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RESEARCH ARTICLE

RESPONSE SURFACE METHODOLOGY FOR OPTIMIZATION OF SORGHUM MALT MEDIA FOR CITRIC ACID PRODUCTION BY IMPROVED STRAINS OF *Aspergillus niger*

M.P.D.Prasad[1], N.V.Surendra Babu [2], *V.Sridevi[3] O.V.S Reddy[4], R.S Prakasam[5]

1. Department of Biotechnology, Institute of Science & Technology, Jawaharlal Nehru Technological University, Kakinada, East Godavari Andhra Pradesh, India.
2. Department of Pharmacy, NRI Group of Colleges, Vijayawada, Krishna, Andhra Pradesh, India.
3. Department of Chemical Engineering, Andhra University, Visakhapatnam-03, Andhra Pradesh, India
4. Department Of Biochemistry, S.V.Univeristy, Tirupathi, Andhra Pradesh, India.
5. Senior Principal scientist, Indian Institute of Chemical Technology, Hyderabad, Andhra Pradesh, India.

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Abstract

Citric acid is a tri carboxylic organic acid is the most important acid produced by fermentation used in the food and pharmaceutical industries. The present work deals with determination of optimum growth conditions for citric acid production by submerged fermentation by using strain of *A.niger MTCC662* respectively. Isolation, screening and mutated the *A.niger* strain induced by UV irradiation, diethylsulphate and Co60 by providing different types of media and the submerged fermentation was carried out under various growth parameters like temperature, pH and media of Sorghum malt as substrate. Strain improvement studies for maximum production of citric acid obtained after the fermentation. By using the response surface methodology has been optimized the production of citric acid. These variables were further optimized using a 24 full factorial CCD (Central Composite Design) and a second order polynomial model equation was obtained. In the present study the value of the regression coefficient R^2 = 0.6238 which indicates that 62.38% of the variability in the response could be explained by the model. The adjusted R^2 value is 0.6238 which is also very high to advocate the significance of the model. Maximum yield was obtained from mutant type of *A. niger MTCC* 662 than wild strains were about 12.0g/100ml and 8.2g/100ml. It could be concluded that the *A.niger MTCC662* mutant strains have produced high yield than wild strain of *A.niger MTCC662* cultures.

Introduction

Citric acid is a tricarboxylic organic acid, soluble in water with pleasant taste, and is the most important in food and other industries. Citric acid derives its name from the Latin citrus, the citron tree, the fruit of which resembles a lemon. Refined sugars such as glucose and sucrose are the most commonly used substrates for commercial production of citric acid by fermentation process; however they are expensive and can be replaced by various cheap and abundant substrates like agro-industrial wastes or by-products1 including starch, cellulose and lignocellulose material. Surface method and submerged process are widely recognized for the production of citric acid. The surface process though commercially profitable for many years, is labored intensive and inefficient in its use of space. The submerged process has become the method of choice in the industrialized countries, because it is less labour intensive, yields higher production rate, and uses less space. Citric acid is used in food, Confectionery and beverages, in pharmaceuticals and industrial fields. Citric acid forms a wide range of metallic salts including complexes with copper, iron, magnesium, manganese and calcium. These salts are used as sequestering agents in industrial processes and as anticoagulants in blood preservation. Citric acid is used as plasticizer in plastic films and as an antioxidant in oil and fats. Sorghum is produced in agro-based countries like India (Abuzeid, A.Z.A, 1981). The present work is focused on these substrates which are rich in starch. Instead of hazardous acid hydrolysis and expensive enzymatic treatment, the flours are hydrolyzed by natural malting process which is economically feasible and enhances the nutrient quality of the malts for fermentation process. Since the citric acid concentration produced by wild strains is too low for economical processes, strain improvement was carried out in order to develop mutants of parent strain for increased production of the products (Adham. N.Z, 2002). However, strain development from wild strains to mutants depends mainly on the process of mutagenesis (physical and chemical mutagenesis). Although, traditional random strain mutagenesis is time-consuming and lacks specificity and precision due to the absence of adequate molecular information i.e., there is no opportunity to expand the gene pool, it is still a very practical approach4, as it is quite simple and rapid method when compared to targeted mutagenesis. Developments of mutant strains which can synthesize higher concentration of citric acid within a short fermentation time and capable of growing at lower pH (Prescott S, 1981).

Materials and Methods

Isolation: A successful fermentation process depends both on an appropriate strain and optimization of fermentation parameters. Samples were collected from sugar cane waste molasses from local industry Empee Sugar and Distilleries, Naidupeta, Nellore district (A.P).

Screening of fungal cultures:

Dye Method: The *Aspergillus niger* was screened qualitatively in Petri plates containing Czapek-Dox agar medium with Bromocresol green as an indicator (Bai DM, 2004).

Preparation of medium: Sorghum (*Sorghum bicolor*) is the cereal crops produced through out the world, and are very important to the Asian countries. Hence the raw material is available perhaps at a cheaper cost. The rate conversion of carbohydrates into fermentable sugars is high in sorghum. The conversion rate of starch into simpler sugars in these substrates is around 80-88% (Domsch.K.H, 1980).

Malting and mashing of substrates: Instead of expensive enzymatic hydrolysis and hazardous acid hydrolysis the natural and safe malting and mashing process is adopted to convert the complex substrates in to readily utilizable simple substrates (Parekh S, 2000).

Estimation of reducing sugar: The reducing sugars in the malted and mashed grains of sorghum and paddy estimated by DNS (Dinitro salicylic acid method). The result showed that paddy and sorghum malts contained a high amount of reducing sugar and can serve as better substrate (Mattey M, 1990). The reducing sugars further qualitatively estimated by paper chromatography with standard reducing sugars.

Estimation of citric acid: Citric acid from the fermentation broth is quantitatively estimated by acetic anhydride, pyridine method using spectrophotometer at 405 nm (Marrier, J.R, 1958).

Strain improvement studies: Mutagenesis is mainly through physical mutagen by UV irradiation, chemical mutagen by diethyl sulfonate (DES) and a mixture of both (UV and DES) mutagens in combination with certain time intervals of exposure (Gupta, S., 2002)

Design of Experiments: Design of experiments is a technique for setting an efficient experiment point parameter. This is equivalent to establishing a parameter for creating a better regression expression. This regression expression itself will be a response surface using the least-square method, as explained in the preceding section. In design of experiments, all variables, even continuous ones, are thought of as being discrete "levels". As a design of experiments, many techniques have been proposed such as full factorial design, orthogonal design, central composite design (CCD).

Orthogonal design: In an orthogonal design, parameter setting involves allocating levels by using an orthogonal array L8 orthogonal array with two levels. In this array, the rows indicate the number of experiments. Parameters can be set easily by allocating variable levels to the individual columns as instructed. An orthogonal array for a function of a third or higher degree can be created. For a multi-level orthogonal array, however, experiments will not be orthogonal and, therefore, for a high polynomial of a second or higher degree, an orthogonal polynomial is required.

Results and Discussion

A successful process depends upon appropriate strain, culture medium and optimization of fermentation parameters. In the present work, cultural conditions such as sugar concentration, time profile of citric acid synthesis, incubation time, temperature, pH and nitrogen source were optimized with the parent isolate and mutant strain on sorghum malt substrates.

Optimization of malting and mashing conditions: Malting is the limited germination of the sorghum grain under very carefully controlled conditions. The steeping conditions (time and temperature) for sorghum were optimized and maximum yield of reducing sugars were obtained at 18 h of time and 31°C temperature for sorghum. The reducing sugar concentration was more with NaOH steeping rather than with distilled water or KOH. The germination process was carried out for 3 days for optimum yield of reducing sugars. In order to convert more amount of starch, mashing process was carried out with different ranges of temperature (55°C-65°C) and the yield obtained was maximum at 59°C for sorghum.

Optimization of media conditions for maximum production of citric acid:

Incubation temperature: The production of citric acid at different temperatures (25°C-35°C) was studied. Maximum production of citric acid was obtained when temperature of the medium was at 30°C.

Effect of pH: The production of citric acid was studied at different pH (4.8 to 6.0) levels. Maximum yield was obtained (9.3g/100 ml). When the pH was maintained at 5.4 with the *Aspergillus niger* and maximum yield 12g/100ml with the mutant strain at pH 4.8.

Substrate concentration: The effect of substrate concentration was also studied with 10%, 15%, 20% of sorghum with parent isolate and mutant strain. The same experiment was also carried out with MTCC culture for comparison of efficiency of the parent isolate for citric acid production. The high yield of citric was reported at 15% sorghum with *Aspergillus niger* isolate and mutant strain.

Other nutrients: The effect of the nitrogen sources (both natural and artificial) was studied for the fermentative production of citric acid was 2.4 mM concentration of ammonium sulphate with the *Aspergillus niger* isolate and mutant strain.

Reducing sugar concentration: The effect of different sugar concentration (12-18g/100 ml) on citric acid production by *Aspergillus niger* isolate was carried out, and the maximum amount of citric acid (9.3g/100 ml) was obtained in the medium containing 15g/100ml.

Time profile of citric acid: The fermentation was carried out with different malt of sorghum from 24 h to 216 h. Maximum yield of citric acid was obtained after 144 h (7 days).

Response Surface Methodology: Further through response surface methodology, the fermentation parameters like temperature, pH, substrate concentration and incubation time and nitrogen concentration were further optimized with stataicsa software with minimum number of experiments with sorghum malt substrates with parent isolate. The mutant strains showed better yield than the parent strain by several folds. Further optimization through RSM with different variables further increased the yield of citric acid. The desirability surface/contour graphs, Pareto chart, critical values, are tabulated.

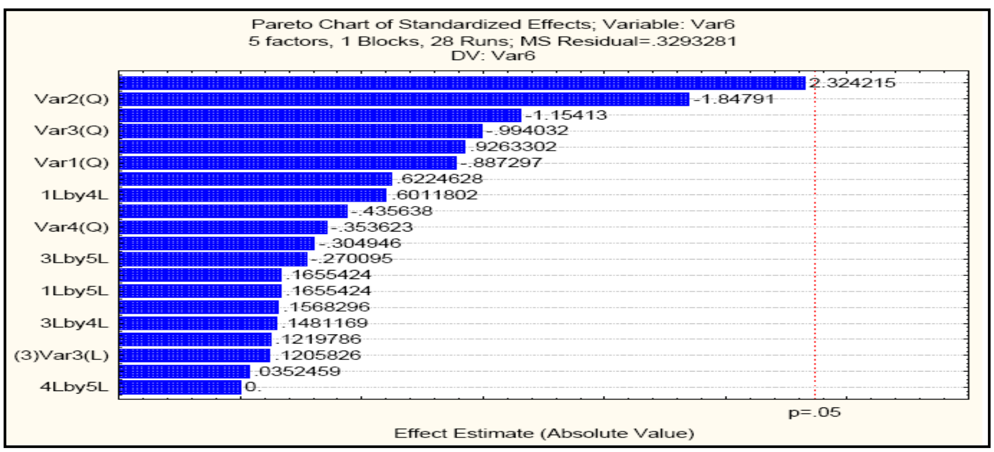


Fig 1: Pareto chart of standardized effects (Sorghum)

The Pareto chart (Figure 1) shows each of the estimated effects, interactions and the standard error of each of the effects, which measures their sampling error. In the experimental design the Pareto chart is a Frequency Histogram that shows the amount of influence each factor has on the response in decreasing order.

Table 2 Critical values (Sorghum)

Factor	Observed minimum	Critical values	Observed maximum
Var 1	23.44963	43.2482	32.5504
Var 2	4.48993	4.3770	6.3101
Var 3	8.62407	28.3208	31.3759
Var 4	41.39554	428.6902	150.6045
Var 5	0.43489	2.1815	3.1651

Critical Values: From the table 1 43.24 temperature, 4.3 pH, 28% Substrate concentatrtion, 428 hrs of incubation, 2.0Mm concentration of nitrogen was needed by yeast in order to increase the ethanol production.

Table 2: Observed, Predicted, residue values (Sorghum).

Case or Run	Observed	Predicted	Residues
1	0.5100	0.698273	-0.188273
2	0.7100	0.900810	-0.190810
3	0.5100	0.490625	0.019375
4	0.6900	1.002377	-0.312377
5	0.4900	0.636546	-0.146546
6	0.6600	1.138298	-0.478298
7	0.5700	0.838113	-0.268113
8	0.7400	1.010650	-0.270650
9	0.5700	0.517835	0.052165
10	1.2600	1.539587	-0.279587
11	0.6300	0.699402	-0.069402
12	1.3200	1.391939	-0.071939
13	0.5400	0.775323	-0.235323
14	0.7200	0.957860	-0.237860
15	0.5100	0.537675	-0.027675
16	1.0500	1.409427	-0.359427
17	1.3800	1.477760	-0.097760
18	1.2500	0.820656	0.429344
19	1.3000	1.137292	0.162708
20	0.5100	0.430009	0.079991
21	0.9600	0.447938	0.371695
22	1.2600	0.888305	0.220357
23	1.1700	0.949643	0.220357
24	0.6900	0.687830	0.0021790

25	2.4600	1.870118	0.5898825
26	0.6000	0.593370	0.00630
27	1.6500	1.064578	0.585422
28	1.6800	1.477760	0.202240

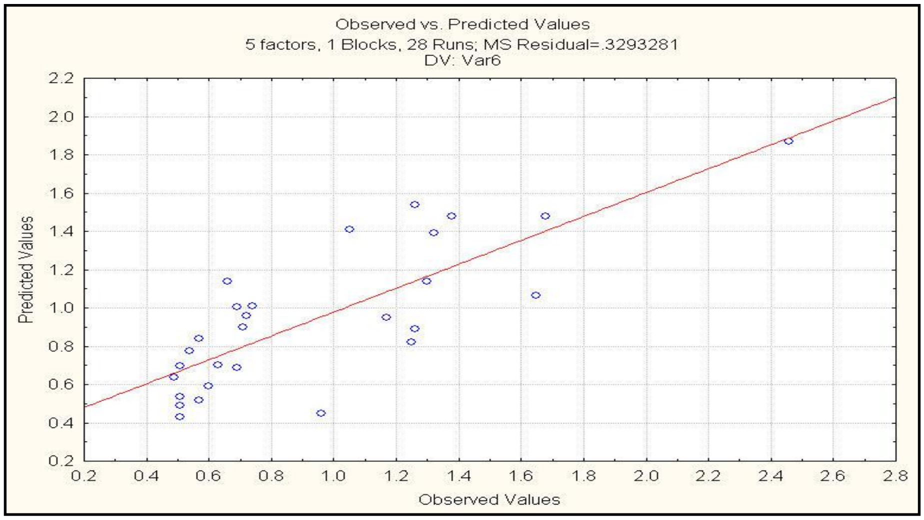


Figure 2: Observed vs. Predicted values (Sorghum)

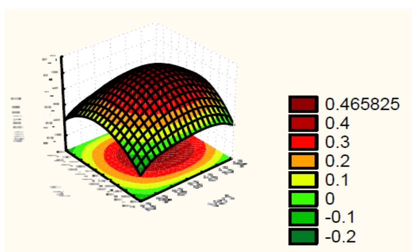


Figure 3: Desirability Surface; method: spline fit (Sorghum)

Figure 3: Response surface and contour plot showing the effect of temperature and pH on production of citric acid. Other variables are held at zero level.

Figure 4: Response surface and contour plots showing the effect of temperature and substrate concentration (above), the effect of temperature and incubation time (below), on production of citric acid . Other variables are held at zero level.

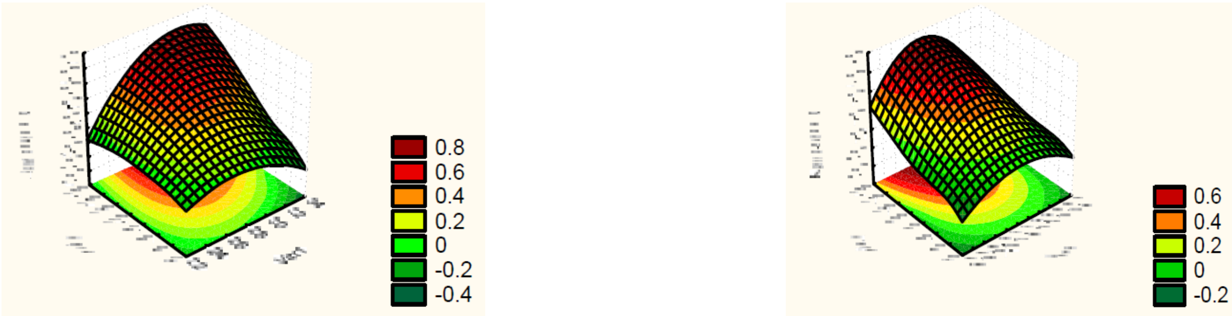


Figure 5: Response surface and contour plots showing the effect of temperature and nitrogen concentration (above), the effect of pH and substrate concentration (below), on production of citric acid . Other variables are held at zero level.

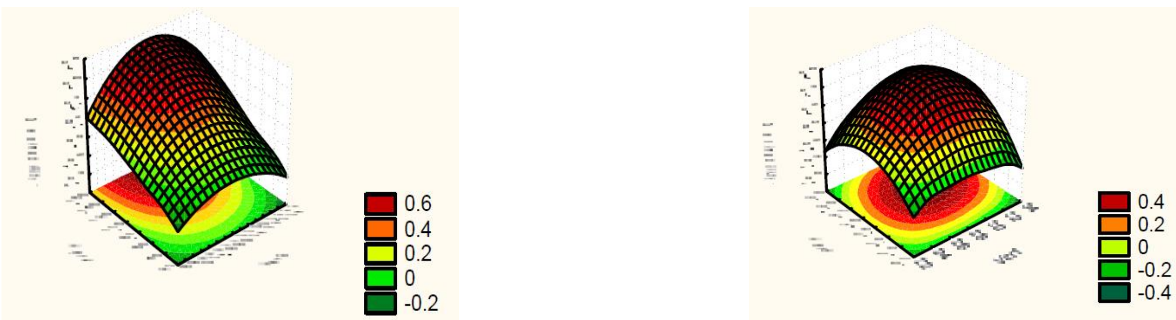


Figure 6: Response surface and contour plots showing the effect of pH and incubation period of fermentation (above), the effect of pH and Nitrogen source (below), on production of citric acid. Other variables are held at zero level.

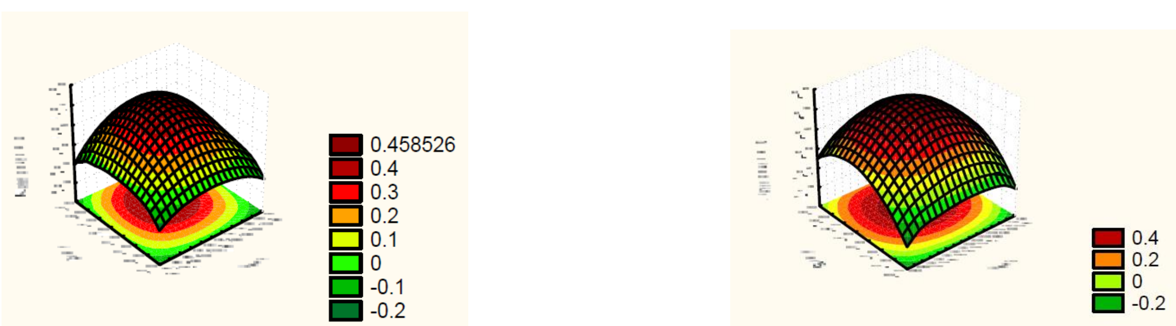


Figure 7: Response surface and contour plots showing the effect of substrate concentration and incubation time (above), the effect of substrate concentration and nitrogen (below), on production of citric acid. Other variables are held at zero level.

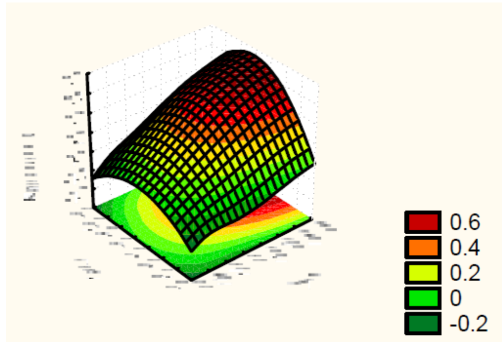


Figure 8: Response surface and contour plot showing the effect of incubation time and nitrogen source on production of citric acid. Other variables are held at zero

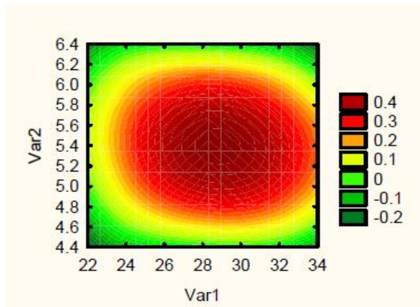


Figure 9: Contour plot showing the effect of temperature and pH on production of citric acid. Other variables are held at zero

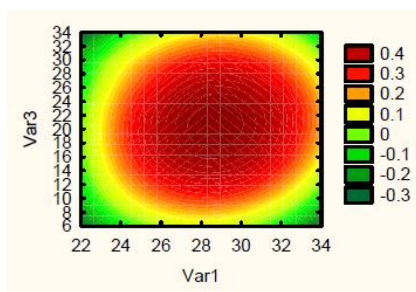


Figure 10: Contour plot showing the effect of temperature and Substrate concentration on production of citric acid . Other variables are held at zero

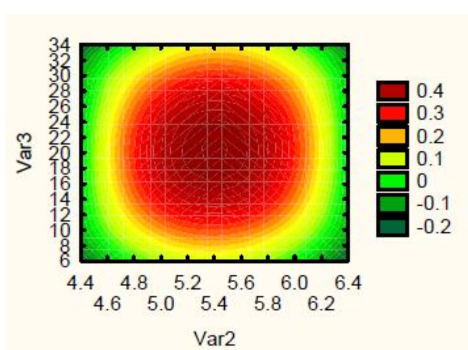
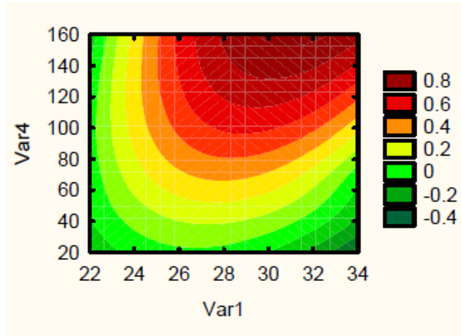


Figure 11: Contour plot showing the effect of pH and Substrate Concentration on production of citric acid. Other variables are held at zero



Figur 12:Contour plot showing the effect of temperature and incubation time of fermentation on production of citric acid . Other variables are held at zero

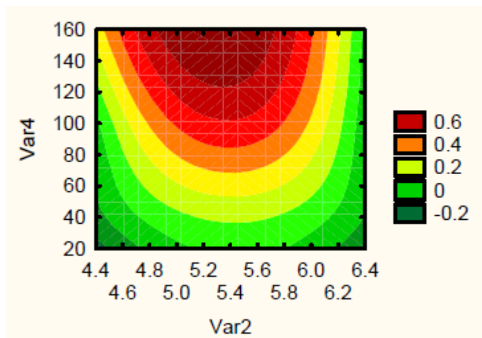


Figure 13: Contour plot showing the effect of pH and Incubation time of fermentation on production of citric acid . Other variables are held at zero

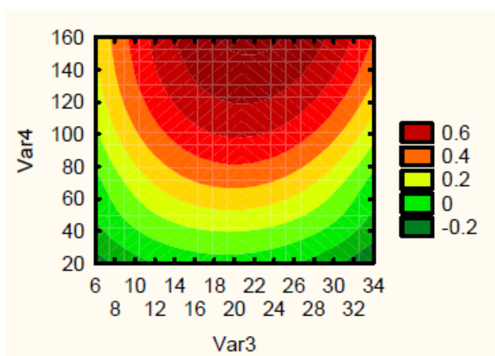


Figure 14 Contour plot showing the effect of Substrate concentration and incubation period of fermentation on production of citric acid. Other variables are held at zero

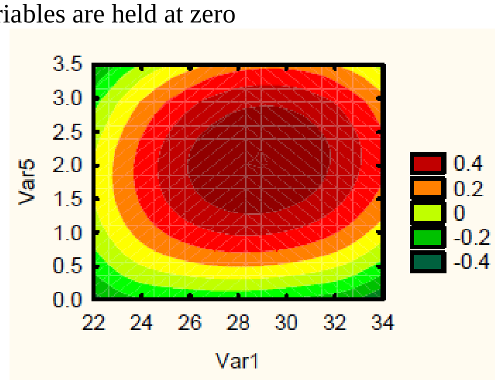


Figure 14: Contour plot showing the effect of Temperature and nitrogen source on production of citric acid. Other variables are held at zero

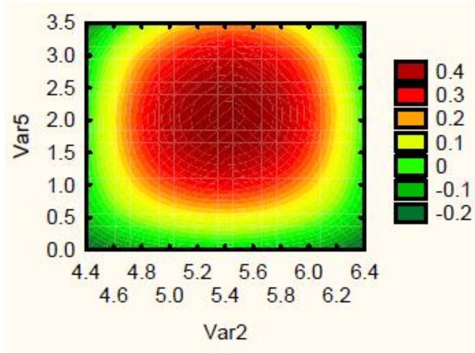


Figure 15 contour plots showing the effect of incubation time and nitrogen source on production of citric acid. Other variables are held at zero

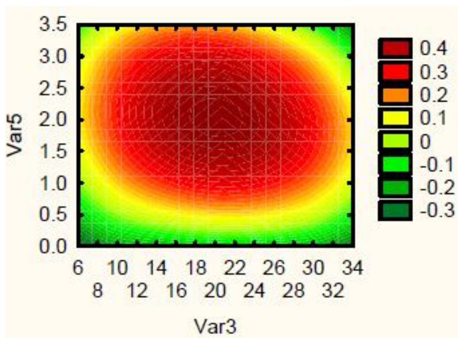


Figure 16: Contour plot showing the effect of Substrate concentration and nitrogen source on production of citric acid. Other variables are held at zero

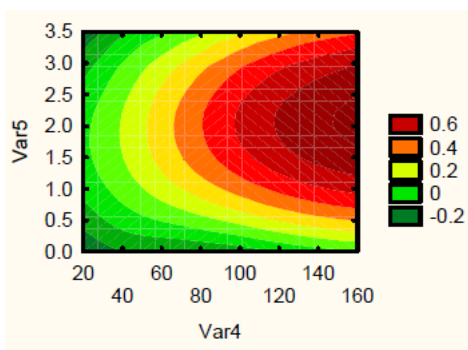


Figure 17: Contour plot showing the effect of Incubation period of fermentation and nitrogen source on production of citric acid. Other variables are held at zero

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