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Microalgal feedstock for the production of omega-3 fatty acid ethyl esters and ε-polylysine

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ABSTRACT

Microalgal omega-3 fatty acids are considered as an efficient alternative for fish-based omega-3 fatty acids. Ethyl esters derived from omega-3 fatty acids are being considered as the drug for hypertriglyceridemia. In this study, omega-3 fatty acids rich *Chlorella* sp. was utilized for the transesterification for the ethyl ester production using a potassium carbonate alkaline catalyst. At the optimized conditions of transesterification, 86.2% ethyl ester yield was achieved with solvent to algae ratio (20 mL/g), water addition (45 %), catalyst (4 %), temperature (75°C), and reaction time (60 min). Additionally, the acid-hydrolysed spent biomass was used for the production of ε-polylysine by fermentation using *Streptomyces* sp. as fermentative organism. The maximum yield of 1.78 g/L was achieved after 90 h fermentation. This study established a biorefinery approach where two highly valuable compounds could be produced from the *Chlorella* sp. by transesterification followed by fermentation.

1. Introduction

Microalgae have been considered as the photosynthetic microorganisms which are able to produce various important biochemical compounds. Microalgae are widely considered for the bioenergy applications such as biodiesel from lipids, and bioethanol from carbohydrates. In addition, microalgae have various valuable components such as antioxidants, polysaccharides, vitamins, pigments and omega-3 and omega-6 fatty acids [1]. In most cases, commercial omega-3 fatty acids synthesis is mainly relying on the fish and it is likely confronting the problem in terms of global demand [2]. Besides, fish-based omega-3 fatty acids have a high risk of contamination due to the heavy metals consumed by the fish and thus raise the concern on the quality of fish-based omega-3 fatty acids. Microalgae can be the good alternative for the fish-based omega-3 fatty acids. Some microalgae are rich in omega-3 fatty acids which are less toxic and more stable towards the oxidation [3]. Omega-3 fatty acids play an important role in animal and human nutrition in the diet. Omega-3 fatty acids have various functions in the cells and act as a precursor for the eicosanoids synthesis [4]. Consumption of omega-3 fatty acids is proven to be a health boosting with a protective role against various illnesses. Omega-3 fatty acids are used in the immune system boosting, retinal and neuronal development, treatment for dementia, arthritis, depression, asthma, headaches, migraine, and schizophrenia [5]. *Chlorella* sp. was rich in omega-3 fatty acids which can be utilized for the omega-3 fatty acid methyl ester production [6]. The microalgal omega-3 fatty acid can be converted into methyl esters by transesterification which can be used as a drug for the treatment of hypertriglyceridemia. Transesterification is the conversion of lipids into methyl esters with the help of catalysts (acid or alkali or enzyme catalysts) [7]. However, enzyme mediated transesterification is a time-consuming process and acid catalysts require more time and high temperatures. Hence, alkaline catalysts are preferably considered for the transesterification which show higher conversion efficiency in a short period of time [8].

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Table 1
Central composite design for the optimization of various parameters for ethyl ester yield.

Run	A:Solvent to algae ratio (mL/g)	B:Water addition (%)	C:Catalyst (%)	D:Temperature (°C)	E:Reaction time (min)	Ethyl ester yield (%)	
						Actual	Predicted
1	15	60	3	60	45	60.5±0.2	62.00
2	15	30	5	60	45	52.7±0.1	53.48
3	20	45	4	75	30	43.1±0.0	43.75
4	10	15	4	75	30	38.6±1.2	37.02
5	15	30	3	60	15	38.4±0.7	35.99
6	20	15	2	45	60	44.8±0.6	45.06
7	20	15	2	45	30	30.3±0.4	32.20
8	15	30	3	30	45	24.1±0.1	25.21
9	20	45	2	75	30	40.1±0.3	40.08
10	10	45	2	45	60	38.7±0.0	40.21
11	20	45	2	75	60	76.2±0.8	75.91
12	15	30	3	60	45	30.0±0.1	28.53
13	10	45	2	75	30	38.4±0.9	38.91
14	15	0	3	60	45	38.2±0.2	38.03
15	15	30	3	60	45	28.4±0.9	28.53
16	15	30	3	90	45	45.3±0.7	45.52
17	20	15	4	45	60	62.7±1.5	62.28
18	20	45	4	45	30	56.3±0.3	57.61
19	10	15	4	75	60	70.6±1.9	72.74
20	10	15	4	45	60	43.8±0.1	43.11
21	10	45	2	45	30	36.2±0.7	35.99
22	10	45	4	75	30	43.5±0.5	43.93
23	5	30	3	60	45	30.9±1.0	32.35
24	20	45	4	45	60	81.6±0.0	78.60
25	20	15	2	75	30	30.1±0.2	31.02
26	20	15	4	75	60	80.3±0.7	79.67
27	10	45	4	75	60	82.3±1.3	79.51
28	20	45	4	75	60	86.2±1.6	87.85
29	20	45	2	45	30	51.4±0.0	49.40
30	10	45	4	45	60	58.8±0.1	58.02
31	20	45	2	45	60	61.8±0.8	62.12
32	15	30	1	60	45	30.7±0.1	31.25
33	10	45	2	75	60	68.1±0.4	66.24
34	10	15	2	75	30	29.1±0.1	31.26
35	10	45	4	45	30	45.3±0.2	45.55
36	20	15	4	45	30	40.5±0.2	41.15
37	20	15	2	75	60	68.5±0.6	66.99
38	10	15	2	45	30	21.6±0.1	20.21
39	10	15	2	45	60	26.6±0.0	24.56
40	25	30	3	60	45	52.8±0.3	52.68
41	20	15	4	75	30	36.3±0.2	35.43
42	10	15	2	75	60	59.4±0.4	58.73
43	15	30	3	60	75	80.7±1.1	84.44
44	10	15	4	45	30	29.7±0.3	30.50

The lipid containing biomass can be utilized in the trans-esterification. However, the carbohydrate content is still present in the spent biomass and can be used for the other valuable component synthesis. Sivaramakrishnan and Incharoensakdi [9] utilized spent biomass carbohydrate for the fermentative production of ethanol. ε-polylysine is the compound which can be synthesized by the utilization of sugar molecules by fermentation. ε-polylysine is a highly valuable component and considered for food-based industries. ε-polylysine contains ε-group and α-carboxyl group in the L-lysine molecule and 20-30 repeating units of this molecule is considered as a homopolymer. This ε-polylysine polymer has strong antibacterial activity against gram-positive and gram-negative bacteria [10].

Several studies reported the production of methyl ester from algal biomass [9, 11]. However, there is very limited study on omega-3 fatty acid-based esters production. Omega-3 fatty acids esters are approved by FDA (Food and Drug Administration – US) for the treatment of hypertriglyceridemia. In the present study, an omega-3 fatty acid rich microalga *Chlorella* sp. was utilized to produce omega-3 fatty acid ethyl esters by using potassium carbonate as catalyst. Statistical experimental design considering five levels and five factors was applied to determine the significant factors and their optimum conditions for the ethyl ester yield. Furthermore, the sugars after acid hydrolysis of the spent biomass were utilized for the fermentative production of ε-polylysine by *Streptomyces* sp. which is an added advantage with respect to biorefinery approach.

2. Materials and methods

2.1. Microalgal strain

The microalga *Chlorella* sp. was obtained from our previous study [6] which was isolated from the stone quarry pond water and its GenBank accession number was KP972095. The organism was maintained and grown in BG11 medium with the culture conditions of 100 rpm shaking, 27 ± 1°C and continuous illumination of 50 μmol photons/m^2/s. The microalgal purity was ensured by the regular microscopic analysis. The 12 days grown cells were collected by centrifugation (2790 g for 10 min). The lipid and carbohydrate contents of *Chlorella* sp. were found to be 30 % and 28 % (DCW) respectively. The omega-3 fatty acid content was enriched in the *Chlorella* sp. by plant hormone treatment as described previously [1].

2.2. Direct transesterification

In this study, wet biomass was used for the direct transesterification as a model to avoid the time-consuming drying process. The wet biomass

Table 2
Chlorella sp. fatty acid profile and its ethyl esters compositions.

Fatty acid compositions	Fatty acid content of lipids from *Chlorella* sp. (%)	Fatty acid content of ethyl esters of *Chlorella* sp. (%)
Myristic acid	0.24 ± 0.01	0.2 ± 0.01
Palmitic acid	16.73 ± 1.02	12.92 ± 0.09
Palmitoleic acid	8.66 ± 0.52	5.62 ± 0.51
Stearic acid	1.03 ± 0.08	1.01 ± 0.09
Linoleic acid	10.11 ± 0.46	7.12 ± 0.47
Linolenic acid	12.75 ± 0.64	11.32 ± 0.08
Arachidic acid	1.12 ± 0.08	0.87 ± 0.06
Eicosapentanoic acid	23.26 ± 0.42	22.22 ± 0.36
Docosahexanoic acid	26.11 ± 0.53	24.94 ± 0.51

was prepared using water and lyophilized biomass. The reaction mixture was prepared using wet biomass, potassium carbonate as catalyst and ethanol as solvent. The reaction mixture was placed in a screw-capped Erlenmeyer flask and shaken at 100 rpm. The dry biomass was added with different water content (%) to create wet biomass.

All the chemicals used were analytical grade. The reaction was carried out using an orbital shaker at 100 rpm. After the reaction was stopped, the sample was mixed with hexane (5:4 v/v) and the samples were washed with distilled water and allowed for phase separation after centrifugation at 2790 g for 5 min. The upper layer was removed and filtered using 0.20 μm syringe. The obtained ethyl ester yield was determined by gas chromatography (GC) [9]. The spent biomass was further washed and used for the hydrolysis to extract the sugars.

2.3. Optimization of ethyl ester production

Central composite design (CCD) was used to investigate the optimization of solvent to algae ratio, percentage of added water, percentage of catalyst, temperature, and reaction time on ethyl ester yield. The experiments were designed using Design-Expert Version 12 (Stat-Ease Inc., Minneapolis, MN. USA). A five-factor and five-level factorial central composite design (small) and two replicates at the center points leading to 44 runs were employed (Table 1). All experiments were carried out in duplicate and average values were reported.

2.4. Fermentative ε-polylysine production from spent biomass

The spent biomass was subjected to acid hydrolysis to obtain the hydrolysate and the hydrolysis conditions were adopted from our previous study [9]. The hydrolysis was performed by using spent biomass in a 125 mL flask with 20 ml of 0.3N H_2SO_4 and 120℃ (in an autoclave with 15 psi) for 20 min. After the hydrolysis, the hydrolysate containing reducing sugars was collected after centrifugation at 5000 g for 10 min. The hydrolysate containing H_2SO_4 was added with 0.1 M phosphate buffer (pH 6.8) until the pH 6.8 was attained. The hydrolysate was used for the fermentative production of ε-polylysine using *Streptomyces albulus* MTCC 503. The fermentative medium composition was prepared according to Bankar and Singhal [12]. A 2.5 ml of the culture (8.0×10^8 cells/ml) was added to the hydrolysate to initiate the fermentation with shaking at 180 rpm, 30℃ and grown for various times up to 130 h. After the reaction was stopped, the cells were harvested by centrifugation (10, 000g for 10 min). The dry cell weight (DCW) was determined according to Sivaramakrishnan and Incharoensakdi [6]. ε-polylysine was determined by the addition of 1 ml of 1 mM methyl orange to 1 ml of supernatant and incubated at 37 ℃ for 1 h with mixing. After the incubation, the samples were centrifuged (10,000g for 10 min) and the supernatant was measured for the absorbance at 465 nm using UV-Vis spectrophotometer [13].

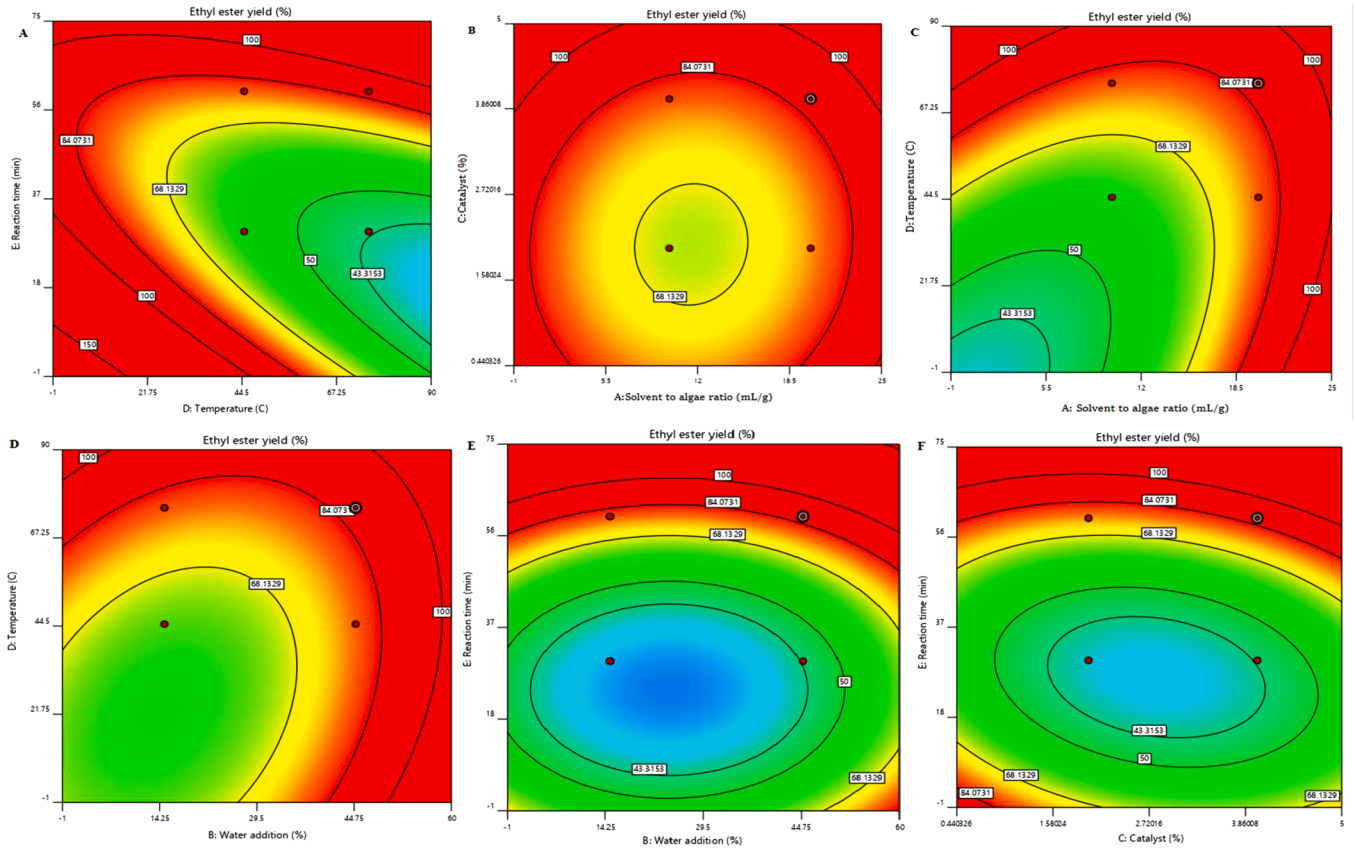

Fig. 1. Contour plot representing the effect of solvent to algae ratio (mL/g), water addition, catalyst, temperature, and reaction time on ethyl ester yield.

2.5. Analytical method

The ethyl ester was analyzed using Agilent gas chromatograph, the instrument is equipped with the carbowax column and flame ionization detector (FID). N_2 was used as a carrier gas at 1ml/min flow rate. H_2 and O_2 were used for the ignition purpose in detector. The initial column temperature was set at 150 ℃ and increased to 240 ℃ at a rate of 10 ℃/min. The ethyl ester yield was determined by injecting 10 µl samples and 230 µl of methyl heptadecanoate (internal standard). The ethyl ester content was determined according to the EN14103 (European Normalization) method. The fatty acid composition and its content were determined as previously described [6]. The fatty acid profiles of the lipids and the ethyl esters of *Chlorella* sp. are presented in Table 2.

The ethyl ester yield was calculated by using the following formula.

$$\text{Ethyl ester yield } (\%) = \frac{\text{Weight of oil ethyl ester (g)}}{\text{Weight of lipid (g)}} \text{X100}$$

2.6. Statistical analysis

The results were obtained from the mean of triplicate values with the error bars showing standard deviation (mean ± SD, $n = 3$). The statistical significance was determined by Minitab (software) and the values were found to be significantly different ($P < 0.05$).

3. Results and discussion

3.1. Central composite design (CCD) for optimal ethyl ester yield

The CCD consisting of 44 experiments with a central point and axial points were used to determine the desirable conditions of solvent to algae ratio (A), percentage of water added (B), percentage of catalyst (C), temperature (D), and reaction time (E) on ethyl ester yield response. The transesterification yields were achieved according to the Design Expert software as a combination of fixed parameters. The design matrix illustrating various factors and its corresponding experimental and predicted values are given in Table 1.

The experimental results were visualized using contour plots to see the influence of the 5 parameters tested. The maximum ethyl ester yield was obtained in the 28th run with the following optimized parameters: solvent to algae ratio (20 mL/g), water addition (45%), catalyst (4%), temperature (75°C), reaction time (60 min). Fig. 1 A,C,D shows the effect of temperature on the direct transesterification determined for the ethyl ester yield where the temperature was varied between 30 and 90 ℃ and reaction time was varied between 15 and 75 min. Generally, alkaline catalyzed transesterification produces maximum methyl esters at 60 ℃ [11]. In the case of wet biomass, high temperature is required to achieve the maximum yield. Water molecules bound on microalgae protect the strong microalgal cell walls which makes it difficult for the access of catalyst and reactant towards lipid molecules [14]. The increase in temperature increases ethyl ester yields as a result of the enhanced mass transfer and diffusion at elevated temperatures. Furthermore, high temperature increases the solubility of oil in the solvent which contributes to efficient transesterification resulting in enhanced yield. In-situ transesterification of Jatropha seeds with heterogeneous alkaline catalysts showed higher yields at 65℃ [15] indicating that the direct transesterification *Chlorella* sp. using alkaline catalysis showed high ethyl ester yield at lower temperature than that of Jatropha seeds. Similarly, it is economically important to determine the optimal reaction time; a short reaction time is crucial to reduce the cost. The ethyl ester yield was initially low during the short reaction time. Later on, the yield increased rapidly, and the maximum yield was attained after 60 min as shown in Fig. 1 A,E,F. A further increase in reaction time did not show any improvement in the yield. Potassium carbonate is highly soluble in the presence of glycerol, the by-product released during transesterification. After 30 min of reaction, the increase of glycerol in the reaction mixture could enhance the ethyl ester yield by increasing the solubility of potassium carbonate.

The effect of solvent/biomass ratio and the effect of catalyst was investigated and shown in Fig. 1B. Increasing solvent/algae ratio up to 20 mL solvent/g of biomass increased the ethyl ester yield. A further increase in solvent/algae ratio decreased the yield. Excessive solvent may dilute the extracted oil leading to a slow rate of the reaction [16]. The optimized amount of biomass/solvent revealed that the excess or lower than the optimum level affects the ethyl ester yield. In the present study, ethanol/biomass at 20 mL/g ratio showed the maximum ethyl ester production in the 28th run.

To investigate the optimal concentration of catalyst in direct transesterification, potassium carbonate addition was varied in the range of 1 – 5 % in ethanol (Fig. 1 B, F). The addition of potassium carbonate concentration of 4% increased the ethyl ester yield at the 28th run. The effect of catalyst at the concentration beyond 4% decreased the ethyl ester yield. Above the optimal level, water phase in the reaction mixture was insufficient to solubilize the catalyst [17]. Addition of water indirectly helps the solubilization of catalyst since it is less soluble in the ethanol and thus increases the ethyl ester yield. The alkoxide ion is formed when alkaline carbonate reacts with an alcohol and this helps to reduce the soap formation [18]. However, increasing catalyst concentration beyond the limits also causes the soap formation [19] and hence appropriate concentration of catalyst is required to produce maximum yield of ethyl esters. The alkaline catalysts are preferred for the faster reaction rate, mild reaction condition and less possibility of inhibition. Moreover, some alkaline catalysts are tolerant to the water. Potassium carbonate is the promising catalyst for transesterification of wet biomass due to the bicarbonate formation instead of soap formation [20]. There are very limited studies with potassium carbonate as catalyst for transesterification. The potassium carbonate is an alkaline catalyst and it can also be used to make jelly candies for children [21]. Hence, it causes no harm even if traces of potassium carbonate are present in ethyl ester. In the present study, the maximum ethyl ester production was achieved at 4 % of catalyst.

The water content in the reaction mixture for the direct transesterification in *Chlorella* sp. biomass was studied. Different water contents in the range between 0 and 60% were used to determine the transesterification efficiency (Fig. 1 D, E). Surprisingly, dry biomass with ethanol showed a lower yield of ethyl esters than the water added samples. In the case of methanol, dry biomass showed higher methyl ester yield (data not shown). In the present study, potassium carbonate was used as a catalyst. Potassium carbonate has poor solubility in ethanol and therefore its catalytic performance was reduced. Water addition plays an important role in solubilizing the catalyst and provides adequate ethoxide formation and gives the high ethyl ester yield without soap formation. Increasing water content up to 40% increased the ethyl ester content. A further increase of water content decreased the yield. It is worth noting that the addition of water increases the yield whereas in most cases the presence of water had the adverse effects in alkaline catalyzed direct transesterification [7].

The fatty acid compositions and contents of *Chlorella* sp. lipids and its ethyl esters are presented in Table 2. The fatty acid profile of *Chlorella* sp. is rich in eicosapentanoic (EPA) and docosahexanoic (DHA) acids which are highly valuable omega -3 fatty acids. The direct transesterification of *Chlorella* sp. lipids into ethyl esters allowed the conversion of most omega-3 fatty acids into ethyl esters. Hence, omega-3 fatty acid ethyl esters obtained from this study can be used for the treatment of hypertriglyceridemia. The ester form of omega-3 fatty acids is approved by FDA (Food and Drug Administration – US). Hypertriglyceridemia causes acute pancreatitis and atherosclerosis. To reduce the effect of hypertriglyceridemia it is necessary to decrease the triglyceride level. Among the various treatment such as therapeutic intake of fibrates, nicotinic acid, omega-3 fatty acid esters, intake of omega-3 fatty acids esters such as EPA or DHA is highly preferable for hypertriglyceridemia [22]. From the optimization process, the high yield of

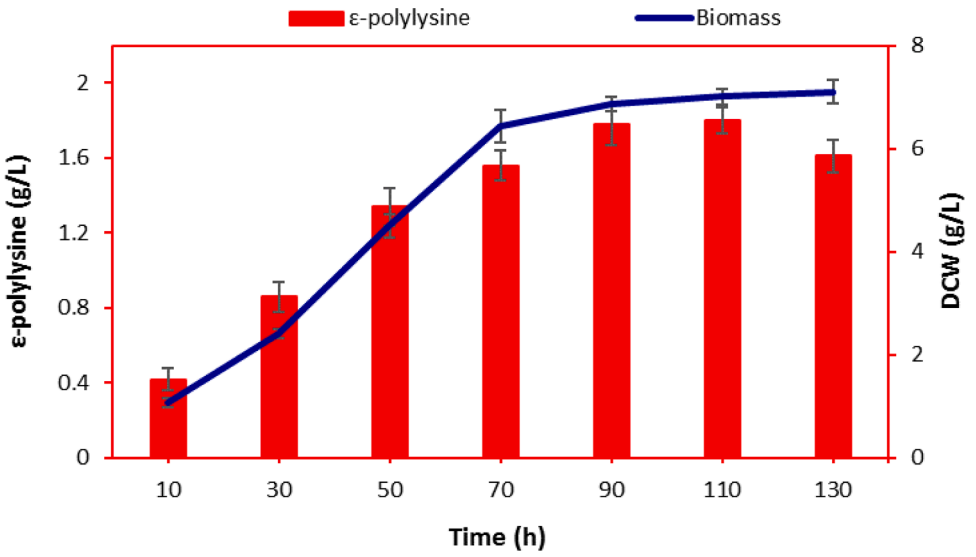

Fig. 2. Time course of fermentative ε-polylysine production and biomass content. Data points are the average of three independent experiments with error bars showing standard deviation.

EPA and DHA esters achieved in this study has the potential to serve as the drug of hypertriglyceridemia.

3.2. Fermentative ε-polylysine production

The spent biomass after direct transesterification was further subjected to acid hydrolysis. The hydrolysate was then utilized for the fermentative ε-polylysine production using *Streptomyces* sp. and the results are shown in Fig. 2. The biomass and ε-polylysine yield was increased with the increase in reaction time. The maximum biomass and ε-polylysine production were achieved at 90 h of fermentation yielding 6.87 and 1.78 g/L respectively. No improvement in the biomass and ε-polylysine production was detected after 90 min. Previous studies reported that the highest ε-polylysine production can be achieved between 48 and 72 h [26] and at 144 h [27] of fermentation when *Streptomyces* sp. was used. Similar results were also observed in another study by the same group when *Streptomyces albulus* PD-1 was used for the fermentation [23]. In the present study, the highest ε-polylysine production was achieved during 90 h which is the intermediate among the previous studies reported in the literature. In this respect, it should be noted that fermentation time longer than 120 h resulted in a decrease of ε-polylysine yield due to its degradation by the enzyme produced by fermentative organism (*Streptomyces* sp.) during post stationary phase [24]. ε-polylysine is biodegradable and can be used in food industries as a food preservative. Other important benefits of ε-polylysine include biochip coating, anticancer agent enhancer, emulsifying agent, gene delivery carrier, dietary agent and drug delivery in nano or micro capsules [25].

4. Conclusions

Direct transesterification of *Chlorella* sp. lipids produces omega-3 fatty acid ethyl esters using potassium carbonate as a catalyst. Various process parameters were optimized using central composite design and the maximum ethyl ester yield of 86.2% was achieved at the optimized conditions of solvent to algae ratio (20 mL/g), water addition (45 %), catalyst (4 %), temperature (75°C), reaction time (60 min). Potassium carbonate is an efficient catalyst for the synthesis of ethyl esters. Although potassium carbonate is not a conventional catalyst, it showed high ethyl ester yield from *Chlorella* sp. lipids. The hydrolysate obtained from the spent biomass was efficiently fermented to produce ε-polylysine which has various industrial benefits. A biorefinery approach in this study, showing the production of two different compounds, omega-3 fatty acids ethyl esters by single step technology (direct transesterification) and ε-polylysine offers a promising option for the commercialization of microalgae derived value-added biochemicals.

Authors' contribution'

Ramachandran Sivaramakrishnan : Conceptualization, Methodology, Investigation, writing- original draft, Writing - review & editing

Govindarajan Ramadoss : Formal analysis, Writing - review & editing.

Subramaniam Suresh : Validation, Data curation

Sivamani Poornima: Investigation

Arivalagan Pugazhendhi : writing-review & Editing

Aran Incharoensakdi : Funding acquisition, Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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