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Production of Citric Acid by *Candida lipolytica* under Fermentation Conditions Using a Plackett-Burman Design

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Abstract Citric acid is one of the most versatile industrial organic acids that are used in food industries, cosmetics and pharmaceuticals products. This work aimed to produce citric acid by *Candida lipolytica* under submerged fermentation conditions using a Plackett-Burman design. Twelve factors including pH, concentration of sodium acetate, magnesium sulfate, Ammonium chloride, potassium phosphate, ferric sulfate, manganese sulfate, zinc sulfate, yeast extract, glucose, aeration ratio and incubation time at a temperature of 30°C were tested as main variables affecting citric acid production using Plackett-Burman design. The results indicated that pH (7), concentration of 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 (5g/L), glucose (150g/L), aeration ratio(75ml medium/ flask250ml) and incubation period of 7 days were the most effective factors for the highest yield of citric acid production. The highest citric acid concentration was 22.8 g/L of the medium under the previously mentioned conditions.

Keywords: *Citric acid- Candida lipolytica-Plackett-Burman design*

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1. Introduction

Citric acid (CA) is one of the most versatile industrial organic acids that are used in food preparations, cosmetics and pharmaceuticals. About 70% of citric acid is utilized in food industry, confectionary and beverages as an acidulant, flavor enhancer, preservative, chelator, buffer, emulsifier, stabilizer and antioxidant. About 10% is used in cosmetics and pharmaceuticals (Kubicek & Rohr, 1986 and Lodhi *et al*., 2001). In the food industry, it is used as an acidulate due to its lower toxicity and high solubility (Kapoor *et al*., 1982). 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. In this case, it is used not only for cleaning metal, but also in domestic detergents. Due to its easy biodegradability, the use of CA expanded and replaced the polyphosphates.

In 2004, the worldwide production of CA was approximately 1.4 million tons, according to the Business Communications Co. (BCC)'s recent studies on fermentation (Soccol& Vandenberghe, 2003; Soccol *et al*., 2006). Moreover, due to its large applications and low price, the CA consumption is expected to grow significantly until 2009, and this raises the need for industries to search for new technological alternatives and for cost reduction in CA production (Vandenberghe *et al*., 2000).

CA is often produced by fermentation using low cost raw materials. The composition of these products varies according to their origin, conservation, and obtaining methods. A great variety of substrates can be used in CA production by solid-state fermentation (SSF) such as some by-products and agro-industrial residues. There are countless possibilities in establishing industrial activities directed to the improvement and/or reprocessing of bioresidues such as sugarcane bagasse, cassava bagasse, and CP (Kolicheski 1995; Soccol, 1996 and Pandey *et al*., 2000) which can cause serious environmental problems.

Citric acid is considered colorless, odorless and easily soluble in water and alcohol with a pleasant taste, solid at room temperature and melts at 153ºC. It exists as an intermediate in the Krebs cycle when carbohydrates are oxidized to carbon dioxide (Haq *et al*., 2002).

The industrial CA production is performed using *A. niger*, due to its higher capacity to accumulate acid when compared to other microorganisms (Yokoya 1992; Pazouki *et al*., 2000 and Crolla & Kennedy, 2001). The main advantages of the use of *A. niger* are: it's easy manipulation, its ability to ferment a great variety of raw materials, the low cost of its fermentation, and its capacity to obtain high yields of CA (Yokoya, 1992).

The main target of the present study was to produce of citric acid by *candida lipolytica* under submerged fermentation conditions using the Plackett-Burman design. Twelve 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 is to contribute a model that can be applied for the optimization of citric acid production by *Candida lipolytica* using the Plackett-Burman screening design.

2. Materials and Methods

2.1. Microorganism

Candida lipolytica used in this study was previously isolated and identified by industrial biotechnology dept. Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City. The strain was selected to test its ability to produce citric acid.

2.2. Maintenance Medium

Yeast-malt agar (YMA) medium, having the following composition (%, w/v): yeast extract 0.3; malt extract 0.3; glucose anhydrous 1.0; agar 1.5, the medium pH (at 25°C) was adjusted to 6.2 ± 0.2.

2.3. Inoculation Medium

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

2.4. 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),

At the end of fermentation time, the broths were centrifuged at 6000 rpm for 15 min. using (Centurion Scientific LTD Model 1020 series) to separate the yeast cells from the culture filtrate. Biomass, pH, glucose content, protein content and citric acid content were estimated in the examined samples

2.5. Glucose Determination

Glucose was determined colorimetrically method using enzyme colorimetric GOD-POD (glucose oxidase-peroxidase) kit (Diamond Diagnostics). Measurement was carried out at 37°C after 10 min of mixing the samples with the reagent, and then the color intensities were measured at wavelength 546 nm versus a standard using a spectrophotometer (UV-200-RS LW Scientific). Procedures of measurement were carried out according to manufacture′s instructions (Kapan *et al*., 1984).

2.6. Chromatographic Conditions

Citric Acid concentration was determined by isocratic HPLC analysis using a AGILENT (1260 HPLC Liquid Chromatography and Waters), Bondapak C18 3.9×300 mm column according to the method described by Hooijkaas et al.(1998). The mobile phase consisted of 0.1 M KH_2PO_4 in distilled deionized water adjusted to a pH of 2.5 with concentrated H_3PO_4. Analysis consisted of a mobile phase flow rate of 0.6 ml min−1, ambient column temperature (25 °C), and injection volume of 20µl. 1.0 ml of cell free supernatant sample was filtered using 0.2 µm Millipore GV-13 filters prior to injection into column. Absorbance readings were taken at a wavelength of 215 nm and citric acid concentrations determined using a standard curve of absorbance at various known citric acid concentrations. However, in this citric acid concentration there are also trace amounts of iso-citric acid. The HPLC retention time cannot differentiate between the two isomers.

2.7. Plackett-Burman Design

The Plackett-Burman experimental design, a fractional factorial design Yu *et al*. (1997) used to reflect the relative importance of various physical and nutritional factors on the citric acid production in liquid cultures. In this design each factor was examined at 2 levels: (-1) for the low level, 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 Plackett & Burman, (1946) and for screening medium components with respect to their main effects and not their interaction effects. Table 1 represents the physical conditions and medium components as well as the higher and lower levels of each factor used in the experimental design, whereas Table 2 represents the Plackett-Burman design with the coded values. The studied factors were: 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 were tested. The Plackett-Burman experimental design was based on the following first-order model: $Y=\beta 0+\Sigma\beta ixi$.

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

Trial	Independent variables	Units	Experimental values	
			Lower level (-)	*Higher 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	Na Acetate	g/L	5	10
X6	Aeration ratio	ml/ 250 ml flask	50	75
X7	$MgSo4H_2O$	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. The Plackett Burman experimental design with coded values for CA 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-

3. Results and Discussion

3.1. Characteristics of Fermentation Process

Optimization of the growth medium for the citric acid production by selecting the best nutritional and physical conditions is important to increase the CA yield. A sequential optimization strategy was applied in this work, where the first phase dealt with screening and identifying the nutritional and physical factors affecting CA production by *Candida lipolytica***.** Once the significant factors affecting CA production were determined, the second phase involved ascertaining the combination that leads to the maximum CA action.

In the first phase, a Plackett-Burman experimental design was applied to reflect the relative importance of various fermentation factors. The examined levels of the twelve culture variables, was studied with twenty different fermentation experiments. All the experiments were performed in duplicated, and averages of the observations were presented in Table 3. The data in this table indicate the analysis of glucose concentrations after 4 and 7 days fermentation. From this data we can conclude that in trial no 10A the concentration of glucose was 300 mg after 4 days but after 7 days it decreased to 194 (mg/dl). From these results there were decreases in glucose concentration during the fermentation process and the maximum concentrations were in treatments 10A after 4 days and in treatment 10A after 7 days. These results indicated that the microorganisms consumed the glucose during the first stage of the cultivation. The obtained results were in agreement with the results obtained by El-baz *et al*., 2012 who demonstrated that during the first stage of the fermentation process; glucose was consumed exclusively as carbon source.

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

Sample No.	Glucose (mg/dl) for 4 days	Glucose (mg/dl) for 7 days
1A	110.0	60.8
2A	109.9	55.6
3A	108.0	24.83
4A	250.0	156.1
5A	85.7	49.4
6A	105.2	62.6
7A	200.3	108.2
8A	220.8	122.8
9A	250.7	105.5
10A	300.8	194.3
11A	152.9	88.3
12A	112.4	62.3
13A	83.6	40.4
14A	250.3	102.9
15A	88.2	40.6
16A	ND	ND
17A	103.5	40.1
18A	65.9	31.3
19A	ND	ND
20A	94.7	33.8

Table 4 presented the results of final pH after 4 and 7 days fermentations of the strain *Candida lipolytica* using the Plackett- Burman design. The data indicated that there were incensements in the value of pH in all the experiments. The highest pH value measured at the end of the four days fermentation period was 6.88 in the trial no. 4A, whereas the lowest was 4.45 in the trial no.7A. The highest pH value measured at the end of the 7 days of fermentation was 6.97 in the trial no. 14A, whereas the lowest was 4 .40 in the trial no. 2A. From the obtained results we can conclude the decrease of the pH values

during the fermentation due to the secretion of organic acids mainly citric acid. The obtained results were in agreement with the results obtained by El-baz *et al*., 2012 who stated that during the death phase, cells were autolyzed and citric acid was synthesized. in addition to GL which has a higher acidity than GA. Glycyrrhizin was consumed by *Aspergillus parasiticus* Speare BGB and leading to the GA accumulation. All of these together made pH value of the fermentation increase gradually.

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

Sample no.	Initial pH	Final pH	
		After 4 days	After 7 days
1A	7	5.73	5.20
2A	4.5	4.65	4 .40
3A	7	6.50	6.65
4A	7	6.88	6.79
5A	4.5	4.58	4.79
6A	4.5	4.58	4.78
7A	4.5	4.45	4.62
8A	4.5	4.51	4.55
9A	7	6.55	6.79
10A	4.5	4.69	4.65
11A	7	5.59	5.50
12A	4.5	4.64	4.69
13A	7	5.48	6.83
14A	7	6.32	6.97
15A	7	5.88	5.50
16A	7	6.21	5.67
17A	4.5	4.65	4.55
18A	4.5	4.56	4.69
19A	7	5.98	5.50
20A	4.5	4.58	4.65

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

Table 5 presented the effect of independent variables on citric acid production after 4 and 7 days of fermentations using Plackett- Burman design by *Candida lipolytica* at 30 °C at 120 rpm. Data showed that the highest yield of citric acid by *Candida lypolitica* was. in trial no. 11 at the pH (7), concentration of 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 (5g/L), glucose (150g/L), aeration ratio(75ml medium/ flask250ml) and incubation period of 7 days. The lowest yield of citric acid production was in treatment no.17. The obtained results were in agreement with the results obtained by Hooijkaas *et al.*, 1998 and Förster *et al*., (2007) who stated that 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. Similar results were obtained by Yadegary *et al*., (2013) who found that the optimum nitrogen concentration and adapted C/N ratio are essential for successful continuous citric acid production. The biomass-specific nitrogen feed rate is the most important factor influencing continuous citric acid production by yeasts. Numerous chemostat experiments showed the feasibility of continuous citrate production by yeasts. On the other hand, found that the sugarcane bagasse is an ideal substrate in producing citric acid and the aforementioned process could be considered as a beneficial and cost-effective method in citric acid production during citric acid production from sugarcane bagasse through solid state fermentation method using *Aspergillus niger* mold and optimization of citric acid production by taguchi method.

Table 5. Effect of independent variables on citric acid concentration after 4 and 7 days fermentations using Plackett Burman design by *Candida lipolytica* at 30°C at 120 rpm

Trail No	Glucose (g)	Na acetate (g)	MgSo4H2O(g)	KH2Po4 (g)	NH4CL (g)	Fe(So4)3 (mg)	MnSo4 (mg)	ZnSo4 (mg)	yeast extract (g)	Aeration ml/250ml flask	pH	Time (Day)	citric acid concentration g/L
1	150	10	0.5	5	1	140	10	20	0.5	75	7	4	21
2	150	5	0.5	5	3	140	50	20	5	75	4.5	4	13.16
3	50	10	1.5	1	1	140	10	20	5	75	7	7	15.1
4	150	5	1.5	5	1	35	50	80	5	50	7	7	20
5	50	5	0.5	5	3	35	10	80	0.5	50	4.5	7	2.7
6	50	10	1.5	5	3	140	50	20	5	50	4.5	4	5.93
7	150	10	0.5	1	3	35	10	80	5	75	4.5	7	13.3
8	150	5	1.5	1	3	35	10	20	0.5	75	4.5	7	4.0
9	50	10	0.5	1	3	140	50	80	0.5	50	7	7	9.7
10	50	10	0.5	5	1	35	10	10	5	50	4.5	7	11.15
11	150	10	1.5	5	3	140	50	80	5	75	7	7	22.8
12	50	10	0.5	1	1	35	50	80	0.5	75	4.5	4	3.8
13	150	10	1.5	1	3	35	50	20	0.5	50	7	4	18
14	50	5	1.5	5	1	35	50	20	0.5	75	7	7	10.8
15	150	10	1.5	1	1	35	10	80	5	50	7	4	13.3
16	50	5	0.5	5	3	35	10	80	0.5	75	7	4	6.4
17	50	5	1.5	1	1	140	50	80	5	75	4.5	4	3.6
18	150	5	0.5	1	1	140	50	20	0.5	50	4.5	7	4.9
19	50	5	0.5	1	3	140	10	20	5	50	7	4	5.9
20	150	10	1.5	5	1	140	10	80	0.5	50	4.5	4	7.5

Another more convenient way of representing the results of the Plackett- Burman design is using a Pareto chart, which displays the magnitude of each estimate; Figure 1 Showed the ranking of the factor estimates in a Pareto chart. On analyzing the chart; the following three variables; glucose, sodium acetate concentration and PH value have the main effect on CA production.

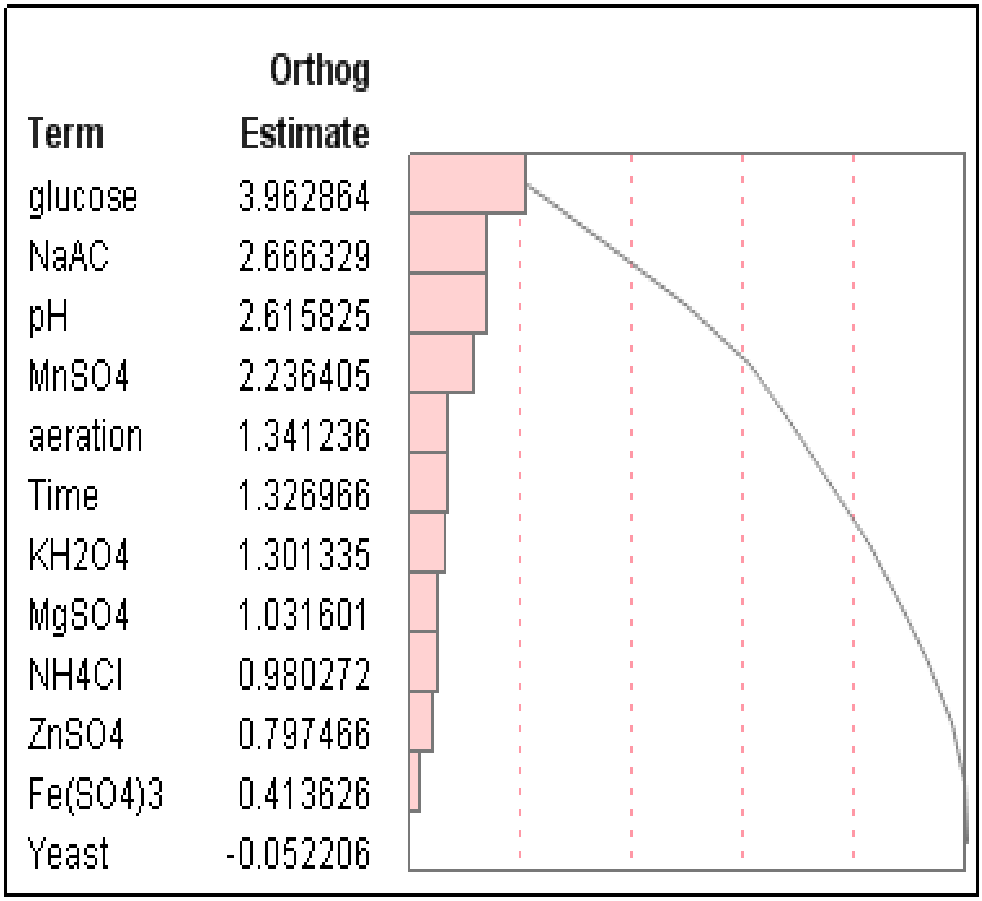

Figure 1. Pareto graph showing effect of various variables on citric acid production based on the observations of Plackett–Burman design

The RSq value for the CA production was 0.92 for the Plackett-Burman design, (Figure 2). This indicated a high degree of correlation between the experimental and predicted values and also indicated an increase in validity of experimental designs results.

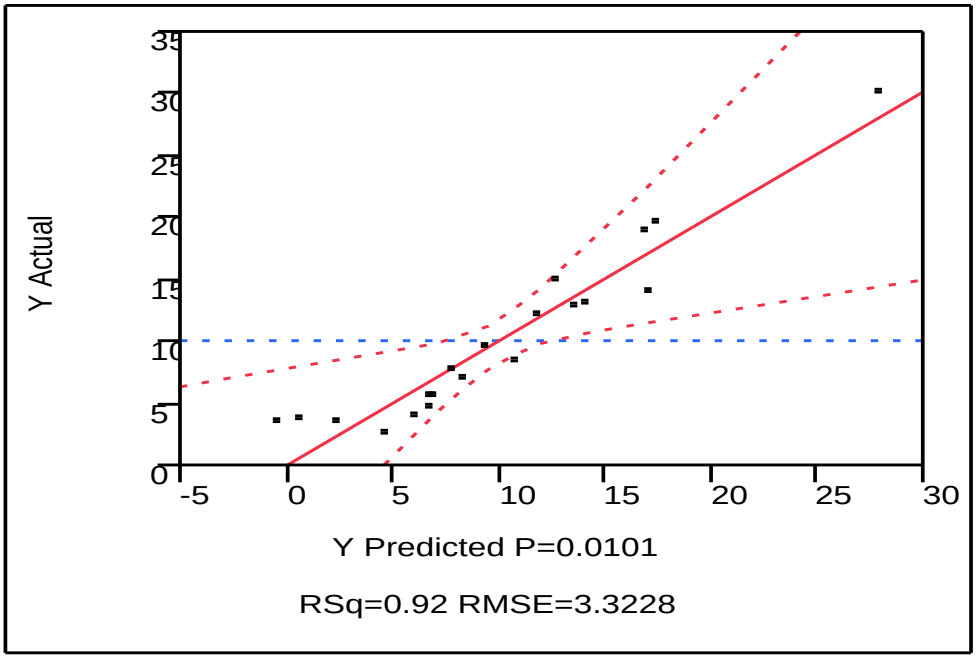

Figure 2. Response Y Actual by Predicted Plot

4. Conclusion

Because of the wide applications of citric acid, its production by fermentation continues to be of interest for extensive study. Over a period of years a lot of substrates, different microorganisms and techniques were introduced for citric acid production in pilot scale so as to enable the industry to scale it up and increase the production to meet the demand for citric acid. At laboratory level scientists try introducing new substrates and methods and confirm their potential with respect to various aspects. The substrates from economical sources certainly can reduce the cost of production but in terms of limiting substances in them and their removal, it needs extensive attention to make the process more successful. It is now realized that conversion of industrial waste with the microorganisms to value added products is profitable provided that the process control strategies are carefully monitored and controlled. The optimization of the conditions and the selection of the suitable strains for the production were so significant factors depending on the results obtained in this investigation.

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