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Biotechnological Production of Biosuccinic Acid (Bio-SA) from Green Microalgae *Chlorella* sp. Hydrolysate: Synergistic Effect of Substrate Concentration and Agitation Assessment Using RSM

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Abstract. This paper evaluates the potential of *Chlorella* sp. microalgae biomass as a feedstock for biosuccinic acid (bio-SA) production via the fermentation process. Generally, the effectiveness of biosuccinic acid production is significantly influenced by various factors, including substrate concentration and agitation. High substrate concentrations may lead to increased viscosity, resulting in low succinic acid production. This study aims to evaluate the interaction effect of substrate concentration and agitation on bioscuccinic acid production. In this study, the biomass was pretreated and hydrolyzed using a mild acid before being fermented with *Actinobacillus succinogenes*. The influence of the initial biomass concentration and agitation rate on bio-SA production during batch fermentation was evaluated using a Response Surface Methodology (RSM) design. The study results indicated that *Chlorella* sp. microalgae biomass contained various types of sugars, with glucose identified as the dominant reducing sugar in the *Chlorella* sp. hydrolysate. According to the RSM analysis, the study showed that initial biomass concentration and agitation rate changes could significantly affect bio-SA production from *Chlorella* sp. hydrolysate. The highest bio-SA concentration of 14.56 g/L with a yield of 0.62 g/g was achieved when fermentation was performed using 10% (w/v) biomass at 150 rpm. Therefore, this study suggests that *Chlorella* sp. hydrolysate can be an alternative and renewable feedstock for efficient bio-SA production.

Keywords: Fermentation, Microalgae, Response Surface Methodology, Succinic Acid

INTRODUCTION

Succinic acid (SA), with a chemical formula $C_4H_6O_4$, is an organic acid widely used as a building block for various industrial chemicals, including adipic acid, 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, and gamma-butyrolactone (Kumar *et al*., 2020; Delhomme *et al*., 2009). Furthermore, SA is a high-value chemical employed in agricultural, food, pharmaceutical, biodegradable polymer industries, and the production of eco-friendly solvents (Raj *et al*., 2023).

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Commercial SA production primarily relies on petroleum-based chemicals, involving the catalytic conversion of n-butane to maleic anhydride, followed by hydrogenation to form succinic acid (Saxena *et al*., 2017). However, this chemical synthesis process is costly and potentially harmful to the environment. Additionally, the heavy reliance on petroleum feedstock hinders the broad utilization of SA for various applications.

In pursuit of sustainable alternatives, various studies have explored the production of SA through fermentation processes using renewable resources. In this approach, sugars derived from renewable feedstocks, such as agricultural waste (e.g., corn, wheat, and rice straw, oil palm trunk, and empty fruit bunch), food waste, fruit and vegetable waste, and aquatic biomass (e.g., microalgae, seaweed, and aquatic plants), are utilized by microorganisms like A*naerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, and *Actinobacillus succinogenes* (Li *et al*., 2010; Putri *et al*., 2023; Akhtar *et al*., 2017; Dessie *et al*., 2018;, Kuglarz *et al*., 2023; Patsalaou *et al*., 2020; Bai *et al*., 2015). Microalgae, as photosynthetic microorganisms, are recognized as potential feedstock for bio-SA production, offering an alternative to purified sugar derived from lignocellulosic and starch-based raw materials. Microalgae biomass is rich in nutrients and active compounds, with significant starch and carbohydrate polymer content that can serve as fermentation feedstock for bio-SA production. Additionally, the use of microalgae biomass offers advantages such as carbon dioxide (CO) consumption during photosynthesis for biomass production, and it doesn't compete with arable land for cultivation (Khan *et al*., 2018).

Bio-SA production through fermentation processes is significantly influenced by several factors, including initial biomass concentration and agitation rate (Kanchanasuta *et al*., 2021; Terboven *et al*., 2021). These factors are crucial for achieving a high yield of bio-SA. Using suitable feedstock concentration and agitation rate is expected to result in high bio-SA production. However, high biomass concentrations can lead to increased viscosity, reducing the mass transfer of nutrients into microbial cells and consequently, reducing bio-SA production. To the best of our knowledge, no literature reports on the investigation of the synergistic effect of initial biomass feedstock concentration and agitation rate on bio-SA production from microalgal biomass.

In this study, Response Surface Methodology (RSM) was applied, using R analysis to assess the impact of agitation rate and initial biomass feedstock concentration, along with examining their interactive effects on the production of bio-succinic acid (bio-SA) utilizing the *Actinobacillus succinogenes* strain.

EXPERIMENTAL

Microalgae Biomass

The green microalga biomass, Chlorella sp. powder (Batch 040122-31) was purchased from A&T Ingredients Sdn. Bhd., Nilai, Negeri Sembilan, Malaysia. All chemicals used in this study were analytical grade and purchased from R&M Chemicals, Malaysia.

Pretreatment and Hydrolysis

Initially, the *Chlorella* sp. biomass sample was soaked in 100 mL of 1% (v/v) dilute sulfuric acid (H_2SO_4) in a 250 mL conical flask. The mixture was then placed in an oven at 140°C for 25 minutes (Harun and Danquah,

2011). Afterward, the sample was centrifuged at 4500 rpm for 10 minutes at 10°C to collect the pellet. The obtained supernatant was discarded, and the pellet was collected for the next process.

The enzymatic hydrolysis process of the pretreated *Chlorella* sp. biomass was carried out by adding 100 mL of sodium acetate buffer. The buffer was then mixed with 20 mg of cellulase (Sigma Aldrich brand), and the mixture was incubated in an orbital shaker at 45°C and 100 rpm for 48 hours. After hydrolysis, the sample was heated to 121°C for 15 minutes to inactivate enzyme activity before proceeding with the fermentation process.

Fermentation Biosuccinic Acid Production

The fermentation process was performed using *Actinobacillus succinogenes*, obtained from the School of Industrial Technology, University Sains Malaysia. Initially, the *A. succinogenes* bacteria were grown in Brain Heart Infusion (BHI) (Merk Millipore, Germany) media and incubated at 37°C with an agitation speed of 150 rpm for 16 hours. After the incubation period, approximately 10% (v/v) of the active inoculum was transferred into a conical flask containing *Chlorella* sp. hydrolysate obtained from hydrolysis. An additional medium was also used to support bacterial growth. The media used in this experiment consisted of the following per liter: 0.2 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 0.2 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 3.0 g of potassium dihydrogen phosphate (KH_2PO_4), 1.0 g of NaCl, 15.0 g of yeast extract, and 40.0 g of magnesium carbonate ($MgCO_3$). The mixture was fermented in an incubation shaker for 36 hours. During fermentation, approximately 5 mL of the broth was sampled at 6-hour intervals. The samples were centrifuged at 10,000 rpm for 10 minutes to remove microbial cells and other suspended solids and then filtered using a 0.22 µm membrane filter. The filtered samples were placed in 1.5 mL vials for subsequent analysis.

Screening Parameters

The screening test was conducted in the R software, utilizing one-way analysis of variance (ANOVA) to determine the significance of variables at a confidence level exceeding 95%. Variables identified as significant in this initial screening, with p-values below 0.05, were subjected to a more comprehensive investigation using RSM through a central composite design (CCD).

Effects of Initial Biomass Concentration (% w/v) on Succinic Acid Production

The first series of experiments aimed at determining the effects of the initial *Chlorella* sp. biomass concentration on succinic acid production. This evaluation ranged from 2.5% to 20% (w/v). Initially, the pretreated microalgae sample underwent hydrolysis at different feedstock concentrations. The fermentable sugars obtained from each concentration were then harvested and analyzed. Subsequently, the enzymatically saccharified microalgae hydrolysate was subjected to fermentation in a shaker at 37°C, with an initial pH of 4.8 and an agitation speed of 150 rpm, for a total incubation time of 36 hours. 1.5 mL samples were harvested at 6-hour intervals for analysis throughout the experiment.

Effects of Agitation Speed (rpm) on Succinic Acid Production

Next, we determined the effects of agitation speed on succinic acid production by conducting experiments at different

agitation speeds, ranging from 50 to 250 rpm, while maintaining a pH of 4.8, an inoculum size of 10%, and an incubation temperature of 37°C. This study used the optimal initial biomass concentration obtained from the earlier experiment. Table 1 shows the overall experiment design during the screening process.

Table 1. Experimental design for screening process.

Experiment	Biomass concentration (% w/v)	Agitation rate (rpm)
1	2.5	150
2	5	150
3	10	150
4	15	150
5	20	150
6	10	50
7	10	100
8	10	150
9	10	200
10	10	250

Optimization Biosuccinic Acid using RSM

Following the completion of ANOVA and regression fitting analyses, the significant independent variables and response were integrated into a second-order polynomial equation as Eq. (1), which serves to predict the optimal conditions for succinic acid production.

$$Y = \beta_0 + \sum_{i-1}^{n} \beta_i X_i + \sum_{i-1}^{n} \beta_{ii} X_i^2 + \sum_{i-1}^{n-1} \beta_{ij} X_i X_j + E \qquad (1)$$

Where, Y is the predicted response while β_0, β_i, β_{ii} and β_{ij} are regression coefficient for intercept, linear, quadratic and interaction coefficient. X_1 and X_2 are the coded values of the independent variables and E is the experimental error.

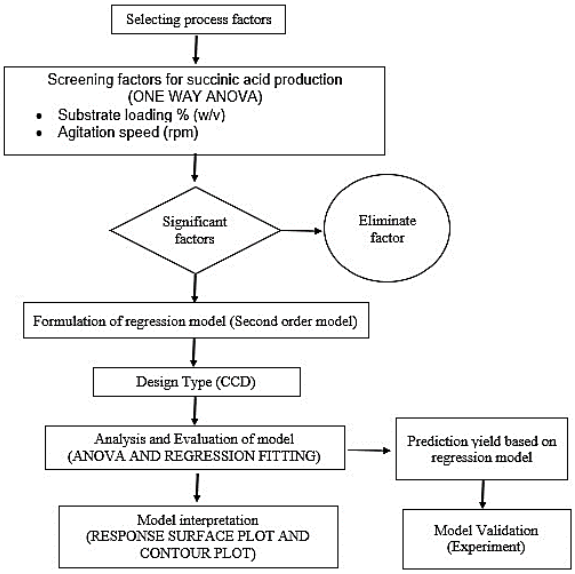

Fig. 1: Overall optimization steps for succinic acid production using RSM in R software.

Analysis

Proximate Analysis

Proximate analysis of the microalgal biomass was performed to determine the sample's carbon, hydrogen, nitrogen, and sulfur content. This analysis used a Perkin Elmer Analyzer (Model 2400, Series II, USA). Two milligrams of the microalgal biomass sample were used for this analysis, which was conducted in triplicate.

Determination of Reducing Sugar by Dinitroalicyclic Acid (DNS) Method

The microalgae hydrolysate sample from fermentation was prepared by centrifuging it at 4500 rpm for 10 minutes. The supernatant was then used in the DNS method to analyze the reducing sugar in the sample. For every 1.5 mL of the sample, 3 mL of DNS reagent was added and boiled for 5 minutes. The solution was cooled down by running cold water over it. After cooling, 5 mL of distilled water was added, and the optical density at 575 nm was measured using a Hitachi U-1900 spectrophotometer. The total amount of

reduced sugar was calculated based on the glucose standard curve and expressed as a percentage of sugar saccharification.

Determination of Biochemical Composition

Approximately 25 mg of dry microalgae sample was added to 2.5 mL of 2.5 N Hydrochloric acid (HCl). The sample was then incubated at 90°C for 3 hours. Sodium carbonate (Na_2CO_3) was added to the sample until the bubbling ceased. Afterward, the sample was brought up to a total volume of 50 mL with distilled water before centrifugation at 3000 rpm for 15 minutes. The supernatant was collected from the sample and mixed with 1 mL of distilled water, 1 mL of 5% phenol solution, and 5 mL of H_2SO_4. The sample was then incubated at 30°C for 30 minutes before being measured at optical density (OD) 485 nm for analysis.

Total lipid content was determined using a modified Bligh and Dyer method. For this analysis, an aliquot of lyophilized biomass was dissolved in 4 mL of chloroform and 2 mL of methanol for 2 hours. Then, 2 mL of methanol and 2 mL of water were added to the mixture. The sample was centrifuged, and the upper layer was removed. The bottom layer containing lipids was collected and evaporated using a rotary evaporator.

Additionally, the protein content of the Chlorella sp. biomass was determined using the Lowry method.

Determination of Succinic Acid by High Pressure Liquid Chromatography (HPLC) system

Succinic acid concentrations in all the fermentation samples of microalgae hydrolysate were analyzed using High-Performance Liquid Chromatography (HPLC) equipped with a UV detector and a Zorbax SB-Aq column (4.6 mm ID x 150 mm, 5 µm). The mobile phase consisted of 1% ACN and 99% mM $NaHPO_4$ with a pH of 2. The flow rate was set at 1.0 mL/min, and the temperature was maintained at 35°C. The retention time for each sample was 10 minutes.

RESULTS AND DISCUSSION

Microalgae Biomass Composition

Table 2 shows the biochemical composition of Chlorella sp. biomass before being subjected to biosuccinic acid production. According to this analysis, protein is the most dominant compound with 38.43±0.08%, followed by carbohydrate with 28.4±0.36%. Lipid fraction only contributed 12.87±0.68% of the total major metabolite in the microalgal biomass. This analysis suggests that high carbohydrate content in the biomass can be potentially used as feedstock for biochemical synthesis via fermentation process.

Table 2. The main chemical composition of microalgae *Chlorella* sp. biomass

	Component	Percentage (%)
Chemical composition	Lipid	12.87±0.68
	Protein	38.43±0.08
	Carbohydrate	28.4±0.36
	Ash	20.23±0.49
Ultimate analysis	Carbon	39.34±0.47
	Hydrogen	6.60±0.43
	Nitrogen	7.91±1.40
	Sulfur	0.65±0.05
	Oxygen	45.50±6.25
	Lipid	12.87±0.68

Pretreatment of Microalgae Biomass

Before the bio-SA fermentation process, the microalgal *Chlorella* sp. biomass was

pretreated and hydrolyzed using a mixture of cellulase cocktails, producing approximately 25.56 mg/mL of reducing sugar. This corresponds to a 90% conversion yield of the total carbohydrate available in the *Chlorella* sp. biomass.

Screening Effect of Solid Loading and Agitation Rate

Initial screening was performed to determine the effects of initial biomass concentration and agitation rate on bio-SA production using a one-variable-at-a-time (OVAT) approach. According to Figure 2 in this study, the highest bio-SA production was achieved in the fermentation using 10% (w/v) of microalgae solid biomass, resulting in a bio-SA concentration of 13.23 mg/mL. This was followed by fermentation using 5% and 15% (w/v), with bio-SA concentrations of 12.73 mg/mL and 11.18 mg/mL, respectively. The study indicated that fermentation with a high initial biomass concentration of 20% (w/v) resulted in the lowest bio-SA concentration of 7.32 mg/mL.

These findings highlight the significant influence of the initial biomass concentration on bio-SA production using pretreated *Chlorella* sp. The observed variations of bio-SA output may be attributed to differences in the concentration of various nutrients in the fermentation broth, such as the carbon-to-nitrogen ratio, inorganic salts, and vitamins. These factors can impact bacterial growth and the accumulation of metabolites (Behera *et al*., 2019).

Furthermore, the lower bio-SA production during fermentation with a high biomass concentration could be explained by the fact that higher biomass produces bio-SA and inhibitors that can affect the fermentation process in microorganisms. According to Phuengjayaem *et al*. (2016), the presence of inhibitors, such as 5-hydroxymethylfurfural and furfural, might have had an adverse effect on cell growth and, subsequently, succinic acid production.

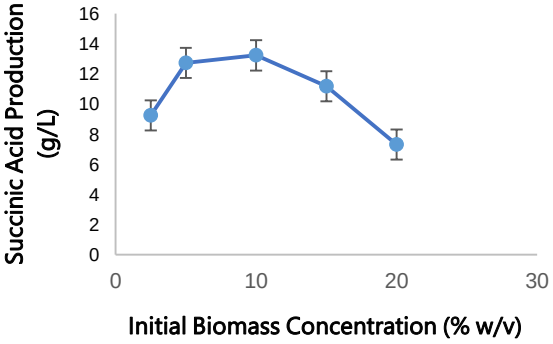

Fig. 2: Succinic acid production by A. succinogenes from different percentage biomass solid loading of microalgae hydrolysate at initial pH 4.8, 37 ℃ and agitated at 150 rpm.

Figure 3 illustrates the bio-SA concentration produced during fermentation using various agitation rates. Determining the most suitable agitation rate during fermentation is crucial to achieving maximum bio-SA yield. Agitation is the process by which cultured microbial cells and nutrients are kept in a homogeneous suspension. The agitator thoroughly mixes nutrients and increases the oxygen transfer rate in the reaction medium. It prevents clump formation and enhances the metabolism rate. According to this study, the highest bio-SA production was observed at 13.12 mg/mL in fermentations performed at 150 rpm. Increasing the agitation rate beyond this level was found to reduce bio-SA production yield. Conversely, fermentation at a low agitation rate, such as 50 rpm, resulted in only 4.32 mg/mL of bio-SA from pretreated *Chlorella* sp. biomass.

The high bio-SA yield observed in fermentations at 150 rpm can be attributed

to the optimal availability of nutrients and their transfer into microbial cells during fermentation. A similar trend was reported by Isar *et al*. (2006), who observed that 1.0 g/L of bio-SA was produced when the fermentation was conducted at 100 rpm. This finding agrees with the results obtained by Oh *et al*. (2009), who noted improved bio-SA production with *Mannheimia succiniciproducens* LPK7 at a high agitation rate of 150 rpm. Higher bio-SA production obtained at high agitation rates can be attributed to relatively high agitation speeds favorable sugar consumption and conversion into bio-SA during the fermentation process (Rodmui *et al*., 2008; Muregi *et al*., 2021). Unfortunately, further increases in agitation speed beyond 150 rpm significantly reduced bio-SA production yield. The result clearly indicated that a high agitation rate could negatively affect the formation of bio-SA from microalgal biomass. The low bio-SA production at high agitation rates can be attributed to the shear forces generated during fermentation under these conditions. These forces influence microorganisms in various ways, including changes in morphology, growth and metabolite formation fluctuations, and potential damage to cell structures (Kassim *et al*., 2022).

The higher the agitation rate, the better the fermentation yield compared to those without agitation or at lower rates. With more vigorous agitation, better cell-medium interaction occurs, enhancing bacterial growth and accelerating nutrient consumption, resulting in higher product yields. Under lower agitation conditions, the microbes may have settled at the bottom of the vessel, reducing its ability to absorb nutrients effectively (Hariz *et al*., 2023). A previous study on bio-SA production by *Aspergillus niger* found that fermentation low agitation rates resulting in lower bio-SA and less contact between enzyme and substrate to produce bio-SA from the available sugar available in the hydrolysate (Alcantara and Mondala, 2021)

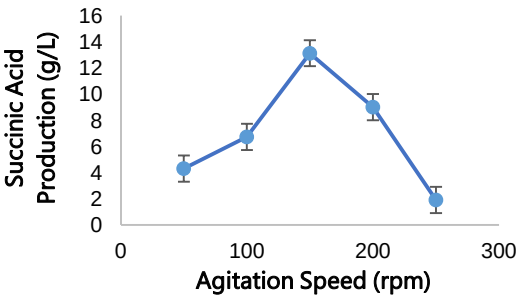

Fig. 3 Succinic acid production by A. *succinogenes* from different agitation speed at initial pH 4.8, 37 ℃ and 10 % (w/v) of biomass solid loading.

Based on Table 3, both parameters exhibited a significant influence on enzyme activity, as indicated by a p-value of less than 0.05, which led to their advancement to the next step of optimization using the R software.

Table 3. One-way Analysis of variance (ANOVA) on effect of initial biomass concentration and agitation speed on succinic acid production

	Df	Sum Sq	Mean Sq	F value	P value
Biomass	4	1132.35	33.09	3.72	0.042 *
Residuals	10	88.95	8.90		
Agitation	4	184.95	46.24	6.23	0.009**
Residuals	10	74.12	7.41		

Optimization

The interaction between the initial biomass concentration and agitation rate was assessed using response surface methodology (RSM) within the R software, employing the Central Composite Design

(CCD) approach. Based on the results obtained during the screening stage, the highest succinic acid production was achieved with a biomass solid loading of 10% w/v (13.23 mg/mL) and an agitation speed of 150 rpm (13.12 mg/mL).

For this interaction analysis, ten experiments with randomly selected biomass solid loading percentages and agitation speeds were conducted (Table 4). The results indicated that the highest succinic acid production was achieved in run 3, with a biomass solid loading of 10% (w/v) and an agitation speed of 150 rpm, resulting in a succinic acid production of 13.06 mg/mL. In contrast, the lowest succinic acid production was observed in run 6, with a biomass solid loading of 10% (w/v) and an agitation speed of 80 rpm, resulting in a succinic acid production of 0.05 mg/mL.

Table 4. Central composite design and experimental results of succinic acid production (mg/ml) at different biomass solid loading and agitation speed.

Run	Biomass solid loading (%)	Agitation speed (rpm)	Succinic Acid Production (g/L)
1	5.00	200	5.05
2	15.00	200	2.76
3	10.00	150	14.56
4	15.00	100	7.95
5	5.00	100	2.56
6	10.00	80	0.05
7	17.07	150	3.64
8	10.00	150	12.29
9	10.00	220	1.15
10	2.93	150	1.31

A pure quadratic model was the most suitable fit for bio-SA production from pretreated *Chlorella* sp. biomass, with a Prob>F value of 0.004 (Table 5). The significant F value obtained from this study suggests that both tested parameters significantly affected bio-SA production from microalgae biomass. Analysis of variance (ANOVA) for the response surface of the pure quadratic model was performed to evaluate the effect of both parameters on bio-SA production (Figure 5). The model's fitness was also expressed by the coefficient of determination (R^2). The predicted R^2 value obtained from this analysis was 0.9345, which is close to 1, indicating that the data obtained reasonably agree with the actual values obtained from the experiments. The model thus is a reliable predictor of bio-SA production within the design boundaries of our experiments. This indicates that the model significantly affects bio-SA production from pretreated *Chlorella* sp. biomass.

Table 5. ANOVA of second order polynomial model.

	Df	Sum Sq	Mean Sq	F value	P value	P value
First Order	2	4.54	2.27	0.61	0.58	0.0419 *
Two-way	1	0.08	0.08	0.02	0.88	
Pure Quadratic	2	204.94	102.47	27.9	0.0044	0.00876 **
Residuals	4	14.68	3.67			
Lack of fits	3	12.11	4.03	1.56	0.51	
Pure error	1	2.57	2.57			

From the experimental results, the fitted equation analyzed by multiple regression (Table 6) for bio-SA production is expressed as:

$$Y = 13.3949 - 5.1870X_1^2 - 6.1436X_2^2 \quad (2)$$

Where Y indicates succinic acid production (mg/mL), X_1 represents the percentage biomass solid loading (% w/v), and X_2 represents the agitation speed (rpm).

The interaction between the initial microalgal biomass concentration and agitation rate on bio-SA production was evaluated using 3D response surface plots (Figure 4 and 5).

Table 6. Estimated regression coefficient of the second order polynomial model.

	Polynomial coefficient	Std. Error	t-value	P value
(Intercept)	13.39	1.3aq5	9.88	0.0005 ***
X_1	-0.09	0.68	-0.13	0.89
X_2	0.75	0.68	1.10	0.33
$X_1 : X_2$	0.95	-0.14	0.88	0.95
X_1^2	0.90	-5.71	0.004 **	0.90
X_2^2	0.90	6.76	0.002 **	0.90
Predicted R^2	0.93			
Adjusted R^2	0.85			
F-statistic	11.41			
P-value	0.017			

*X_1: biomass solid loading of microalgae hydrolysate; X_2: agitation speed of fermentation; *Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

According to Figure 4, an increase in both biomass solid loading and agitation speed led to an increase in succinic acid production. As the agitation rate increased from 50 to 150 rpm, the bio-SA concentration produced increased significantly, from 2.56 mg/mL to 14.56 mg/mL. Further increases in agitation rate beyond this optimum condition resulted in a significant reduction in bio-SA production.

Similarly, better bio-SA production was observed for fermentations using higher initial biomass concentrations. The highest bio-SA production of 14.56 mg/mg was achieved when the fermentation was conducted using 10 g/L at 150 rpm. Fermentation using an initial biomass concentration higher than 10 g/L significantly lowered the bio-SA production. This finding agrees with Escanciano *et al*. (2023), who investigated bio-SA production using potato waste and found that the initial substrate concentration could significantly affect bio-SA production. Their study indicated that fermentation at concentrations beyond the optimum substrate levels caused inhibition effects due to the substrate used during the fermentation process. The low product yield observed in fermentations with high substrate concentrations may also be attributed to increased viscosity, which hampers efficient mass transfer between bacteria and substrate (Kassim *et al*., 2011).

The contour plot in Figure 5 illustrates the relationship between biomass solid loading and agitation speed in 2D. The darker or reddish zone in the center indicates that bringing the independent variables closer to the center results in increased enzyme activity.

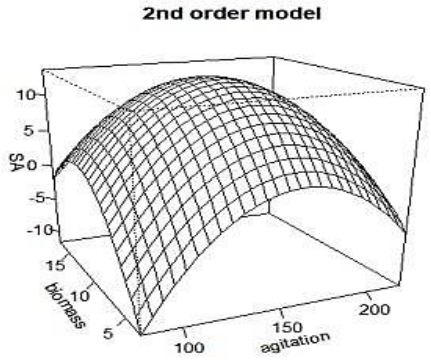

Fig. 4: 3D Surface plot of bio-SA production using different initial biomass concentration and agitation rate.

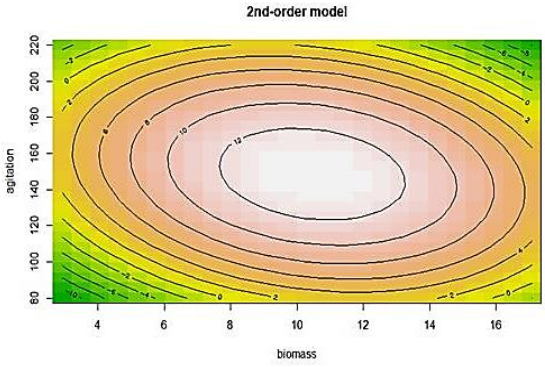

Fig. 5: Contour plot of succinic acid production.

The optimal conditions for maximum succinic acid production were calculated using one of the R software packages, the desirability function. The desirability function combines an objective ranging from zero outside the boundaries to one at the maximum target. The obtained desirability value is 0.9194, and the optimal conditions calculated by the desirability mechanism were 11.21% (w/v) percentage biomass solid loading and an agitation speed of 147 rpm to achieve succinic acid production of 13.39 g/L.

Table 7 compares the bio-SA production obtained in this study to those reported in other findings on SA production from different biomass sources. The bio-SA concentration obtained from the fermentation of *Chlorella* sp. hydrolysate is higher than that from carrot pods and rice husks. However, the bio-SA obtained is lower than the bio-SA produced from the fermentation of duckweed, corn straw, and EFB. According to this analysis, it is clear that several factors, such as pretreatment, the mode of fermentation, and initial sugar concentration influence the production of bio_SA from different biomass feedstocks.

CONCLUSIONS

In conclusion, selecting an appropriate feedstock emerges as a pivotal determinant in the viability of bio-succinic acid (bio-SA) production through fermentation. This study meticulously examined the potential of microalgae *Chlorella* sp. biomass as a bio-SA feedstock, employing a comprehensive CCD design. The results were compelling, revealing a noteworthy production yield of approximately 14.56 g/L with an impressive glucose conversion yield of 0.62 g/g, achieved through the mild acid pretreatment of *Chlorella* sp. hydrolysate. Notably, our investigation underscored the substantial impact of fermentation conditions, particularly the initial biomass concentration and agitation rate, on the ultimate bio-SA output. identifying optimal conditions, set at 10% (w/v) initial biomass concentration and 150 rpm agitation, further refines the understanding of maximizing bio-SA production. Importantly, utilizing *Chlorella* sp. hydrolysate as a renewable bio-SA feedstock emerged as a promising strategy, presenting potential enhancements to the overall economics of the fermentation process. This study contributes valuable insights into the intricate dynamics of bio-SA production, emphasizing the significance of feedstock choice and process optimization for sustainable and economically viable biotechnological endeavors.

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Table 7. Comparison of bio-SA production from different biomass hydrolysate

Biomass	Pre-treatment agent	Hydro-lysis enzyme	Fermentation configuration	Micro-organisms	Initial substrate concentration	Bio-SA concen-tration	SA yield	References
Mischantus straw	Organosolv 1.25% H_2SO_4	Viscozyme® L, Carezyme 1000L®, β-Glucanase, Cellic® CTec2, Cellic® HTec2	Batch	A. succinogenes	-	24 g/L	82	Dabkowska *et al.*, 2019
EFB	Autohydrolysis		SHF	A. succinogenes		23.5 g/L	32.6	Pasma *et al.*, 2013
EFB	Dilute acid-alkaline	Cellulase	SSF	A. succinogenes	70 g/L	42.9 g/L	61%	Akhtar *et al.*, 2019
Rice husks	Acid hydrolysis		SHF	A. succinogenes	100 g/L	12.5 gL	59.9	Bevilagua *et al.*, 2015
Carob pod	-	-	Batch	A. succinogenes		1.61 g/L	55	Carvalho *et al.*, 2014
Jerusalem Artichoke tuber	0.2% H_2SO_4	-	Batch	A. succinogenes	10% (w/v)	48.9 g/L	83	Gunnarsson *et al.*, 2014
Corn Straw	Dilute alkaline treatment	Cellulase	Batch	A. succinogenes	176.5 gL sugar	33.7 gL	80.7	Pu Zheng *et al.*, 2009
Duckweed	-	Enzyme mixture (celluclast, pullulanase and Viscozyme)	SSSF	A. succinogenes	160 gL	75.46 gL	82.87	Naikun Shen *et al.*, 2018
Sugarcane baggase	-	Multi-enzyme hydrolysis	Batch	A. succinogenes	55 gL	39.9 g/L	82.0	Pengcheng Chen *et al.*, 2016

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