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Advanced Statistical Optimization for Enhanced Medium-Chain Fatty Acid Production

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ABSTRACT

In recent years, the development of feed ingredients with natural additives has gained significant importance in increasing the health and quality of animal products, as well as in promoting weight gain in animals. Since *Salmonella* infection is a significant disease that transmits from animals to humans, the inhibition of Salmonella species can be achieved particularly through the improvement of gastrointestinal metabolism in chickens. At this point, the effectiveness of using MCFA (Medium Chain Fatty Acids) as a feed additive has been proven. MCFA are composed of a mixture of various fatty acids, including acetic acid, butyric acid, hexanoic acid, etc. Highest portion of MCFA are hexanoic acid. Besides feed additives hexanoic acid play a crucial role as primary resources in various industries, including the chemical, food, agricultural, and biofuel sectors. It is typically obtained from petrochemical-based solutions but there has been a growing focus on biotechnological production and natural sources in recent years. One of the mostly known bioprocess to produce MCFA is chain elongation (conversion of acetate and ethanol into MCFA by β oxidation reaction) by *Clostridium kluyveri.* However, as in most biotechnological processes, there are low yields and high costs in these reactions as well. In this study, Box-Behnken Design, a statistical experimental design method, was used to optimize the concentrations of acetate, ethanol (the two primary components of chain elongation reactions) and pH for MCFA production via chain elongation reactions with *Clostridium kluyveri*. Batch experiments were performed at 30°C and 37°C to also see the effect of temperature. Higher values of hexanoic acid and bacterial growth were observed at 37°C. From an economic perspective, a 14% reduction in costs has been observed with optimized components.

Keywords: Hexanoic acid, *Clostridium kluyveri*, acetate, ethanol, chain elongation reactions, Box-Behnken Design of Experiments

Orta Zincirli Yağ Asidi Üretiminin Artırılması İçin İleri İstatistiksel Optimizasyon

ÖZ

Son yıllarda, doğal katkı maddeleri içeren yem bileşenlerinin geliştirilmesi, hayvan ürünlerinin sağlığını ve kalitesini artırmanın yanı sıra hayvanlarda kilo alımını teşvik etmede önemli bir rol oynamıştır. Salmonella enfeksiyonları, hayvanlardan insanlara bulaşan önemli bir hastalık olduğundan, Salmonella türlerinin inhibe edilmesi özellikle tavukların gastrointestinal metabolizmasının iyileştirilmesiyle sağlanabilir. Bu noktada, orta zincirli yağ asitleri (MCFA) kullanımının bir yem katkı maddesi olarak etkinliği kanıtlanmıştır. MCFA, asetik asit, bütirik asit, hekzanoik asit vb. dahil olmak üzere

çeşitli yağ asitlerinin karışımından oluşur. MCFA karışımının en büyük kısmını hekzanoik asit oluşturmaktadır. Yem katkı maddesi olarak kullanımının yanısıra hekzanoik asit, kimya, gıda, tarım ve biyoyakıt sektörleri de dahil olmak üzere çeşitli endüstrilerde birincil kaynak olarak önemli bir rol oynamaktadır. Son yıllarda biyoteknolojik üretim ve doğal kaynaklara giderek daha fazla odaklanılmasına rağmen, tipik olarak petrokimya bazlı çözümlerden elde edilmektedir. Ancak, çoğu biyoteknolojik süreçte olduğu gibi, bu reaksiyonlarda da düşük verim ve yüksek maliyetler söz konusudur. Bu çalışmada, *Clostridium kluyveri* ile asetat, etanol (zincir uzama reaksiyonlarının iki ana bileşeni) ve pH konsantrasyonlarını optimize etmek için istatistiksel bir deneysel tasarım yöntemi olan Box-Behnken Tasarımı kullanılmıştır. Sıcaklığın etkisini de görmek için 30°C ve 37°C'de toplu deneyler gerçekleştirilmiştir. Daha yüksek hekzanoik asit değerleri ve bakteri büyümesi 37°C'de gözlenmiştir. Ekonomik açıdan ise ortam bileşenlerinin optimizasyonu proses maliyetlerinde %14'lük bir düşüş sağlamıştır.

Anahtar Kelimeler: Hekzanoik asit, *Clostridium kluyveri*, Asetat, Etanol, Zincir uzama reaksiyonları, Box-Behnken Dizaynı

INTRODUCTION

In recent years, biotechnological production methods have gained increasing significance because to their environmentally friendly nature, utilization of waste materials, less dependence on chemicals, and ability to operate at lower temperatures [1, 2]. Carboxylate based chemical platforms are considered a promising alternative to anaerobic digestion to produce sustainable fuels and chemicals from biomass and organic waste [3].

Medium chain fatty acids, so-called MCFAs, are composed of a mixture of various fatty acids, including butyric acid, pentanoic acid, hexanoic acid, and others. MCFA play a crucial role as primary resources in various industries, including the chemical, food, agricultural, and biofuel sectors. MCFA is typically obtained from petrochemical-based solutions, although there has been a growing focus on biotechnological production and natural sources in recent years. The process of obtaining MCFA from coconut oil or goat milk is referred to as biobased manufacturing. However, this method constitutes a challenge in terms of food security. The production of MCFA through bacterial chain elongation has gained importance in recent years [4, 5]. MCFA mixture rich in hexanoic acid can be synthesized through chain elongation process using *Clostridium* species. A large portion of the MCFA produced as a result of the chain elongation reaction is composed of hexanoic acid. There are significant studies in the literature showing that when hexanoic acid is used as a feed additive in livestock, it can improve the gastrointestinal systems of animals and prevent Salmonella infections, which is a major issue [6-7]. By chain elongation reactions during anaerobic biotechnological process, volatile fatty acids (VFAs) and the source of electrons can be transferred into MCFA, which are more valuable bioproducts [8]. Conversion of short chain acids and alcohols to MCFAs with ethanol which acts as electron donor is performed by chain elongation reaction microorganisms (i.e., *Clostridium kluyveri*) using the reverse β-oxidation pathway. The pathway includes oxidation of ethanol to form acetate for every five chain elongation reactions (Equation 1) [9].

Reverse β-oxidation reaction:

$$5\ C_xH_{2x}{}^{-1}\ O_2{}^- + 6\ C_2H_6O \rightarrow 5C_x + 2H_{2x} + 3O_2{}^- + C_2H_3O_2{}^- + 4H_2O + H^+ + 2H_2 \quad (1)$$

MCFA can be produced by hydrolysis and acidogenesis of organic wastes. The electron carriers for MCFA production such as ethanol, hydrogen [10], methanol [11] and lactic acid [12] can also be generated from organic wastes via biochemical and thermochemical conversion of organic wastes. The most significant disadvantages of chain elongation reactions are high process costs and low yields.

Given the low yields of chain elongation reactions and the high cost of process inputs, it is necessary to optimize production conditions to both increase production and decrease cost. Experimental design methodologies are very useful for optimization processes. Experimental design is a systematic process that involves the careful selection and control of significant components using various designs to acquire crucial answers. This approach is typically followed by statistical analysis [13]. A Box-Behnken design (BBD) is a type of fractional factorial design consisting of three levels. It was established by Box and Behnken and is used to analyze the response surface within a specific experimental zone. It has many advantages over factorial design such as lower experiment needs. The design is a hybrid of a two-level factorial design and an incomplete block design. In each block, subset of components undergoes all possible combinations, while the remaining factors are held constant at their center levels. This design has multiple benefits, including the ability to code three levels as -1 (low), 0 (middle), and +1 (high), the creation of an independent quadratic design, and a simplified approach to organizing and interpreting the results. The design includes an incomplete block design, with each block comprising the highest and lowest values, factorial design values, and the core values of the factors A polynomial equation can be used for prediction of optimized result (Equation 2).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

Y is the predicted optimized value, β values are regression coefficients and Xi and Xj are independent variables (from 1 to 999) [14].

In this study, the statistical experimental design method has been used for the first time in the literature for MCFA production with *Clostridium kluyveri.* Box Behnken Design was used to achieve both product maximization and cost minimization in the costly chain elongation reaction by *Clostridium kluyveri*. Three independent variables were selected as pH, acetate and ethanol concentrations. The relevant statistical experimental design method was conducted at two different temperatures (30°C and 37°C), to observe the effect of temperature.

MATERIALS and METHODS

Growth Conditions and Activation of *Clostridium kluyveri* DSM 555

Clostridium kluyveri (DSM 555) bacteria purchased from DSMZ culture collection and medium 52. The components of the medium is as follows: (for 1000 mL); K-acetate, 10 g, K_2HPO_4,0.31 g, KH_2PO_4, 0.23 g, NH_4Cl, 0.25 g, $MgSO_4$ $7H_2O$, 0.20 g, Trace element solution, 1 mL, Selenite-Tungtate solution, 1 mL, yeast extract, 1 g, Sodium resazurin (%0.1), 0.5 mL, Ethanol-absolute, 20 mL, Na_2CO_3, 1 g, seven vitamins solution, 1 mL, L-cysteine, 0.25 g, Na_2S $9H_2O$, 0.25 g. Trace element solution in 1000 mL HCl (%25) 10 mL, $FeCl_2$ $4H_2O$, 1.5 g, $ZnCl_2$ 70 mg, $MnCl_2$ $4H_2O$, 100 mg, H_3BO_3, 6 mg, $CoCl_2$ $6H_2O$, 190 mg, $CuCl_2$ $2H_2O$, 2 mg, $NiCl_2$ $6H_2O$, 24 mg, Na_2MoO_4 $2H_2O$, 36 mg . Selenite Tungtate solution in 1000 mL; NaOH, 0.5 g, Na_2SeO3 $5H_2O$, 3 mg, Na_2WO_4 $2H_2O$, 4 mg. Seven vitamins in 1000 mL; Vitamin B12, 100 mg, p-aminobenzoic acid, 80 mg, D(+) Biotin, 20 mg, Nicotinic acid, 200 mg, Ca-pantothenate, 300 mg, Thiamine HCl_2H_2O, 200 mg, Pyrodoxine hydrochloric acid, 300 mg. To validate viability of the bacteria, a gram staining procedure was applied [15]. The medium was sterilized at autoclave (HİRAYAMA) and inoculated by 10% in 50 mL serum bottles. After inoculation the head space was flushed with 20% CO_2, 80% N_2. The serum bottles were kept at 37°C. The bacteria were transferred into fresh medium every 2 weeks for keeping active. Since the growth of bacteria is maximized on 48 th hour, the experiments were conducted after 48 h growth of bacteria.

Box Behnken Experimental Design Methodology

Box Behnken methodology was applied for optimization of concentrations of K-acetate and ethanol for chain elongation reaction. The experimental conditions for each batch reactors are as in Table1. The same set of experiments was also repeated at 30°C and 37°C to evaluate the effect of temperature.

Table 1. The experimental conditions for Box-Behnken design*

	K-acetate Concentration (g/L)	Ethanol Concentration (g/L)	pH
Low (-1)	2	8	5
Medium (0)	6	24	6.5
High (+1)	10	40	8

*: The results were analyzed using Design Expert 7.0 trial version.

Batch Reactors

50 mL serum vials were used as batch reactors. All reactors were capped with rubber stopper and sealed with aluminum ring. The reactors were inoculated by 10% using 48 h growth *Clostiridum kluyveri*. The headspace of the reactors was flushed with 20% CO_2 and 80% N_2 gas mixture. Each design has 15 reactors with different medium compositions. All experiments were performed in duplicates for 30°C and 37°C. The reactors were kept at temperature-controlled incubator (Thermoscience).

Analytical Methods

Samples were collected from the reactors daily by removing 2 mL of reaction medium. Firstly, the OD at 600 nm values were measured using UV spectrophotometer (Thermoscience). The pH values were determined using pH strips (Merck). The samples were centrifuged for 10 mins at 5000 rpm to remove the biomass. After centrifugation the supernatant was filtered through syringe filter with 0.20 µm pore size PTFE filter. The filtered samples were collected in 1.5 mL vials for chromatographic analysis. The MCFAs (acetic acid, butyric acid, hexanoic acid and ethanol) in the medium were analyzed by a High-Pressure Liquid Chromatography (HPLC) (Thermo Fisher Scientific, USA). A solution of 5 mM H_2SO_4 was used as the mobile phase in the HPLC system. After a 5-minute purge to remove air bubbles, the column was heated, and the RID was purged for 30 minutes at 0.8 ml/min and set to 55°C. Filtered samples were diluted 1:10 with ultrapure water and placed in vials for analysis. Calibration standards (100, 500, 25, 12.5, 6.25 mM) were run in triplicate for calibration [16].

RESULTS and DISCUSSIONS

Following the activation of *Clostridium kluyveri*, studies were conducted using statistical experimental design in batch reactors at temperatures of 30°C and 37°C. The measurement findings obtained from the experimental settings were evaluated using a statistical software.

Following the analysis, the results were analyzed, and cost comparisons were also carried out.

MCFA Production Optimization at 30°C

The optimal growing temperature for *Clostridium kluyveri* has been reported to be 37°C. Besides in this study, 30°C, which is the optimum temperature for activity of *Clostridium* species, was also applied investigate the production of MCFA at lower temperatures. Subsequent tests were conducted accordingly.

In order to investigate the growth of bacteria in the samples collected from reactors over 15 different medium compositions, optical density (OD) measurements were conducted using a UV spectrophotometer at 600 nm wavelength. A significant rise in optical density (OD) values was recorded during trials 3 and 10 where values remained constant after the second day. In reactors 1, 8, 9, 14 there was a quick lag phase followed by an exponential growth phase that lasted until day 4, after then the stationary phase was observed. No growth was found in tests 5, 8, 10, 11, and 15. Based on the bacterial growth performances, it is evident that certain media compositions and temperature values of 30°C are unsuitable for the rapid development of *Clostridium kluyveri.* Because the maximum growth values are 0.35-0.40 The positive effect of medium composition was observed in several tests (Figure 1).

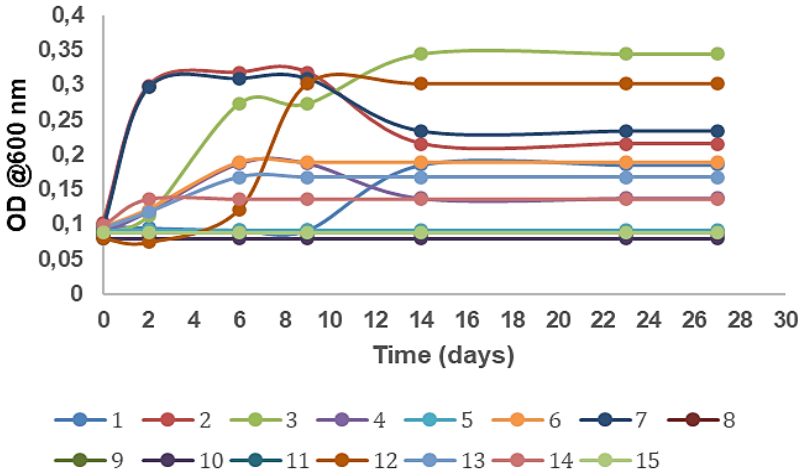

Figure 1. Growth performances of *Clostridium kluyveri* at 30°C

According to the results of pH analysis conducted on the final day of the experiments, it was noticed that the pH remained constant in the reactors without growth, whereas the pH reduced in the reactors with growth because of organic acid production (Table 2). The main goal was to optimize hexanoic acid production from the volatile fatty acid values produced in the reactors, as it is the key component used as an additive in MCFA production. Table 2 also shows the hexanoic acid production results used in statistical optimization analysis. As it can be seen from the results hexanoic acid production is directly related with bacterial growth. These results were analyzed in Design Expert software and the ANOVA results are on Table 3.

Table 2. Experimental results for hexanoic acid production and final pH values.

Exp. No	Acetate Concentration (g/L)	Ethanol Concentration (g/L)	Hexanoic Acid Concentration (g/L)	Initial pH	Final pH
1	6	40	6.72	5.0	5.0
2	6	8	3.12	5.0	4.5
3	2	24	3.17	5.0	4.5
4	10	8	4.05	6.5	5.0
5	2	24	0.07	8.0	6.0
6	2	40	1.99	6.5	5.0
7	10	24	3.66	5.0	4.5
8	6	8	0.09	8.0	6.0
9	6	40	7.48	8.0	6.0
10	10	40	0.06	6.5	6.5
11	2	8	0.31	6.5	5.0
12	10	24	8.33	8.0	5.0
13	6	24	3.80	6.5	6.5
14	6	24	7.50	6.5	5.0
15	6	24	0.05	6.5	6.5

Table 3. ANOVA (partial sum of squares-Type III) results of Box Behnken Design for 30°C

ANOVA for Response Surface Quadratic Model						
Source	Sum of square	df	Mean square	F value	p value Prob>F	
Model	112.76	9	12.529	5.020	0.045	significant
A-Acetate conc.	0.11	1	0.106	0.042	0.845	
B-Ethanol conc.	0.49	1	0.490	0.196	0.676	
C-pH	33.46	1	33.456	13.406	0.015	
AB	0.93	1	0.931	0.373	0.568	
AC	0.06	1	0.055	0.022	0.888	-
BC	3.29	1	3.294	1.320	0.303	
A^2	33.04	1	33.037.-	13.238	0.015	
B^2	18.55	1	18.547	7.432	0.042	
C^2	33.93	1	33.927	13.595	0.014	
Residual	12.48	5	2.496	-	-	
Lack of fit	12.01	3	4.002	17.0100	0.056	not significant
Pure error	0.47	2	0.235	-	-	-
Core total	125.24	14	-	-	-	

Figure 2 shows that predicted and experimental values of the model are quite close to each other. Since the R^2 value was close to 1, shows that results have strong regression. The close alignment between the predicted and actual values indicates the significance of the model. The fact that the predicted and actual values are very close to each other shows that the results of the experiments are significant.

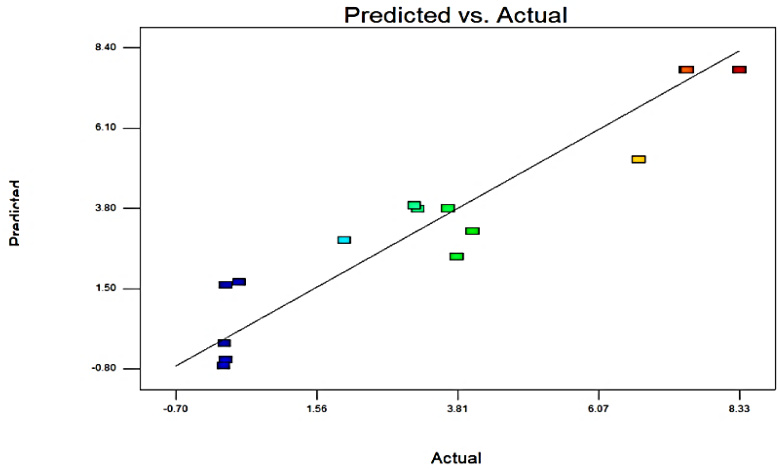

Figure 2. The relationship between the actual and the predicted values at 30°C

Figure 3 shows the results of Box-Behnken Design. The 3D plot and contour plot derived from the Box-Behnken Design (BBD) experiments visually represents the relationship between acetate concentration (A) and ethanol concentration (B) in the process of optimizing hexanoic acid production. The color gradient ranging from green to red signifies different production levels, with the central red area denoting the maximum yield. The middle region indicates the ideal circumstances for achieving the highest possible production of hexanoic acid, indicating that certain levels of acetate and ethanol are very efficient. The progressive increase in production towards the optimal point is indicated by the smooth, concentric contour lines, which illustrate the considerable interaction effects between the two variables. The experimental data points, depicted as red dots, are evenly dispersed throughout the design space, ensuring the reliability of the response surface model. According to Figure 3 and also as reported in Table 3, the statistical optimization experiments were successful in terms of a fit to model, and a significant surface response model was acquired. Figure 3 depicts the conditions with the highest hexanoic acid concentration are for acetate (4-8 g/L) and for ethanol (16-32 g/L). The BBD technique showcases efficacy by minimizing the number of experimental trials needed, while also offering a thorough comprehension of the system's behavior. The results emphasize the significance of focusing on the specific ideal area to attain the highest output of hexanoic acid, thereby validating the effectiveness of the BBD methodology in optimizing biochemical production processes. According to Figure 3, a surface response model was statistically significant and optimization experiments were successful. According to this graph, the conditions with the highest hexanoic acid concentration are for acetate (4-8 g/L) and for ethanol (16-32 g/L). The optimum condition for hexanoic acid

production is suggested as; 5.94 g/L acetate, 24.23 g/L ethanol and pH=5.99 with a desirability value of 0.974. Accordingly, optimum pH value is 6 and the Acetate/Ethanol concentration ratio is 1/4 for maximizing hexanoic production. A similar observation Acetate/Ethanol concentration ratio was also in line with literature [17]. On the other hand, this ratio can vary between different strains of bacteria. According to the optimization results, a maximum yield of 4 g/L of hexanoic acid can be produced at 30°C.

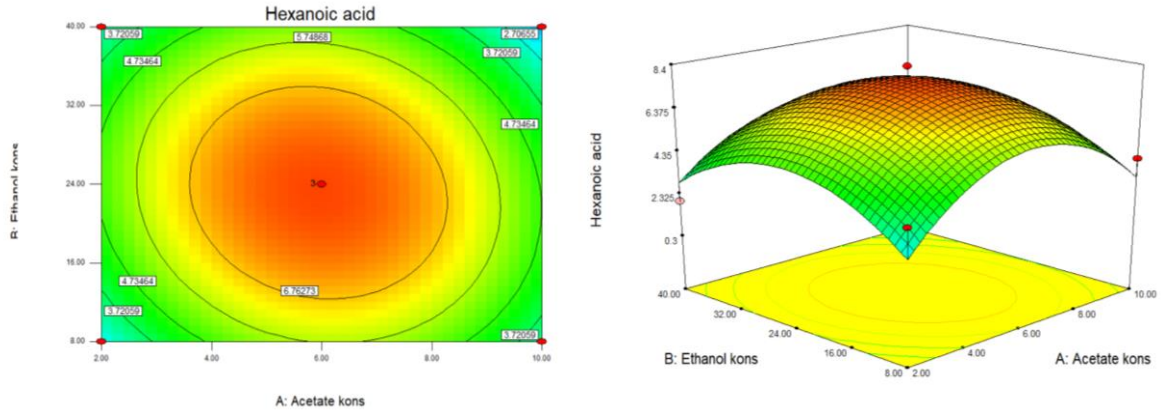

Figure 3. Effect of acetate and ethanol concentrations on hexanoic acid production values (30°C, pH:5.5)

MCFA Production Optimization at 37°C

Figure 4 shows the optical density (OD) at a wavelength of 600 nm as a function of time (in days) for various reactors with different medium compositions at a temperature of 37°C. In most of the runs, there was a fast increase in OD, indicating exponential growth. The growth data for reactors 3 and 10 indicate a significant increase within the initial 10 days. Once the reactors reached a maximum optical density (OD) mostly at the end of 10-15 days, their growth stabilized for each run. Certain reactors consistently exhibit a low optical density (OD) for the whole duration, revealing that there was a slow or no bacteria growth. For example, reactors 6, 9 and 15 showed no growth. Reactors 3, 4 and 13 exhibited the highest optical density (OD) values, reaching approximately 0.6, whilst reactor 1 and others had OD values below 0.2. Some reactors showed a long lag phase characterized by a minimal or nonexistent increase in optical density (OD) at the first stage. For example, reactor 13 showed a gradual increase beginning on day 8.

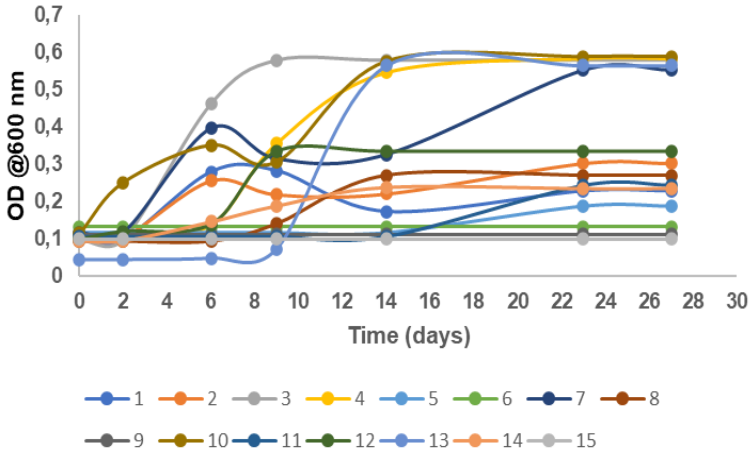

Figure 4. Growth performances of *Clostridium kluyveri* at 37°C

The two figures represent a comparative evaluation of OD measurements at 600 nm over a period of 30 days (Figure 1 and 4). In the initial plot, the OD values remained constant around day 14, with the maximum recorded OD being approximately 0.35 and an estimated mean OD of 0.2. The highest growth values were obtained between days 6 and 14.
On the other hand, the second plot exhibited notably elevated OD values, characterized by peaks at roughly 0.6 and an average OD of around 0.3, suggesting more advantageous growing circumstances. The higher average optical density (OD) indicated higher microbial growth, indicating that the second set of experimental settings or strains was more successful in increasing cell density. Figure 4 highlights the significance of choosing ideal conditions for higher optical density (OD) values, indicating improvement of product yields and overall efficiency of the process. Therefore 37°C is more productive for growth of *Clostridium kluyveri,* as also suggested in literature [17].

The results obtained in the experiments performed at 37°C were also significant with a high significance value (p=0.0001<0.05). The experimental results are significant, and the model is strong. Accordingly, the optimization results are also significant values (Table 4).

Table 4. ANOVA (partial sum of squares-Type III) results of Box Behnken Design for 37°C

ANOVA for Response Surface Quadratic Model						
Source	Sum of square	df	Mean square	F value	p value Prob>F	
Model	132.12	9	14.679	60,486	0.0001	significant
A-Acetate conc.	0.17	1	0.168	0,693	0.4431	
B-Ethanol conc.	14.91	1	14.906	61,418	0.0005	
C-pH	27.98	1	27.975	115,270	0.0001	
AB	0.86	1	0.865	3,564	0.1177	
AC	23.81	1	23.814	98,125	0.0002	
BC	2.02	1	2.016	8,308	0.0345	-
A^2	22.79	1	22.786	93,886	0.0002	
B^2	32.77	1	32.771	135,030	< 0.0001	
C^2	16.04	1	16.038	66,085	0.0005	
Residual	1.21	5	0.243	-	-	
Lack of fit	1.12	3	0.372	7,737	0.1166	not significant
Pure error	0.1	2	0.048	60,486	0.0001	-
Core total	133.33	14	-	0,693	0.4431	

Figure 5 exhibits the correlation between the values obtained from the experimental set and the values predicted by the model. It was observed that the values were quite close to each other.

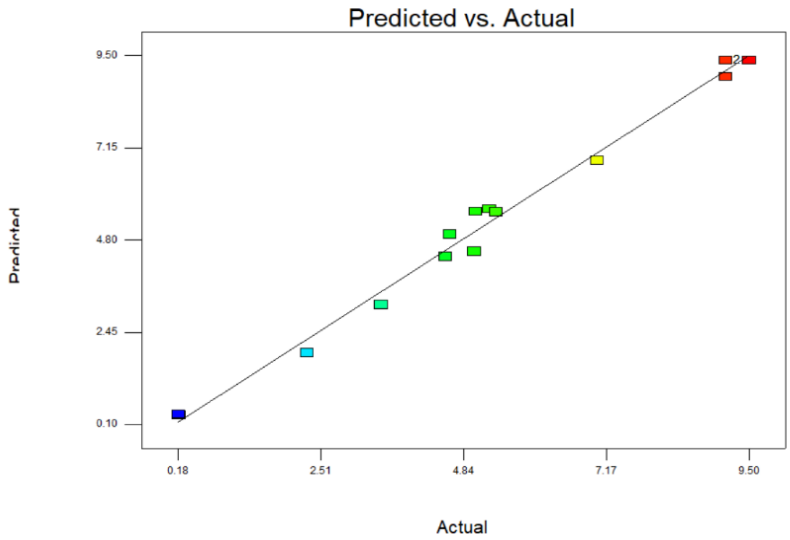

Figure 5. Relationship between values obtained in MCFA production and predicted values (37°C)

R^2 value was calculated as 0.9909. Figure 6 shows the three-dimensional graphics of the model. According to the graph, the ranges where the acetate concentration is 6-8 g/L and ethanol concentration 14-20 g/L.

The highest concentration of hexanoic acid, roughly 9.7 g/L, was achieved when the acetate concentration was around 8.0 g/L and the ethanol concentration is at 24.0 g/L. For optimization, the two independent variables are adjusted in a way that achieves the maximum yield of hexanoic acid. At the lowest and highest levels of acetate and ethanol concentrations, the concentration of hexanoic acid decreased. On the other hand, the hexanoic acid concentration reached its lowest point at approximately 3.65 g/L when the acetate concentration was 2 g/L and the ethanol concentration was 8 g/L. This indicated that both excessively low and excessively high concentrations of the substrates had a detrimental effect on the synthesis of hexanoic acid. The minimum and maximum acetate concentrations were 2.0 g/L to 10.0 g/L; ethanol concentrations were from 8.0 g/L to 32.0 g/L. The hexanoic acid production values are found to be between 3.65 g/L to 9.7 g/L.

The optimization was performed using the equation derived from the experimental results in the model. In numerical optimization, maximization of hexanoic acid production while keeping other variable ranges at intermediate values. The production of 9 g/L hexanoic acid was achieved at 37°C with concentrations of 6.12 g acetate /L and 24.45 g ethanol /L, and a pH value of 5.43.

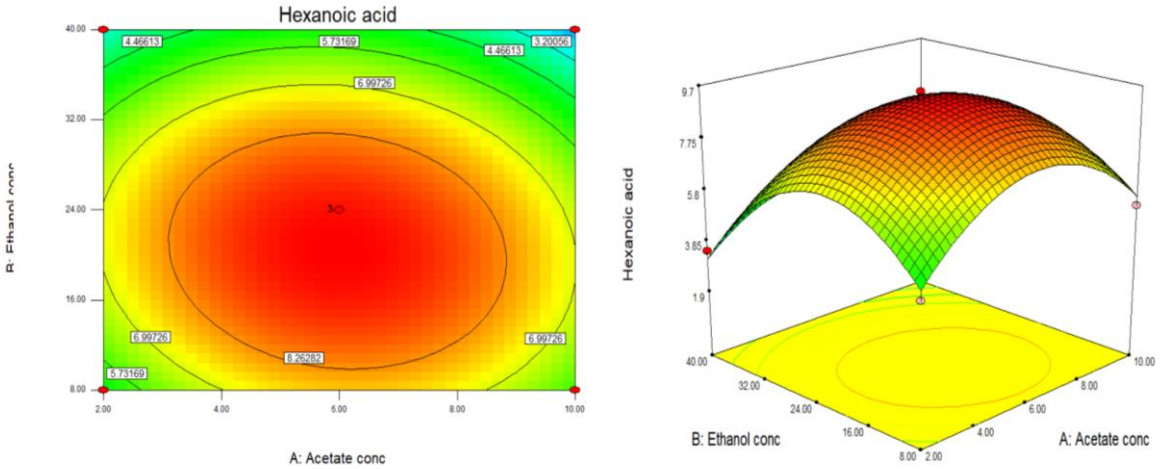

Figure 6. Effect of acetate and ethanol concentrations on hexanoic acid production values (37°C, pH 5.5)

Hexanoic acid production from ethanol and acetate using Clostridium kluyveri was also investigated in previous studies in literature. In another study, the highest hexanoic acid concentration of 8.42 g/L was achieved with an ethanol/acetate ratio of 10:1 (550 mM total carbon). The production rate increased with higher ethanol concentrations, but concentrations above 700 mM inhibited the biosynthesis process. Optimal conditions for caproate production including a defined acetate/ethanol ratio and controlled substrate concentrations proved to be significant on yields and efficiencies [17]. In current study, acetate/ethanol ratio was reduced by 2.5 times while higher concentrations resulted in inhibition of microorganism activity. Therefore, continuous bioreactors can be used to maintain microbial activity without loss of performance.

In a study by Ge et al. [18], spent yeast fermentation beer waste was processed using an anaerobic sequential batch reactor at pH 5.5. An average hexanoic acid production rate of 3.38 g/L/d (based on COD) was achieved with a yield of 70.3 % and an hexanoic acid/ethanol ratio of 1.19 after 55 days. The peak production rate was reached by increasing the organic loading rates, boosting the capacity of the extraction system, and altering the complex feedstock batch [18]. In another study by Grootscholten et al [19], using acetic acid and ethanol in a continuous reactor with a low hydraulic retention time (HRT) of 17 h in an anaerobic filter at pH 7.0 resulted in a high production rate of 15.7 g/L/d and a yield of 11.1 g/L of hexanoic acid.

The same group also reported that lowering the HRT to 4 hours under identical operational conditions resulted in a threefold improvement in the production rates of MCFA [20]. Moreover, the MCFA production can be improved by using two stage reactors. Different types of organic source can be used for MCFA production. For instance, 12.6 g/L/d of MCFA production was achieved by using municipal solid waste [21, 22]. First step can be the application of chemical inhibition for anaerobic bacteria to prevent the competition between anaerobic mixed culture and chain elongating culture. A real time study with syngas fermentation effluent by integrating the carboxylate platform to the syngas platform using initially Clostridium ljungdahlii strain ERI-2 for the production of precursors of the chain elongation reactor in the first reactor and then using the second reactor to produce MCFA with *Clostridium kluyveri*. The highest concentration 1g/L of hexanoic acid was produced at a pH 5.44 with a production rate of 1.7 g/L/d [23]. It can be generally observed that batch reactors generally show moderate production rates and yields. Therefore, continuous production of hexanoic acid with optimized medium conditions is suggested to improve the yields and reduce the costs. San Valero et al. 2022 [24] also focused on the effects of pH, yeast extract and addition of $NaHCO_3$ with continuous bioreactor and achieved 21.4 g/L hexanoic acid production at a pH value between 5.5-6.5, which is similar value observed in current study.

Cost Analysis

The Table provides the cost analysis comparative values while utilizing optimal recipes at a temperature of 37°C. The data in this table was obtained from the official website of Merck. The price of one liter of the media components suggested by DSMZ was determined to be 9.92 €, but the price of the optimized media components was determined to be 8.47 €. Upon comparing the media expenses, it becomes evident that a savings of around 15% in costs has been accomplished. Although this value may seem negligible for laboratory scale experiments, it becomes considerably more significant when applied to larger-scale and commercial applications.

Table 5. Results of cost analysis

	DSMZ medium	Optimized medium
Macro chemicals (including K-acetate)	€ 6.67000	€ 4.59470
Trace elements	€ 0.00147	€ 0.00147
Sodium selenate	€ 0.00009	€ 0.00009
7 vitamins	€ 0.08611	€ 0.08611
Ethanol	€ 3.16000	€ 3.79200
Total cost	€ 9.91767	€ 8.47437
Cost advantage	-	14.55%

CONCLUSION

In this study, optimization of hexanoic acid production from acetate and ethanol via chain elongation reaction was performed by using a statistical experimental design method. In the production of hexanoic acid by chain elongation reactions, a temperature of 37°C is required for the growth and active production of *Clostridium kluyveri*. Statistical experimental design is a crucial technique that efficiently reduces both time and money in microbiological processes. The ideal ratio of acetate to ethanol concentration for the synthesis of hexanoic acid with *Clostridium kluyveri* in batch reactors was determined to be 1:4 by statistical experimental design. An enhanced ambient medium composition resulted in a 14% decrease in process costs.

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