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Citric Acid Optimization by *Candida tropicalis* under Submerged Fermentation Conditions Using a Plackett-Burman Design

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Abstract

Citric acid production by fermentation is the most widely used way of obtaining it. The effects of some medium components were evaluated for Citric acid fermentation during the 1930s and 1940s.This work aimed to optimize citric acid by *Candida tropicalis* under submerged fermentation conditions using Plackett-Burman design. Some factors were tested as main variables affecting citric acid production using Plackett-Burman design. The results showed that incubation period of 7 days and pH 7; sodium acetate (10g/L), magnesium sulfate (1.5g/L), potassium phosphate (5g/L), ammonium chloride (3g/L), ferric sulfate(140mg/L), manganese sulfate (50 mg/L), zinc sulfate (80 mg/L), yeast extract (5.0g/L), glucose (150g/L), aeration ratio (75ml medium/ flask 250ml) were the most effective conditions for the highest yield of citric acid. The highest citric acid concentration was 30.0 g/L of the medium under the aforementioned conditions.

Keywords: Citric acid optimization *Candida tropicalis* - Plackett-burman design, Fermentation

Contents

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1. Introduction

Citric acid is one of the most flexible industrial organic acids that are used in food preparations, makeup and pharmaceuticals. Nearly 70% of citric acid is used in food industry, candy and beverages .About 10% is used in cosmetics and pharmaceuticals [1, 2] and 18% in other industries. In the food plants, it is used as an acidulate due to its low toxicity and good solubility [3]. This property has led to an increase in its use in the cleaning process of special boilers and installations. In some cases, phosphate is replaced by citrate in detergents in order to increase its power.

Plackett–Burman design [4] is well established and widely used statistical technique for screening of different variables. The traditional 'one-factor at a time' technique used for optimizing a multivariable system is a waste of time and also misses the interactions between the variables. Also, this method requires carrying out a large number of experiments to determine the optimum levels when the interactions are significant. The disadvantages of single factor optimization process can be overcome by optimizing all the affecting factors community by central composite design [5] using response surface methodology (RSM). Basically, this optimization process involves three main steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response with checking the adequacy of the model [6]. Many biotechnology researchers have used these techniques for optimization of several parameters [7-10]

Most of the studies have been done using single factor optimization which has limitations to maximize the products due to neglect of the interaction effects of the factors. Statistical optimization is a better solution to optimize the operating factors of the processes considering the linear, quadratic, and interaction effects in the treatment. Several researches have been done using statistical optimization for different bioprocess engineering applications [11].

Therefore, this work was planned to study theeffect oftwelve factors including pH, concentration of sodium acetate, magnesium sulfate, sodium phosphate, potassium phosphate, ferric sulfate, manganese sulfate, zinc sulfate, yeast extract, glucose, aeration ratio and incubation time on the production of citric acid by *Candida tropicalis*. On the other hand, application of Plackett-Burman screening designs to optimize the more effective factors on the production of citric acid.

2. Materials and Methods

2.1. Source of Microorganisms

Candida tropicalis used in this study was previously isolated and identified by Department of Microbiology, Faculty of Science; Ain shams University, Egypt by personal communication with Prof. Dr. Yousria Hassan Sheteia. The strain was selected to study its ability to produce citric acid. The strains were cultivated on yeast and mould agar and subculture were done every month. The isolate was maintenance in Yeast-malt agar (YMA) medium.

2.2. Medium of Inoculation

Yeast-malt broth (YMB) medium, having the following composition (%, w/v): yeast extract 0.3; malt extract 0.3; glucose anhydrous 1.0; with the final pH (at 25˚C) was adjusted to 6.2±0.2.

2.3. Production Medium

Production medium consisting of the following composition (w/v) sodium acetate (5,10g/L), magnesium sulfate (0.5,1.5g/L), potassium phosphate (1,5g/L), ammonium chloride (1,3g/L), ferric sulfate(35,140mg/L), manganese sulfate (10,50 mg/L), zinc sulfate (20,80 mg/L), yeast extract (0.5,5g/L), glucose (50,150g/L), the media was inoculated by the strain (10%) from the total volume of the media then incubated at 30°C for 7 days. At the end of growth time, the cultures were centrifuged at 6000 rpm for 15 min using (Centurion Scientific LTD Model 1020 series) to separate the yeast cells from the culture filtrate.

2.4. Chromatographic Conditions

Isocratic HPLC analysis using an AGILENT (1260 HPLC Liquid Chromatography and Waters) was used in the determine citric acid concentration, Bondapak C18 3.9×300 mm column according to the method described by Crolla and Kennedy [12]**.** The mobile phase content 0.1 M KH_2PO_4 solved in distilled deionized water pH of 2.5. The mobile phase flow rate of 0.6 ml min^{-1}, ambient column temperature (25°C) and injection volume of 20µl. 1.0 ml of supernatant sample after filtered using 0.2 µm Millipore GV-13 filters. Absorbance was taken at a wavelength of 215 nm and citric acid concentrations were detected using a standard curve of absorbance at different known citric acid concentrations

2.5. Plackett-Burman Design

The fractional factorial design Yu, et al. [13] was used to reflect the relative importance of various factors on the citric acid production in liquid cultures. Twelve independent variablesas shownin Table 1 (as mention previously by Abonama, et al. [14] were screened in twenty trials organized according to the Plackett-Burman design matrix as shown in Table 2, including: initial pH, concentration of sodium acetate, magnesium sulfate, sodium phosphate, potassium phosphate, ferric sulfate, manganese sulfate, zinc sulfate, yeast extract, glucose, aeration ratio and incubation time . In this design each factor was examined at two levels: (-1) for the lowlevel, and (+1) for the high level. This design is especially practical in the case of a large number of factors and when it is unclear which settings are likely to be nearer to the optimum responses and for screening medium components with respect to their main effects and not their interaction effects. The Plackett-Burman experimental design was based on the following first-order model:$\mathbf{Y=\beta 0+\Sigma \beta ixi}$.

3. Results and Discussion

3.1. Fermentation Process

Citric acid was the principal organic acid produced. Its maximum concentration was significantly influenced by optimization of the growth medium; selecting strains and selecting the best nutritional and environmental conditions. A sequential optimization strategy was applied in this work, where the first phase dealt with screening and identifying the nutritional and environmental factors affecting citric acid production by*Candida tropicalis.* Once the significant factors affecting CA production were determined, the second phase involved ascertaining the combination that leads to the maximum citric acid production.

Plackett-Burman experimental design was reflect the relative importance of various factors in the fermentation process, in the first phase. The examined levels of the twelve culture variables were studied with twenty different fermentation experiments. All the experiments were performed in duplicates, and averages of the observationswere presented in Table 3. The data in this table indicated the analysis of glucose concentrations after 4 and 7 days fermentation. From this data we can conclude that in trial no 7B the concentration of glucose was 132 mg after 4 days but after 7 days it decreased to 108.2 (mg/dl). The results showed decrees in glucose concentration during the fermentation process and the maximum concentration was in treatments 7B after 4 days and the minimum concentration in treatment 10B after 7 days. These results showed that the organisms consumed the glucose during the first stage of the enumeration. The obtained results were similar with the results obtained by El-Baz, et al. [15].

Table 4 showed the results of the final pH after 4 and 7 days of fermentations for the *Candida tropicalis* using the Plackett- Burman design. The results showed that the highest pH value was observed at the end of the four days fermentation was 6.25 in the trial no. 16B, while the lowest was 4.36 in the trial no. 5B. The highest pH (6.40) was measured at the end of the 7 days of fermentation in the trial no. 15B, while the lowest was 4.46 in the trial no. 7B. From these results there were decreases of the pH values.Most of published in citric acid production by yeasts [16, 17] were detected that the best pH between 4.5 and 5.5.

3.2. Effect of Independent Variables on Citric Acid Production after 4 and 7 Days Fermentations using the Plackett -Burman Design by *Candida Tropicalis* at 30°C at 120 rpm

Table 5 showed the effect of independent variables on citric acid production after 4 and 7 days of fermentations by *Candida tropicalis* at 30 °C at 120 rpm using Plackett- Burman design. Data showed that the highest yield of citric acid by *Candida tropicalis* in trial no. 11 at the pH (7) was 30.0 g/L .The lowest yield of citric acid production was in treatment no.16. at the pH (7), concentration of sodium acetate (5g/L), magnesium sulfate (0.5g/L), potassium phosphate (5g/L), ammonium chloride (3g/L), ferric sulfate(35mg/L), manganese sulfate (10 mg/L), zinc sulfate (80 mg/L), yeast extract (0.5g/L), glucose (50g/L), aeration ratio(75ml medium/ flask250ml) and incubation period of 4 days. The obtained results were in agreement with results detected by Hooijkaas, et al. [18]**;** Förster, et al. [19] who stated that the maximum concentration of citric acid produced was 9.8 g $L^{-1}$and the optimum levels of each parameter for citric acid production were, 10–12% volume for initial biomass concentration, 10–15% volume for *n*-paraffin concentration, 10 mg L^{-1} for ferric nitrate concentration, and 26–30°C for temperature. The same results were observed by Yadegary, et al. [20]. The biomass-specific nitrogen feed rate is the most factor influencing in the production of citric acid by yeasts.

4. Conclusion

Due to the applications of citric acid, the conditions of production by fermentation to be of interest for many studies. At laboratory level relatively amount of this acid had been produced by isolated *Candida tropicalis*. Optimization the conditions and the selection of the suitable strains for the production was so significant factor depending on the results obtained in this investigation.

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Table-1. Factors and coded levels examined as independent variables affecting citric acid production by *Candida tropicalis* and their levels in the Plackett-Burman design experiment.

Trial	Independent variables	Units	Experimental values	
			Low level (-)	***High level (+)***
X1	Initial pH	pH	4.5	7
X2	Incubation time	Day	4	7
X3	Glucose	g/ L	50	150
X4	Yeast extract	g/L	0.5	5
X5	Sodium acetate	g/L	5	10
X6	Aeration ratio	ml/ 250 ml flask	50	75
X7	$MgSo_4H_2O$	g/L	0.5	1.5
X8	KH_2PO_4	g/L	1	5
X9	NH_4CL	g/L	1	3
X10	$Fe(So_4)_3$	mg/L	35	140
X11	$MnSo_4$	mg/L	10	50
X12	$ZnSo_4$	mg/L	20	80

Table-2. Plackett Burman design with coded values for Citric acid production

Trial no.	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12
1	1+	1+	1-	1+	1-	1+	1-	1-	1-	1+	1+	1-
2	1+	1-	1-	1+	1+-	1+	1+	1-	1+	1+	1-	1-
3	1-	1+	1+	1-	1-	1+	1-	1-	1+	1+	1+	1+
4	1+	1-	1+	1+	1-	1-	1+	1+	1+	1-	1+	1+
5	1-	1-	1-	1+	1+	1-	1-	1+	1-	1-	1-	1+
6	1-	1+	1+	1+	1+	1+	1+	1-	1+	1-	1-	1-
7	1+	1+	1-	1-	1+	1-	1-	1+	1+	1+	1-	1+
8	1+	1-	1+	1-	1+	1-	1-	1-	1-	1+	1-	1+
9	1-	1+	1-	1-	1+	1+	1+	1+	1-	1-	1+	1+
10	1-	1+	1-	1+	1-	1-	1-	1-	1+	1-	1-	1+
11	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+
12	1-	1+	1-	1-	1-	1-	1+	1+	1-	1+	1-	1-
13	1+	1+	1+	1-	1+	1-	1+	1-	1-	1-	1+	1-
14	1-	1-	1+	1+	1-	1-	1+	1-	1-	1+	1+	1+
15	1+	1+	1+	1-	1-	1-	1-	1+	1+	1-	1+	1-
16	1-	1-	1-	1+	1+	1-	1-	1+	1-	1+	1+	1-
17	1-	1-	1+	1-	1-	1+	1+	1+	1+	1+	1-	1-
18	1+	1-	1-	1-	1-	1+	1+	1-	1-	1-	1-	1+
19	1-	1-	1-	1-	1+	1+	1-	1-	1+	1-	1+	1-
20	1+	1+	1+	1+	1-	1+	1-	1+	1-	1-	1-	1-

Table-3. Results of glucose concentration after 4 and 7 days fermentations by Placket Burman design for the strain *Candida tropicalis*

No. of sample	Glucose (mg/dl) after 4 days	Glucose (mg/dl) after 7 days
1B	110	85.4
2B	109.9	74.2
3B	46.5	24.83
4B	123	96
5B	32	25
6B	33	21
7B	132	108.2
8B	126	65.3
9B	31	14
10B	23	19
11B	123	88.3
12B	32.0	19
13B	83.6	52
14B	33.3	21.3
15B	88.2	45
16B	45.2	24.0
17B	34	15
18B	125	52
19B	32	25
20B	94.7	54.1

Table-4. Results of final pH after 4 and 7 days fermentations for Plackett Burman for the strain *Candida tropicalis*

Sample no.	Beginning pH	Final pH	
		After 4 days	After 7 days
1B	7	5.76	6.13
2B	4.5	4.61	6.19
3B	7	5.89	6.17
4B	7	5.70	6.85
5B	4.5	4.36	4.69
6B	4.5	4.59	4.85
7B	4.5	4.80	4.46
8B	4.5	4.90	4.64
9B	7	5.69	6.18
10B	4.5	4.56	4.68
11B	7	5.88	5.42
12B	4.5	4.70	4.88
13B	7	5.70	6.12
14B	7	5.77	6.15
15B	7	5.52	6.40
16B	7	6.25	6.18
17B	4.5	4.63	4.58
18B	4.5	4.50	4.59
19B	7	5.42	6.01
20B	4.5	4.49	4.59

Table-5. Effect of independent variables after 4 and 7 days of fermentations using Placket Burman design on citric acid production concentrations by *Candida tropicalis* at 30 °C at 120 rpm

No	Glucose g/L	Na acetate g/L	$MgSo_4H_2O$ g/L	KH2Po4 g/L	NH_4CL g/L	Fe(So4)3 mg/L	$MnSo_4$ mg/L	$ZnSo_4$ mg/L	yeast extract g/L	Aeration	pH	Time	citric acid concentration g/L
1	150	10	0.5	5	1	140	10	20	0.5	75ml	7	4	19.0
2	150	5	0.5	5	3	140	50	20	5	75	4.5	4	12.1
3	50	10	1.5	1	1	140	10	20	5	75	7	7	7.0
4	150	5	1.5	5	1	35	50	80	5	50	7	7	14.0
5	50	5	0.5	5	3	35	10	80	0.5	50	4.5	7	3.4
6	50	10	1.5	5	3	140	50	20	5	50	4.5	4	4.7
7	150	10	0.5	1	3	35	10	80	5	75	4.5	7	13.0
8	150	5	1.5	1	3	35	10	20	0.5	75	4.5	7	5.7
9	50	10	0.5	1	3	140	50	80	0.5	50	7	7	12.9
10	50	10	0.5	5	1	35	10	10	5	50	4.5	7	3.4
11	150	10	1.5	5	3	140	50	80	5	75	7	7	30.0
12	50	10	0.5	1	1	35	50	80	0.5	75	4.5	4	4.0
13	150	10	1.5	1	3	35	50	20	0.5	50	7	4	19.5
14	50	5	1.5	5	1	35	50	20	0.5	75	7	7	9.5
15	150	10	1.5	1	1	35	10	80	5	50	7	4	14.9
16	50	5	0.5	5	3	35	10	80	0.5	75	7	4	2.5
17	50	5	1.5	1	1	140	50	80	5	75	4.5	4	3.8
18	150	5	0.5	1	1	140	50	20	0.5	50	4.5	7	7.8
19	50	5	0.5	1	3	140	10	20	5	50	7	4	5.7
20	150	10	1.5	5	1	140	10	80	0.5	50	4.5	4	8.5