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An Experimental Design Approach for Determining Optimum Nutrient Medium Composition for Citric Acid Production from Pineapple Peels

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Abstract

In this study, citric acid was produced from pineapple peels via solid state fermentation using *Aspergillus niger* and the optimum composition of the medium consisting of magnesium sulphate ($MgSO_4.7H_2O$) and potassium dihydrogen phosphate (KH_2PO_4) was determined using statistically designed experiments. Potassium ferrocyanide ($K_4Fe(CN)_6$) and ammonium oxalate ($(NH_4)_2C_2O_4$) were used to reduce the effect of inhibitory metals such as calcium, iron, zinc, copper and magnesium. A four variable Box-Behnken design (BBD) coupled with response surface methodology (RSM) was adopted to develop a statistical model for obtaining the maximum citric acid concentration as well as the optimum levels of the medium components. The results obtained revealed that the model was statistically significant ($p<0.0001$) with a low standard deviation (0.29) and did not show lack of fit ($R^2=0.996$). Furthermore, all the medium components apart from magnesium sulphate had an overall positive effect on citric acid production. These effects were determined to be statistically significant ($p<0.0001$). Statistical optimization of the medium composition revealed that maximum citric acid production (36 g/l) was obtained at levels of 2.0 g/l ($K_4Fe(CN)_6$), 25 g/l ($(NH_4)_2C_2O_4$), 0.20 g/l ($MgSO_4.7H_2O$) and 2.26 g/l (KH_2PO_4). Results of validation experiments carried out showed that the optimum conditions obtained were similar to those predicted by the statistical model.

Keywords*:* Citric acid, Pineapple peels, *Aspergillus niger*, Fermentation, Response surface

Introduction

Citric acid is one of the most important organic acids which have a never ending demand in the world. Citric acid finds a lot of use in the food and beverage industry as a flavouring agent in fruit juices, candy ice cream, and marmalade. In the pharmaceutical industry, citric acid is used as a preservative for stored blood, tablets, ointments, and cosmetic preparations. In the chemical industry, it is used as an antifoam agent and for the treatment of textiles (Kuforiji et al., 2010). There has been a rapid increase in the global production of citric acid with an estimated annual production of about 1.7 million tons recorded in 2008. This figure has been projected to increase by as much as 5% annually in order to meet the growing demand for citric acid. Microbial fermentation appears to be the most viable route for citric acid production compared to chemical synthesis (Hayder, 2012).

Citric acid could be produced through submerged fermentation or solid state fermentation (SSF). However, most industrial scale production of citric acid is achieved by solid state fermentation using *Aspergillus niger* because this option has lower energy and water requirements, less risk of bacterial contamination, less wastewater generation and less environmental concern as a result of the use of solid waste for the production of value added products like citric acid (Amenaghawon et al., 2014).

Nigeria ranks as one of the leading producers of pineapple in the world. The consumption of pineapple and its processing into canned pineapple slice, chunk and dice, pineapple juice etc results in the generation of solid pineapple wastes particularly the peels which account for about 10% w/w of the weight of the original fruit (Kareem et al., 2010). These wastes have very little reuse capacity and are often disposed of inappropriately especially in less developed countries thereby causing serious environmental problems. This has led to studies on ways to utilize this wastes for the production of value added products. Amongst these, pineapple peels was established to be a suitable solid substrate for the production of citric acid via SSF (Imandi et al., 2008; Kareem et al., 2010; Amenaghawon et al., 2014).

In a previous study, Amenaghawon et al. (2014) established and optimised the physical conditions that could influence citric acid production from pineapple peels. However, it has been reported that the fermentation process is influenced by the composition of the fermentation medium. Furthermore, the presence of some metals such as calcium and zinc in significant amount in the solid carbon substrate could inhibit citric acid production. The addition of ammonium oxalate and potassium ferrocyanide has been proposed as a means of precipitating these undesirable metals from solution (Hauka et al., 2005). Hence, in this study, the focus was to optimise the concentration of some of the fermentation medium components as well as the amounts of ammonium oxalate and potassium ferrocyanide needed for optimum citric acid production. It is believed that by optimizing the concentration of these compounds, the yield of citric acid could be increased. An important means of achieving this is to combine statistical experimental design with response surface methodology. This method is very effective and has been successfully applied to the optimisation of many bioprocesses (Montgomery, 2005; Anupama et al., 2010; Fang et al., 2010; Tian et al., 2011; Amenaghawon et al., 2015). The aim of this study was to optimise the fermentation medium components ($MgSO_4.7H_2O$, KH_2PO_4, $K_4Fe(CN)_6$ and $(NH_4)_2C_2O_4$). The optimum levels of these components were determined using a four variable Box-Behnken design coupled with response surface methodology.

Materials and Methods

Feedstock preparation

Pineapple peels used as solid carbon substrate were obtained from a local market in Benin City, Edo State, Nigeria. The peels were washed with clean water to remove any adhering dirt after which they were dried in an oven at 60 ^oC until constant weight is obtained. The dried peels were then milled to obtain 1 mm particles.

Microorganism and inoculum preparation

Aspergillus niger ATCC 9167, obtained from Microbiology Department of the University of Benin, Benin City, Edo State, Nigeria was used throughout the study as the fermenting organism. Conidia suspensions of fungal strains were obtained from cultures grown on potato dextrose agar slants at 30 ^oC for 5 to 7 days. The spores were washed with sterilised 0.8% Tween 80 solution by shaking vigorously for 1 minute. Spores were counted with a haemocytometer to obtain approximately 2 x 10^7 spores/ml (Amenaghawon et al., 2014).

Solid state fermentation

Solid state fermentation of pineapple peels for the production of citric acid was carried out in 250 ml Erlenmeyer flasks. The composition

($FeCl_3$, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.0012; $CaCl_3$, 0.015 g/L) of the unoptimized fermentation medium used for citric acid production was as described by Lotfy et al. (2007). The concentration of the other components ($K_4Fe(CN)_6$, $(NH_4)_2C_2O_2$, $MgSO_4.7H_2O$ and KH_2PO_4) were determined according to the experimental design. The solid substrate (10 g) was added in the flask and wetted with the nutrient medium to 80% moisture. The contents were thoroughly mixed, cotton plugged and autoclaved at 121°C and 15 psi for 15 minutes. After cooling, the autoclaved substrate including the media was inoculated with 2 ml of inoculum of density $2x10^7$ spores/ml and then incubated at 30°C for 5 days. At the end of fermentation, the medium was diluted with 100 ml of distilled water, filtered, and the filtrate was used for subsequent analysis.

Analytical methods

The concentration of citric acid produced during fermentation was determined using the pyridine/acetic anhydride method as reported by Marrier and Boulet (1958). This was accomplished by adding 1 ml of the filtered fermentation broth along with 1.30 ml of pyridine and 5.7 ml of acetic anhydride in a test tube. The test tube was then placed in a water bath at 32 ^oC for 30 minutes. The absorbance of the sample was measured at 405 nm using a UV-Vis spectrophotometer (PG Instruments model T70). The concentration of citric acid in the sample was determined from a citric acid calibration curve which was prepared from known concentrations of citric acid.

Experimental design

A four variable Box-Behnken design was used to develop the experiments for analysing the effect of the medium components on citric acid production. The range of the variables that were optimised are shown in Table 1.

Table 1: Experimental range and level of the independent variables

Medium components	Unit	Symbol	Coded Levels		
			-1	0	+1
$K_4Fe(CN)_6$	g/l	X_1	0	1	2
$(NH_4)_2C_2O_4$	g/l	X_2	0	12.5	25
$MgSO_4.7H_2O$	g/l	X_3	0.05	0.175	0.3
KH_2PO_4	g/l	X_4	0.1	1.3	2.5

The design is suitable when exploring quadratic response surfaces and construction of a second order polynomial model (Amenaghawon et al., 2014), the response of the surface to the various inputs was used in optimizing the process using few experimental runs. The design makes use of a number of experimental runs given by:

$$N = k^2 + k + c_p \qquad (1)$$

Where k is the factor number and c_p replicate number of central point. Design Expert® 7.0.0 (Stat-ease, Inc. Minneapolis, USA), which is a statistical software was used to develop the experimental design. The coded and actual values of the independent variables were calculated using Equation (2).

$$x_i = \frac{X_i - X_o}{\Delta X_i} \qquad (2)$$

Where x_i and X_i are the coded and actual values of the independent variable respectively. X_o is the actual value of the independent variable at the center point and ΔX_i is the step change of X_i. The polynomial equation generated by this experimental design is of the form:

$$Y_i = b_o + \sum b_i X_j + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i \qquad (3)$$

Where Y_i is the response to be predicted, X_i and X_j are the independent variables, b_o is offset term, b_i and b_{ij} are the single and interaction effect coefficients and e_i is the error term. Multiple regression and graphical analysis of the experimental data was carried out using the Design Expert software to obtain a statistical model for describing citric acid production. Analysis of variance (ANOVA) and coefficient of determination (R^2) were used in the evaluation of the goodness of fit of the model for citric acid production. The optimum values of the variables tested were obtained by numerical optimisation based on the criterion of desirability (Amenaghawon et al., 2014).

Results and Discussion

Statistical analysis

The coded and actual values of the independent variables X_1 ($K_4Fe(CN)_6$), X_2 ($(NH_4)_2C_2O_4$), X_3 ($MgSO_4.7H_2O$) and X_4 (KH_2PO_4) as obtained from the Design Expert software with the corresponding values of citric acid concentration obtained from experiment and those predicted by the statistical model (Equation 4) are shown in Table 2.

$$Y = 16.94 - 9.56X_1 - 0.44X_2 + 97.01X_3 + 1.67X_4 + 0.36X_1X_2 - 2.52X_1X_3 + 4.55X_1X_4 + 1.32X_2X_3 + 0.13X_2X_4 + 12.54X_3X_4 + 1.62X_1^2 - 0.00642.519X_2^2 - 380.67X_3^2 - 3.672.519X_4^2 \qquad (4)$$

Table 2: Box-Behnken design matrix for citric acid production

Runs	Factors								Response	
	Coded Levels				Actual Values				Citric acid conc.	
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	Experiment	Predicted
1	1	1	-1	1	1.5	18.75	0.11	1.9	24.34	24.67
2	0	0	-2	0	1.0	12.50	0.05	1.3	16.65	16.49
3	-1	1	-1	1	0.5	18.75	0.11	1.9	16.01	15.86
4	1	1	-1	-1	1.5	18.75	0.11	0.7	20.96	21.26
5	1	-1	-1	1	1.5	6.25	0.11	1.9	20.45	20.40
6	-1	-1	1	-1	0.5	6.25	0.24	0.7	17.67	17.57
7	0	0	0	0	1.0	12.50	0.18	1.3	21.76	21.70
8	1	-1	1	1	1.5	6.25	0.24	1.9	18.98	19.41
9	-1	-1	-1	1	0.5	6.25	0.11	1.9	15.99	16.10
10	0	0	0	0	1.0	12.50	0.18	1.3	21.77	21.70
11	-1	1	-1	-1	0.5	18.75	0.11	0.7	18.11	17.92
12	-1	1	1	-1	0.5	18.75	0.24	0.7	17.32	17.43
13	0	0	0	0	1.0	12.50	0.18	1.3	21.46	21.70
14	-1	-1	-1	-1	0.5	6.25	0.11	0.7	19.81	20.13
15	2	0	0	0	2.0	12.50	0.18	1.3	27.34	26.98
16	-1	1	1	1	0.5	18.75	0.24	1.9	16.99	17.25
17	0	0	0	0	1.0	12.50	0.18	1.3	21.99	21.70
18	0	0	2	0	1.0	12.50	0.30	1.3	15.12	15.01

19	0	0	0	2	1.0	12.50	0.18	2.5	17.10	17.04
20	0	2	0	0	1.0	25.00	0.18	1.3	22.88	22.76
21	1	-1	-1	-1	1.5	6.25	0.11	0.7	18.99	18.96
22	0	0	0	0	1.0	12.50	0.18	1.3	21.57	21.70
23	1	1	1	1	1.5	18.75	0.24	1.9	26.01	25.75
24	1	-1	1	-1	1.5	6.25	0.24	0.7	15.89	16.09
25	0	-2	0	0	1.0	0.00	0.18	1.3	18.79	18.63
26	1	1	1	-1	1.5	18.75	0.24	0.7	20.33	20.46
27	0	0	0	0	1.0	12.50	0.18	1.3	21.64	21.70
28	-1	-1	1	1	0.5	6.25	0.24	1.9	15.68	15.43
29	0	0	0	-2	1.0	12.50	0.18	0.1	16.01	15.78
30	-2	0	0	0	0.0	12.50	0.18	1.3	19.57	19.65

From the ANOVA results (Table 3), it was observed that the statistical model fitted well with the experimental data and can be considered as statistically significant, since the F value is large (238.81) and p value is very small ($p<0.0001$). The model terms representing the concentrations of $K_4Fe(CN)_6$, $(NH_4)_2C_2O_4$, $MgSO_4.7H_2O$ and KH_2PO_4 i.e. X_1, X_2, X_3, X_4 respectively were statistically significant indicating that changes in the values of these variables could significantly affect citric acid production.

An R^2 value of 0.996 means that 99.6% of the variability in the response could be explained by the model as shown in Table 4. It is generally desirable for the predicted R-squared and the adjusted R-squared values to be within 0.20 of each other, otherwise there may be a problem with either the data or the model. For this study, the values of 0.977 and 0.991 obtained for the predicted R-squared and the adjusted R-squared values respectively satisfied this requirement indicating that the model was adequate to represent the experimental data. A low standard deviation of 0.29 means that there was very little deviation of the individual values of the response from the mean. The coefficient of variation (CV) is the standard deviation expressed as a percentage of the mean and experimental data is considered reproducible if the CV is not greater than 10%. In this study, the CV obtained was 1.49%. Adequate precision value measures signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 57.96 was obtained in this study which indicates an adequate signal.

Table 3: Analysis of variance for statistical model of citric acid concentration

Source	Sum of Squares	df	Mean Square	F Value	P-value
Model	284.98	14	20.36	238.81	< 0.0001
X_1	80.44	1	80.44	943.67	< 0.0001
X_2	25.63	1	25.63	300.69	< 0.0001
X_3	3.28	1	3.28	38.4	< 0.0001
X_4	2.39	1	2.39	27.93	< 0.0001
X_1X_2	20.38	1	20.38	239.03	< 0.0001
X_1X_3	0.1	1	0.1	1.17	0.2970
X_1X_4	29.86	1	29.86	350.23	< 0.0001
X_2X_3	4.28	1	4.28	50.14	< 0.0001
X_2X_4	3.88	1	3.88	45.41	< 0.0001
X_3X_4	3.54	1	3.54	41.49	< 0.0001
X_1^2	4.49	1	4.49	52.62	< 0.0001
X_2^2	1.73	1	1.73	20.23	0.0004
X_3^2	60.65	1	60.65	711.52	< 0.0001

X_4^2	47.81	1	47.82	560.96	< 0.0001
Residual	1.28	15	0.09		
Lack of Fit	1.11	10	0.12	3.26	0.1021
Pure Error	0.19	5	0.04		
Cor Total	286.25	29			

Table 4: Statistical information of Box-Behnken design

Parameter	Value
Standard deviation	0.29
Mean	19.58
C.V. %	1.49
R-Squared	0.996
Adj R-Squared	0.991
Pred R-Squared	0.977
Adeq Precision	57.96

Analysis of response surface plots

The results were plotted to produce three-dimensional (3D) response surface plots which were then used to optimize the variables being investigated. The plots were obtained by keeping two variables constant at the center points and varying the others within the experimental range. Figure 1 shows the effect of magnesium sulphate and ammonium oxalate on citric acid production. Citric acid production increased with increase in the concentration of ammonium oxalate. This trend was observed both at low and at high concentrations of magnesium sulphate. These results show that the addition of ammonium oxalate enhanced citric acid production. This is important because the presence of metals such as calcium in the carbon substrate can be inhibitory to citric acid production. Hence the addition of ammonium oxalate was used to transform the calcium to the unavailable form. This was achieved by the ammonium oxalate decreasing the absorption of calcium by binding with the metal and decreasing its availability in solution thereby reducing its inhibitory effects (Hauka et al., 2005). Similar observations have been reported by previous investigators (Mashhoor et al., 1987; Rasmy, 1999). Figure 1 also shows that intermediate concentration of magnesium sulphate was appropriate for citric acid production. This is seen from the fact that in the absence of ammonium oxalate, citric acid production increased from about 12 g/l to about 17 g/l when the concentration of magnesium sulphate was increased from 0.05 g/l to 0.175 g/l. The increase in citric acid production was however more significant when 25 g/l of ammonium oxalate was added to the substrate. It is also seen from Figure 1 that increasing the concentration of magnesium sulphate beyond 0.175 g/l did not favour citric acid production. The increase in citric acid production resulting from the addition of magnesium sulphate could be attributed to the fact that magnesium is essential for the action of a variety of enzymes in the microbial cell and is required for both growth and citric acid production. Magnesium sulphate contains the sulphate anion ($(SO4)^{-2}$) and the magnesium cation (Mg^{2+}) and the joint effect of both are necessary for citric acid formation (Max et al., 2010). The decline in citric acid production observed beyond 0.175 g/l of magnesium sulphate could be attributed to the fact that the metal ion is needed in a limited concentration for microbial growth and citric acid production but in excess, it becomes detrimental to the process (Hauka et al., 2005).

Figure 2 shows that the addition of potassium ferrocyanide enhanced citric acid production. This could be attributed to the metal ions chelating properties possessed by $K_4Fe(CN)_6$, as it forms complexes with metal ions present in the medium to promote microbial growth and citric acid production by minimizing the availability of these metal ions which tend to inhibit citric acid production. The effect of potassium ferrocyanide was more significant at high concentrations of ammonium oxalate and this could be attributed to the synergistic effects of these two chelating agents (Chanda et al., 1990). Maximum citric acid concentration (36 g/l) was obtained at the highest $K_4Fe(CN)_6$ concentration of 2 g/l which is in line with results obtained by Kapoor et al. (1989), Chanda et al. (1990), Ali et al. (2002) and Hauka et al. (2005).

The presence of phosphate in the medium had a significant effect on citric acid production as shown in Figure 3. Potassium dihydrogen phosphate has been reported to be the most suitable phosphorous source and Figure 3 shows that citric acid production was positively influenced by the presence of phosphate. The presence KH_2PO_4 which serves as the source of phosphorus for *Aspergillus niger* has been reported by Mirminachi et al. (2002) to enhance citric acid yield when its concentration is limited. This is because phosphate is known to be essential for the growth and metabolism of *Aspergillus niger* (Shankaranand and Lonsane, 1994). Excess of phosphate has been reported to negatively impact citric acid production as a result of the formation of certain sugar acids, a decrease in the fixation of CO_2, and the stimulation of growth. This was observed when the concentration of KH_2PO_4 exceeded a value of 1.81 g/l and the citric acid concentration showed a decline.

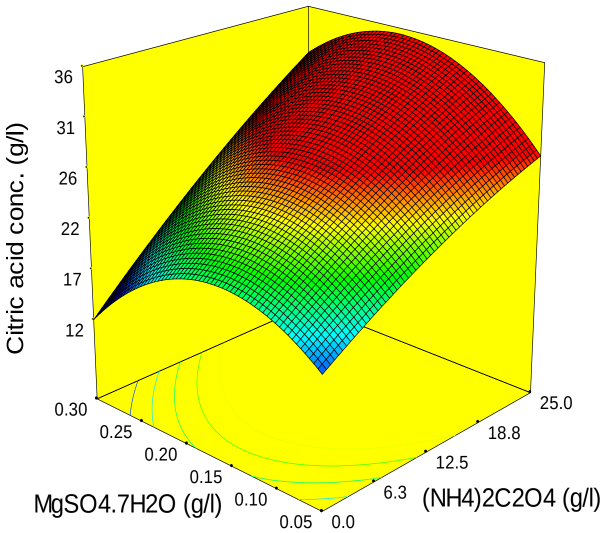

Figure 1: Effect of $MgSO_4.7H_2O$ and $(NH_4)_2C_2O_4$ on citric acid production

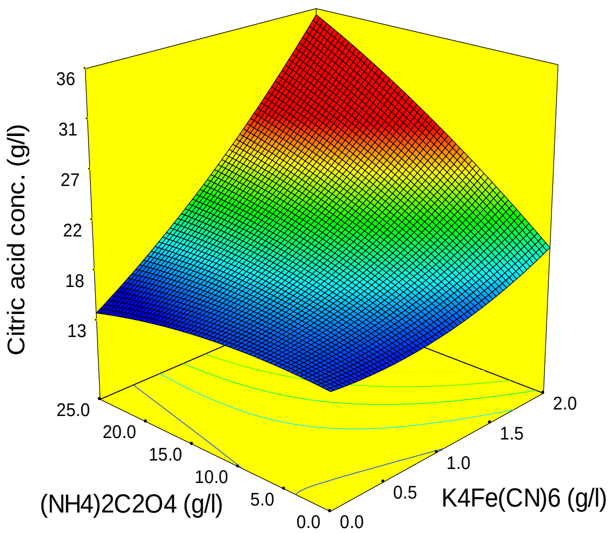

Figure 2: Effect of $K_4Fe(CN)_6$ and $(NH_4)_2C_2O_4$ on citric acid production

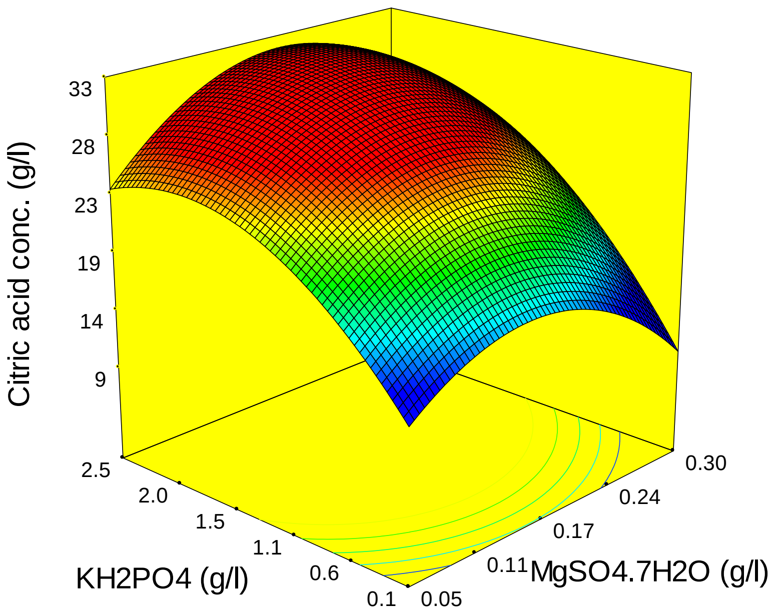

Figure 3: Effect of $MgSO_4.7H_2O$ and KH_2PO_4 on citric acid production

Numerical optimisation

Results obtained from numerical optimisation carried out using the Design Expert software revealed that the optimal citric acid concentration was 36 g/l. This was obtained with a nutrient medium having a composition of $MgSO_4.7H_2O$ 0.20 g/l and KH_2PO_4 2.26 g/l. The optimum concentration of the chelating agents were $K_4Fe(CN)_6$ 2 g/l and $(NH_4)2C_2O_4$ 25 g/l.

Validation of Statistical Model

To confirm the validity of the statistical model for citric acid production, triplicate confirmation experiments were performed at the specified optimum conditions. The average of the results showed that the citric acid concentration obtained (36.2 g/l) was close to the predicted value (36.0 g/l). The good correlation between predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point.

Conclusions

Production of citric acid from pineapple waste is enhanced by the use of chelating agents ($K_4Fe(CN)_6$ and $(NH_4)_2C_2O_4$). Citric acid production is also enhanced by the presence of nutrients ($MgSO_4.7H_2O$ and KH_2PO_4) in limiting amounts in the fermentation medium. The use of statistical tools like Box-Behnken design coupled with response surface methodology helped in the optimization of the citric acid production process. The concentration of citric acid produced during fermentation is related to the concentrations of $K_4Fe(CN)_6$, $(NH_4)_2C_2O_4$, $MgSO_4.7H_2O$ and KH_2PO_4 by a validated statistical regression model. A maximum citric acid concentration of 36 g/l was obtained with optimum combination of the components as follows: $K_4Fe(CN)_6$ concentration of 2.0 g/l, $(NH_4)_2C_2O_4$ concentration of 25 g/l, $MgSO_4.7H_2O$ concentration of 0.20 g/l and KH_2PO_4 concentration of 2.26 g/l.

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