.5 µL water in a 13 mm x 100 mm test tube. In addition, add 0.5 mL of water and 0.5 mL of 0.125 g/L DNPH 2M HCl solution. Place the sample in a water bath, remove the sample after 10 minutes and add 2.5 M 0.6ml NaOH. Next, determine the absorbance at 420 nm. Add 25-200 µl 1 mM sodium pyruvate and reduce the amount of water in the assay accordingly to prepare the standard (Schwimmer and Weston 1961) [11].

2.4 Screening of culture conditions and nutrient composition
The Plackett–Burman design was used to identify factors that significantly influence pyruvate production. A 12-run Plackett–Burman design (Plackett & Burman, 1946) [9] was used to screen 11 variables including nutrients, buffer and physical parameters at high (+1) and low (−1) levels. Maple syrup and yeast extract were tested as carbon and nitrogen source for their impact on BC production. Their concentrations were set based on our experience using fructose as carbon source. The design is useful in screening culture conditions and media components for final experiments. The design was employed with glycerol as standard carbon source with corn steep liquor as nitrogen source with maximum ratio of 50:50 with distilled water. The optimisation studies determined final culture conditions to be used in formulations.

2.5 Data analysis
Statistical experimental designs were generated and analysed using Numerical optimisation was carried using Design-Expert 10.0.8.0 statistical software in Plackett-Burman design. One factor plots were constructed for visualization of independent significant variables and their optimal values. All experiments were carried out independently in triplicates and the average values are presented.

3. Results
3.1 Effect of independent variables on pyruvate production
Plackett-Burman design was employed for screening culture conditions during standardization of media formulation. According to the 12 run-11 factor experimental data, pyruvate concentration ranged from 32.3 to 42.6 g/L. Maximum pyruvate concentration was found 42.6 g/L for experiment number 11, with the combination of carbon source 25g, nitrogen source 12g, time 72h, temperature 27℃, pH 5.0, agitation speed 200rpm, KH_2PO_4 1.2g, thiamine 10µg, biotin 15µg, $MgSO_4.7H_2O$ 0.75g and $CaCO_3$ 30g. Minimum pyruvate concentration was found 32.3 g/L for experiment number 6, with the combination of carbon source 25g, nitrogen source 8g, time 24h, temperature 27℃, pH 5.5, agitation speed 200rpm, KH_2PO_4 1.2g, thiamine 20µg, biotin 5µg, $MgSO_4.7H_2O$ 0.75g and $CaCO_3$ 50g. It can be concluded that independent nutrient concentrations are necessary during processing conditions for optimum pyruvate production (Coote *et al*. 1973) [1].

3.2 Regression analysis
Table 2 shows the regression analysis of the experimental response to the 11 culture parameters in terms of pyruvate concentration. It can be seen that within the tested range; carbon source, nitrogen source, time, temperature, pH, KH_2PO_4, thiamine, biotin, and $MgSO_4.7H_2O$ had significant effect on pyruvate production at a confidence level $P < 0.1$.

The statistical analysis of pyruvate concentration is given in Table 2. The model of pyruvate concentration was found significant ($P < 0.05$) because it had high F-value (665.42). It was also observed that the effect of all independent variables on pyruvate concentration was significant except $CaCO_3$ alongside agitation as a dummy factor within the model. The effects of individual independent variables *viz*. carbon source, nitrogen source, time, pH, thiamine and biotin were found significant ($p< 0.05$) and temperature, KH_2PO_4, and $MgSO_4.7H_2O$ were found significant ($p<0.1$). It can be noted that the presence of carbon, nitrogen and vitamins employed in the culture media are highly essential for pyruvate production.

Table 2: Regression analysis for pyruvate concentration

Source	Coefficient value	df	SS	MS	F-value	p-value
Model	36.89	10	138.63	13.86	665.42	0.0302**
A-Carbon	1.06	1	13.44	13.44	645.16	0.0251**
B-Nitrogen	1.41	1	23.80	23.80	1142.44	0.0188**
C-Time	-0.78	1	7.21	7.21	345.96	0.0342**

D-Temperature	0.53	1	3.31	3.31	158.76	0.0504***
E-pH	-1.29	1	20.02	20.02	961.00	0.0205**
G-KH_2PO_4	0.39	1	1.84	1.84	88.36	0.0675***
H-Thiamine	-0.76	1	6.90	6.90	331.24	0.0349**
J-Biotin	2.22	1	59.41	59.41	2851.56	0.0119**
K-$MgSO_4.7H_2O$	-0.41	1	2.00	2.00	96.04	0.0647***
L-$CaCO_3$	-0.24	1	0.70	0.70	33.64	0.1087
Residual		1	0.021	0.021		
Total		11	138.65			
R^2	0.9998					
Adj-R^2	0.9983					
Pred-R^2	0.9784					
C.V. (%)	0.39					

'*'significant represents 1% level of significance,
'**' significant represents 5% level of significance,
'***'significant represents 10% level of significance, the individual representations and their units are presented in previous chapter accordingly.

The proposed model was tested using a statistical method using the coefficient of determination R^2, adjusted R^2, predicted R^2 and then represented as equation (4.1). R^2 represents the proportion of variance for the pyruvate production in terms of independent variables, also known as coefficient of determination. The model was developed with values of R^2 higher than 90% and the goodness of fit of model was high showing high R^2 (0.9998) for pyruvate concentration, with least residual error (0.021) in the fitted proposed model. Similarly, Adj-R^2 (0.9983) value adjusted for the number of predictors in the model represents a satisfactory adjustment of the proposed model to the experimental data and indicates the best fit of the model. The adjusted R-squared increases only if the new term improves the model more than would be expected by chance. It decreases when a predictor improves the model by less than expected by chance. The Pred-R^2 (0.9784) value predicts the efficacy of the model for its ability to predict the response (pyruvate concentration) for new observations.

Predicted regression equation for pyruvate concentration is as follows:

Pyruvate concentration = 36.89 + 1.06A + 1.41B - 0.78C + 0.53D - 1.29E + 0.39G - 0.76 H + 2.22J – 0.41K - 0.24L …… (1)

Predicted regression equation having significant terms is given below

Pyruvate concentration = 36.89 + 1.06A + 1.41B - 0.78C + 0.53D - 1.29E + 0.39G - 0.76 H + 2.22J – 0.41K …… (2)

the coded factors used in the regression equation are the independent variables with individual coefficient of estimates to determine the response in the experimental procedure. Model equations can be used to predict a particular level of response for each factor. By default, high factor levels are coded as +1 and low factor levels are coded as -1. Coding formulas can be used to identify the relative effects of factors by comparing the coefficients. In Equation 1 the positive coefficient terms indicate a synergistic effect while the negative coefficients shows the antagonistic effect to the production of pyruvate. In other words, the positive coefficient of the model indicate an increase in responses with increase in the level of its independent variables and vice versa. The equation 2 represents predicted regression equation in terms of coded factors and the only term not incorporated in the regression equation is the $CaCO_3$ content used in the media formulation due to low significance (>10%).

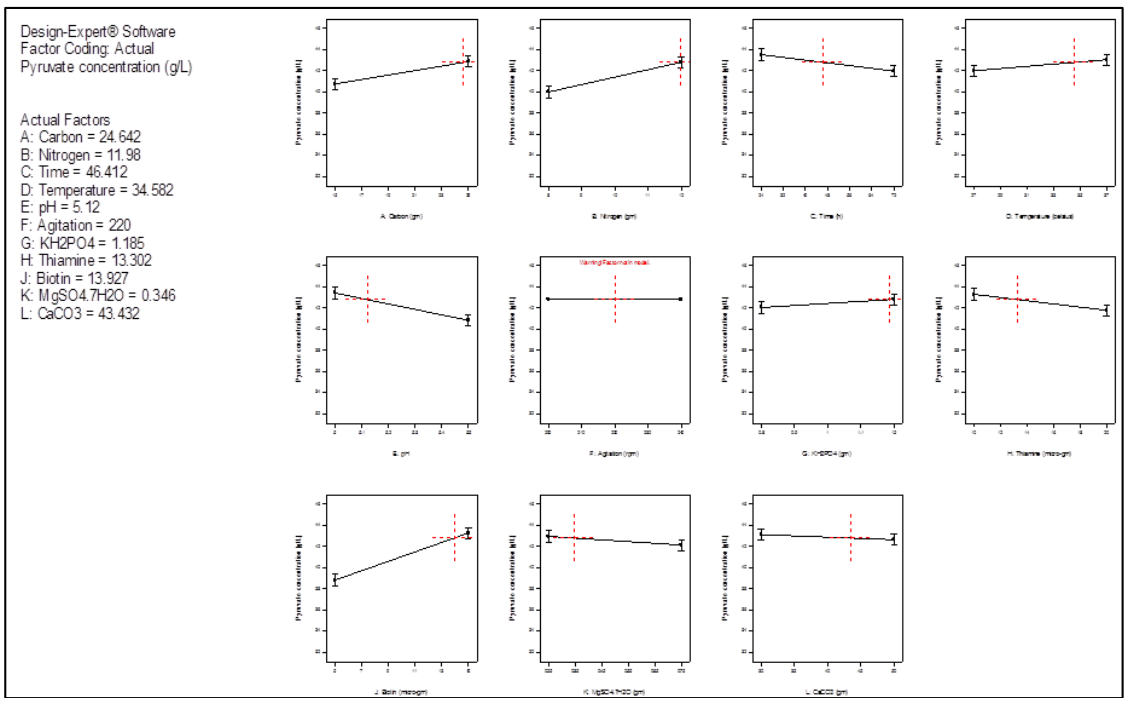

Fig 1: Effect of all factors vs pyruvate concentration

3.3 Graphical analysis

Using the analysis of the data in Table 2, all factors and predicted vs actual graphs (Figs. 1 and 2) were plotted using design expert to visualize the results. Fig. 1 shows the all factor effects on the production of pyruvate for the optimum yield and depicts the optimum values of culture parameters which will be used as constant parameters in main experiments except time and temperature. Fig. 2 displays the predicted vs actual graph over the given set of results and represents a good fit for the model when compared with the null model as it is close to the fitted line with narrow confidence bands. This figure is also a visualization of the ANOVA table. The difference is that the existence of each observation provides more information than a hypothesis test. In F-test, the confidence intervals for the entire line of the model show that all parameters except the intercept are zero. Hypothesis testing makes sense if the confidence intervals do not contain zero-level model lines. The factors compromised for main optimization experiments were screened as follows: carbon source 25g, nitrogen source 12g, pH 5.0, agitation speed 220rpm, KH2PO4 1.1g, thiamine 13µg, biotin 14µg, MgSO4.7H2O 0.3g and CaCO3 43g.

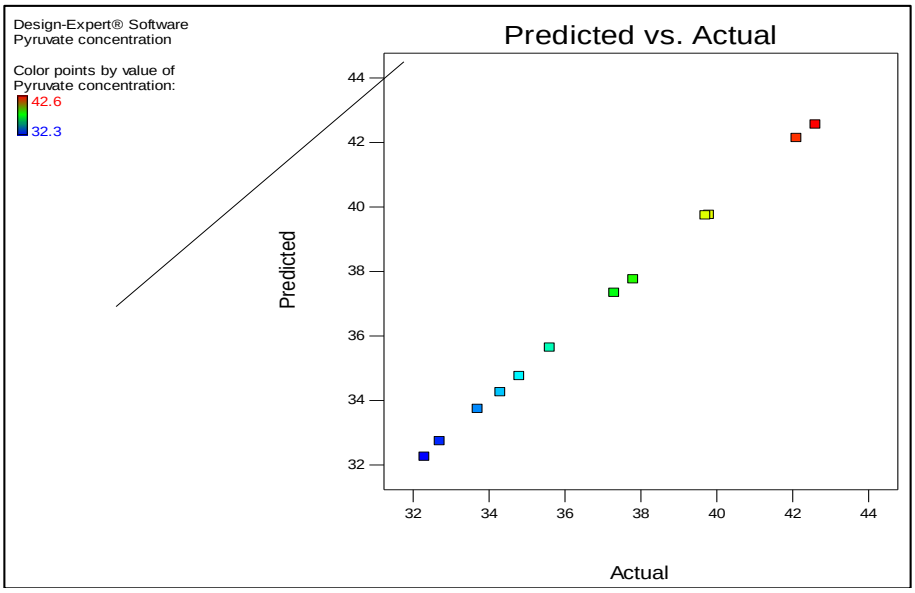

Fig 2: Actual vs predicted plot for pyruvate production

4. Discussion

The media characteristics and culture conditions are presented among the screened optimum levels using Plackett-Burman design. Based on the desired criteria, optimisation was done for maximum pyruvate production in the culture media. Total 24 possible solutions were obtained in all cases, out of which the one possible solution that suited the criteria and was most desirable among other solutions was selected and is presented in Table 3.

Table 3: Screened optimum levels of independent variables

Independent variables	Coded level	Actual level
Carbon	0.928	24.642
Nitrogen	0.945	11.89
Time	-0.066	46.412
Temperature	0.516	34.582
pH	-0.521	5.120
Agitation	0.000	220
KH_2PO_4	0.927	1.185
Thiamine	-0.340	13.302
Biotin	0.785	13.927
$MgSO_4.7H_2O$	-0.617	0.346
$CaCO_3$	0.343	43.432

The media composition is hereby benchmarked for the optimization of process protocol during the microbial production of pyruvate using corn steep liquor as nitrogen source. The amount of carbon source is also standardized with the context of total carbon in any organic biomass to be studied henceforth. The ability of *candida glabrata* to naturally accumulate pyruvate content in the deficiency of vitamins has been considered during the study (Li *et al*. 2016) [4]. Altogether, the inoculum concentration (10%w/w) and age of inoculum (3 days) has been kept constant throughout this study.

5. Conclusion

The Plackett-Burman design has been considered as an effective screening design for reducing the number of experiments and garnering the idea of significance of multiple independent culture parameters. As per this study, we have demonstrated that, using the Plackett-Burman design screening and optimisation of individual parameters can be achieved during the microbial production of pyruvate. The approach for optimization, with corn steep liquor as nitrogen source and several media components proved the need of optimum levels in production of pyruvate. Therefore, these optimum levels can be instrumental in determining the novel media formulations. The corn steep liquor producers can significantly harness the marketable value of the said industrial waste and can prove as an attractive alternative to nitrogen source. Thus the effective use of a plentiful and renewable resource in the form of corn steep liquor can be harnessed for the production of a metabolism enhancer with several medical benefits suitable for a broad range of biomedical and clinical applications.

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