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Optimization of Cryopreservation Process Using Response Surface Methodology for *Chlorella saccharophila* and *Chlorella zofingiensis*

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Abstract

The preservation of microalgae in a stable state is a fundamental requirement in pharmaceutical, agricultural, environmental sciences and different industries. Cryopreservation is widely stabilized for achieving long-term storage and has been applied to an increasingly diverse range of microalgae and cell cultures. The continuous storage of actively growing microalgae strains by routine serial subculture is relatively time-consuming and this technique has possible contamination risks. In this study, the optimization of cryopreservation process was carried out for two different *Chlorella* strains using response surface methodology (RSM) with three factors (cryoprotectant concentration, incubation time and cryopreservation time) including 19 runs. The optimal cell viability of *C. zofingiensis* was found at the dimethyl sulfoxide (DMSO) concentration of 12.89% at the incubation time of 8.14 min and with the cryopreservation time of 93.45 day, while *C. saccharophila* was found at the DMSO concentration of 12.86 % at the incubation time of 7.99 min and at cryopreservation time of 95.17 day.

Keywords: Cryopreservation; *Chlorella saccharophila*; *Chlorella zofingiensis*; response surface methodology.

1. Introduction

Microalgae are foremost biomass sources for health foods, feed additives, cosmetics and biodiesel production due to their benefits, such as not competing for arable land with crops, high influent in capturing sunlight, and decreasing CO_2 emissions compared to terrestrial flora [1].

Long-term stability of microalgae culture collections in serial sub-culturing using liquid or solid media cannot be preserved because there are labor intensive, costly and a risk of contamination and genetic alterations [2]. Therefore cryopreservation, the preservation of cellular viability at low sub-zero temperature, provides a crucial option for conserving a microalga for weeks or even years [3]. Cryopreserved cells are protected from any genetic changes and required minimum maintenance and labor, believing in stored during suitable conditions; contamination risk of other microorganisms should decreased [4]. Although, the most important problem about cryopreservation process of microalgae is formed severe osmotic stress and/or ice crystal damage throughout the process both freezing and thawing [5]. It is reported that metabolic changes lead to cryopreservation can figure out intracellular free radicals by cryoprotectant exposure and cooling process [6]. Thus, microalgae cell viability after cryopreserved was analyzed by response surface methodology (RSM) used to evaluate optimal conditions for cryoprotectant concentration, incubation time with cryoprotectant at room temperature and cryopreservation time. For the optimization of algal cell viability, it is necessary to optimize these factors with an optimization method. In that, RSM is empirical statistical modeling techniques on the multivariate non-linear model-based that is able to work out interactions among all parameters [7].

In this work, the information was given on changes in viability of cryopreserved two different *Chlorella* harvested from late exponential of culture. In this study, optimization of cryopreservation conditions were conducted with 3 different parameters of cryoprotectant concentration (0-25%), pretreatment (1-15 min) the duration of cryopreservation (7-180 day) for *C. saccharophila* and *C. zofingiensis* by central composite design (CCD) using response surface methodology (RSM) to statistically utilize findings.

2. Material and methods

2.1 Cultivation of algae

Two freshwater strains, *Chlorella saccharophila* (Krüger) Migula (EGEMACC 13) and *C. zofingiensis* Dönz (EGEMACC 20) were supplied from Ege Microalgae Culture Collection (EGEMACC-http://www.egemacc.com/). The microalgae cells were monoalgal and growth in 100 mL Bold Basal Medium (BBM) [8] of 250 mL flask, at 22±2 °C under 100 µmol

photons $m^{-2}s^{-1}$ until cells reached to latter part of the logarithmic growth phase. Then, cells harvested with centrifuge, resuspended with fresh BBM and cell concentration were counted with Neubauer hemocytometer, and then adjusted to 10^7 cell/mL.

2.2 Evaluation of design and data analysis

The optimization of cryopreservation process was identified by Response Surface Methodology (RSM) based on Central Composite Design (CCD) by the aid of software package Design Expert (version 7.0.0; Stat-Ease Inc., Minneapolis, MN, USA). Dimethyl sulfoxide (DMSO) was used as a cryoprotectant and prepared percent concentrations (Table 1) in the study. CCD analysis was used to determine the impact on three independent parameters (DMSO percent concentration, incubation time in room temperature, cryopreservation time) in 19 runs (Table 2).

Table 1. Calculations for preparing percent concentration.

Concentration (%)	Cryoprotectant DMSO (µL)	Cell suspension (µL)
0	-	1500
5	75	1425
13	195	1305
20	300	1200
25	375	1125

Table 2. The experimental response surface design matrix with the actual model-based

Treatment	DMSO %	Incubation time (min)	Cryopreservation time (day)
1	13	8	94
2	20	4	145
3	20	4	42
4	13	8	94
5	5	12	42
6	5	4	145
7	5	4	42
8	13	8	94
9	13	1	94
10	13	15	94
11	13	8	94
12	13	8	7
13	5	12	145
14	20	12	145
15	13	8	180
16	0	8	94
17	20	12	42
18	25	8	94
19	13	8	94

ANOVA was utilized to peruse the statistical significance of regression coefficients via performing the F-test. The most accurate model formed and exhibited in graphical representations with contour plots of factors that represent their relative influence and optimal parameter values. A quadratic polynomial empirical model was in use stated optimum conditions for cryopreserved *C. saccharophila* and *C. zofingiensis*. The second order quadratic polynomial empirical model was used to identify models (2.1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (2.1)$$

where Y represents the response (750 nm and 665 nm for *C. saccharophila* and *C. zofingiensis*, respectively), β_0 is the interception coefficient; βi and βii are the first and second order quadratic design coefficients, respectively; βij is the linear design coefficient for the interaction between factors *i* and *j*; X is the factor.

2.3 Cryopreservation Process

In this study, the percent concentrations of DMSO are shown in Table 1. DMSO and cell suspension (the concentration of DMSO percentage) were added into cryogenic vials, incubated in room temperature (incubation time) according to the set of experiments (Table 2). After prepared all cryogenic vials, the cells were firstly incubated at -20ºC for 30 min, then -80 ºC for an overnight and put into liquid nitrogen (-196ºC) for cryopreservation time. Defrost process was

performed by immersing cryo-vials in a 35°C water bath. To remove cryoprotectant, defrosted cell suspensions were centrifuged and supernatant was removed [9]. After that cells were resuspended with 5 mL of fresh BBM and incubated under 20 µmol photons m^{-2} s^{-1} at 22±2º C for 1 week, subsequent to incubation in the dark for 24 h.

2.4 Viability assay

After one day thawing, cell viability was measured by the most common staining protocol using fluorescein diacetate (FDA). FDA stock solution was prepared by dissolving of 1 mg flourescein diacetate in 1 mL of methanol. 50 µL of FDA stain solution was added to 1 mL culture, incubated at 22±2 ºC for 5 min, and observed by blue-light fluorescence microscopy. Viable cells fluoresce green (positive control) and nonviable cells appeared red or non-color. The images of living cells were taken under 485/535 excitation/emission nm with fluoresce microscope (Olympus BX53, Japan) at 60X magnification [2].

Cell viability was calculated by the equation (2.2);

$$Viability\ (\%) = \frac{cryopreserved\ live\ cell\ number}{non{-}cryopreserved\ live\ cell\ number} \times 100 \quad (2.2)$$

2.5 Measurement of microalgal growth

Microalgal cell growth was monitored by optical density and cell counting using Neubauer chamber. The optical turbidity was measured at 665 nm and 750 nm in spectrophotometer (GE Healthcare Ultrospec 1100 pro, London, UK).

3. Results and Discussion

Several factors can potentially influence the success of cryopreservation such as the state and density of the culture, the nature and concentration of the cryoprotectant, the pretreatment with cryoprotectant, the composition and osmolarity of the medium, the cryopreservation time, the rate of cooling, thawing and post-thawing [10]. The most important factors, which effects on algal viability, are the type, concentration and timing of cryoprotective agents, pretreatment with cryoprotectant and duration of cryopreservation [11]. Salas-Leiva and Dupré [12] reported that concentration, temperature and time of exposure are related to the use of cryoprotectant in additionally there are various methods of handling them emphasized in the literature. The cryoprotectant agent as DMSO is used for the treatment of microalgae because it easily permeates cell membranes and its low hydrophilicity [13]. In order to obtain optimum cell viability after cryopreservation, it is necessary to optimize of three factors (cryoprotectant concentrations, incubation time and cryopreservation time) by using response surface methodology (RSM) in analytical optimization [14].

The experimental data were calculated by the Design-Expert software, and the results of each range and level variance analysis were given in Table 3. The range of variables was selected on test experiences to summarize in databases related to algal cultivation. Response surface methodology (RSM) based on three variables at five level central composite design (CCD) was applied to determine the effect of the cryoprotectant concentration, incubation time and cryopreservation time. As shown in Table 3, 5 different cryoprotectant concentrations; X_1 – % (0, 5.07, 12.5, 19.93, 25), 5 different incubation times; X_2 – min (1, 3.84, 8, 12.96, 15) and 5 different cryopreservation times; X_3 – day (7, 42.07, 93.5, 144.93, 180) were tested. Total of 19 experiments were used to optimize the range and levels of selected variables. Besides, the order of treatments was arranged randomly. As seen from Table 3, viability (%) of the cells are in compatible with the spectrophotometric results and the most vial cells were obtained in the set 1 (75%) and set 11 (100%) for *C. saccharophila* and *C. zofingiensis*, respectively.

Table 3. Experimental data and levels of the independent variables error in the response surface design.

Independent variables	Symbol	Levels				
		-α	**-1**	**0**	**+1**	**+α**
Cryoprotectant concentration (DMSO %)	X_1	0	5.07	12.5	19.93	25
Incubation time (min)	X_2	1	3.84	8	12.16	15
Cryopreservation time (day)	X_3	7	42.07	93.5	144.93	180

Run	X_1 (%)	X_2 (min)	X_3 (day)	*C. zofingiensis* (665 nm)	Viability (%)	*C. saccharophila* (750 nm)	Viability (%)
1	12.5	8	93.5	0.65	80	0.105	75
2	19.93	3.84	144.93	0.1	42	0.029	40
3	19.93	3.84	42.07	0.15	14	0.033	60
4	12.5	8	93.5	0.63	86	0.1	25

5	5.06	12.16	42.07	0.08	29	0.014	20
6	5.06	3.84	144.93	0.1	15	0.029	50
7	5.06	3.84	42.07	0.056	7	0.018	25
8	12.5	8	93.5	0.665	57	0.102	25
9	12.5	1	93.5	0.4	18	0.04	20
10	12.5	15	93.5	0.44	29	0.04	25
11	12.5	8	93.5	0.68	100	0.1	25
12	12.5	8	7	0.06	13	0.02	30
13	5.06	12.16	144.93	0.08	18	0.029	35
14	19.93	12.16	144.93	0.138	25	0.038	50
15	12.5	8	180	0.05	12	0.02	20
16	0	8	93.5	0.017	12	0.029	10
17	19.93	12.16	42.07	0.121	33	0.025	20
18	25	8	93.5	0.09	11	0.035	35
19	12.5	8	93.5	0.65	11	0.103	75

Based on the experimental results of *C. saccharophila* (3.1) and *C. zoffingiensis* (3.2), the following response surface model was explained in the form for the symbol factors (X_1; X_2; X_3):

$$Y=+0.042+1.116*10^{-3} *X_1-4.222 *10^{5} *X_2+8.364 *10^{-4} *X_3-0.012* X_1^2-0.011 *X_2^2-0.013 *X_3^2 \quad \textbf{(3.1)}$$

$$Y=+0.66+0.023 * X_1+5.878 *10^{-3} * X_2-4.260 *10^{-4}* X_3-0.22* X_1^2-0.092* X_2^2-0.22 *X_3^2 \quad \textbf{(3.2)}$$

The Analysis of Variance (ANOVA) for the model Equivalent (2, 3) of two *Chlorella* strains observed a good fit between the models and the experimental data. As depicted in Table 4 and 5, the cellular viability of *C. saccharophila* at 750 nm and *C. zofingiensis* at 665 nm had second-degree nominal effect by the three process variables. Tables of ANOVA p-value was measured significance of variable. When both model p>F value of the variable was less than 0.0001, it represented that the variable had highly significant effects on the response value. The model lack of fit value of 2 implies was significant for *C. saccharophila*, while the F-value of 3.20 implies was found for *C. zofingiensis.* The statistically significant of each coefficient was served by the values of F and p. The values of p > F using the design was less than 0.05, indicated that this design was a significant and desirable [15]. In this study, the squared values of the variables (X_1^2, X_2^2, X_3^2) found to have significant effects on the viability of the cells ($p < 0.05$), even though the first order values may not seem to have. However, the design was significant and the lack of fit value is insignificant suggesting a good fit of the model with experimental results with negligible errors. Similar results were also reported by Malakar et al., 2012 [16].

Table 4. Analysis of variance for response surface design of cryopreserved *C. saccharophila* on viability at 750 nm.

Source	***SS**	***DF**	***MS**	**F-value**	**p-value Prob>F**
Model	$4.494*10^{-3}$	6	$7.49*10^{-4}$	305.94	<0.0001
Concentration of DMSO ($\boldsymbol{X_1}$)	$1.7*10^{-5}$	1	$1.7*10^{-5}$	6.94	0.0218
Incubation time ($\boldsymbol{X_2}$)	$2.434*10^{-8}$	1	$2.434*10^{-8}$	$9.942*10^{-3}$	0.9222
Cryopreservation time ($\boldsymbol{X_3}$)	$9.555*10^{-6}$	1	$9.555*10^{-6}$	3.90	0.0717
$\boldsymbol{X_1^2}$	$1.867*10^{-3}$	1	$1.867*10^{-3}$	762.51	<0.0001
$\boldsymbol{X_2^2}$	$1.541*10^{-3}$	1	$1.541*10^{-3}$	629.63	<0.0001
$\boldsymbol{X_3^2}$	$2.349*10^{-3}$	1	$2.349*10^{-3}$	956.62	<0.0001
Residual	$2.938*10^{-5}$	12	$2.448*10^{-6}$		
Lack of fit	$2.35*10^{-5}$	8	$2.938*10^{-6}$	2	0.2628
Pure error	$5.876*10^{-6}$	4	$1.469*10^{-6}$		
Corrected total	$4.523*10^{-3}$	18			
Std. Deviation	$1.565*10^{-3}$		R-Squared	0.9935	
Mean	0.016		Adj R-Squared	0.9903	
C.V. %	9.55		Pred R- Squared	0.9814	

Press	$8.396*10^{-5}$	Adeq Precision	40.552
*SS, sum of squares; DF, degrees of freedom; MS, mean square			

Table 5. Analysis of variance for response surface design of cryopreserved *C. zofingiensis* on viability at 665 nm.

Source	***SS**	***DF**	***MS**	**F-value**	**p-value Prob>F**
Model	1.20	6	0.20	230.99	<0.0001
Concentration of DMSO (X_1)	$7.301*10^{-3}$	1	$7.301*10^{-3}$	8.46	0.0131
Incubation time (X_2)	$4.718*10^{-4}$	1	$4.718*10^{-4}$	0.55	0.4740
Cryopreservation time (X_3)	$2.478*10^{-6}$	1	$2.478*10^{-6}$	$2.870*10^{-3}$	0.9582
X_1^2	0.67	1	0.67	779.44	<0.0001
X_2^2	0.12	1	0.12	135.17	<0.0001
X_3^2	0.67	1	0.67	775.72	<0.0001
Residual	0.010	12	$8.634*10^{-4}$		
Lack of fit	$8.961*10^{-3}$	8	$1.120*10^{-3}$	3.20	0.1381
Pure error	$1.400*10^{-3}$	4	$3.500*10^{-4}$		
Corrected total	1.21	18			
Std. Deviation	0.029		R-Squared	0.9914	
Mean	0.27		Adj R-Squared	0.9871	
C.V. %	10.83		Pred R- Squared	0.9686	
Press	0.038		Adeq Precision	37.393	
*SS, sum of squares; DF, degrees of freedom; MS, mean square					

The value of prediction was in good agreement with adjusted R^2 emphasizing the significance of this model. Moreover, the closer the R^2 value is to 1, the stronger the design is and the higher it predicts the response [17]. *C. saccharophila* of regression analysis revealed a coefficient of determination value of 0.9935 and only 0.65% of the total variations were not explained by this design. Meanwhile, the adjusted correlation coefficient (0.9903) and the predicted correlation coefficient (0.9814) values ratified that the design was good. As an analysis of variance for *C. zofingiensis* is given in Table 5, the R^2, adjusted R^2, and predicted R^2 values were 0.9914, 0.9871, and 0.9686, respectively, which implied that experimental values could not be enough explained by the design.

These results indicated that the precision and general reliability of the second-order model was quite well and analysis of the response using the model was related to the variation of the factors. The three-dimensional (surface) graphs exhibited the common graphical representation of the regression equation and were shown the optimal values of each dependent variable in Figure 1. Three surface and contour plots were shown to indicate influence of the interaction of cryoprotectant concentration, incubation time and cryopreservation time on cell viability of *C. saccharophila* (1-a,b,c,d) and *C. zofingiensis* (2-a,b,c,d), respectively. Figure 1-1 is shown that incubation time and DMSO concentration for *C. saccharophila* followed a concave trend. An increase in the incubation time with increasing the DMSO concentration increased the turbidity gradually up to very specific values and after that point, the viability was decreased dramatically. It was also presented that all variables have significant interactions with each other. As shown Figure 1-2, the response surfaces of *C. zofingiensis* having circular contour lines stated that the interaction between the corresponding variables was less significant than the ones of *C. saccharophila*. Furthermore, a weak effect on the response was observed at the maximum and minimum levels of incubation and cryopreservation times.

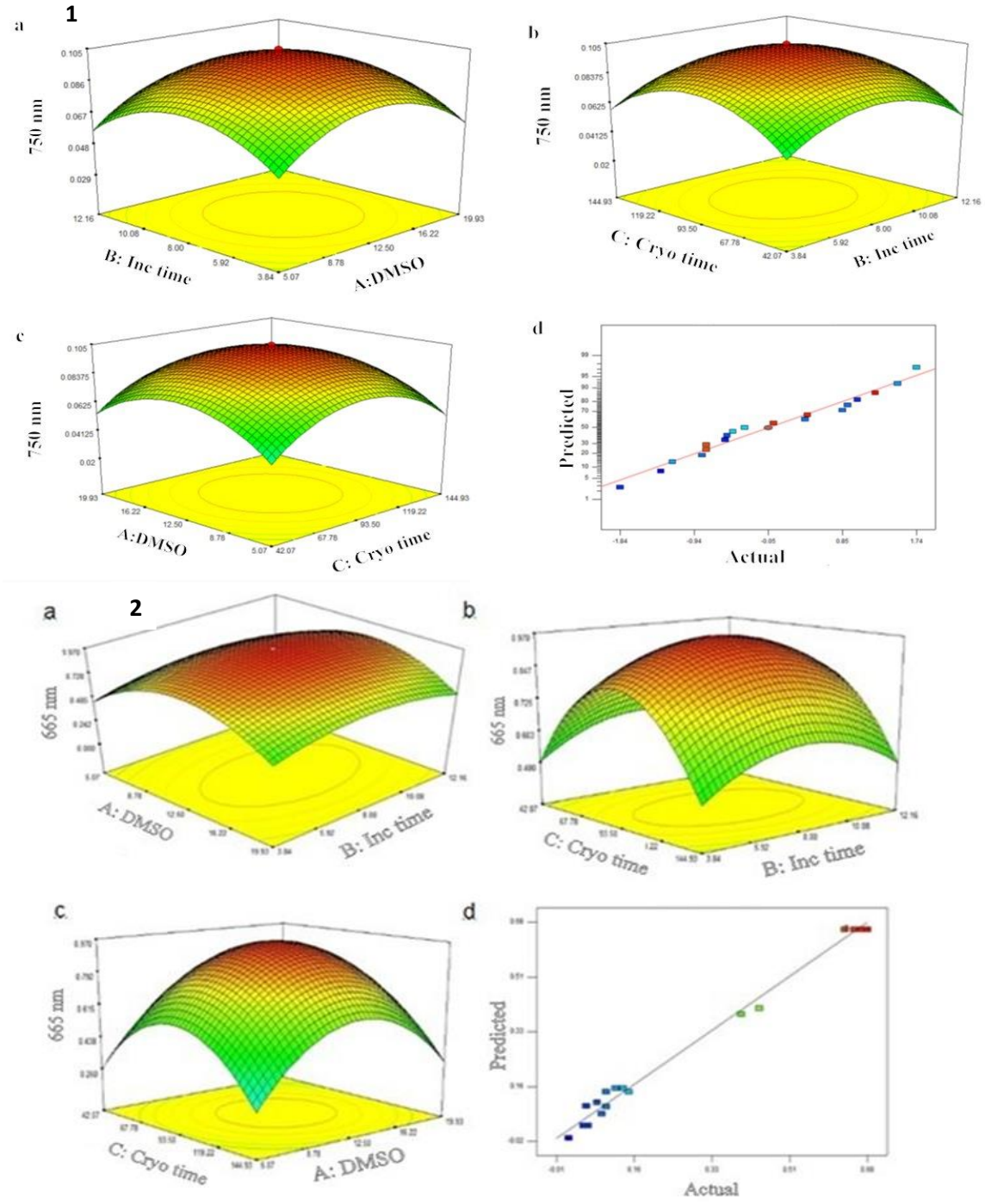

Figure 1. 3D response surface plot of CCD showing the mutual effects of cryoprotectant concentration (DMSO), incubation time (Inc time) and cryopreservation time (Cryo time) on cell viability of *C. saccharophila* (1) and *C. zofingiensis* (2), respectively.

As shown Figure 2, fluorescent dyes fluorescein diacetate (FDA) stains the cytoplasm of live cells, as also visualizing the vacuoles, viable cells fluoresce green and dead and damage cells appeared red or colorless. FDA dye was formed by intracellular hydrolysis, reporting the intact vacuolar and plasma membranes [18]. Figure 2E of *C. saccharophila* and Figure 2J of *C. zofingiensis* cells were observed red because chlorophyll autofluorescence was lower in bad physiological state of the cell. Joseph [19] reported that the highest concentration of DMSO where *Tetraselmis gracilis*, *Chaetoceros calcitrans* and *Chlorella marina* cells were viable and found between at 30% and 40%. Although in DMSO, three strains were viable up to 30% concentration. Moreover, the effective viability of *C. marina* was also stated at 5% DMSO. Whereas, in this study and investigation of Canavate and Lubian [20]

have emphasized that algae could be grown well after cryopreserving in nearly 15% DMSO. In additional, the study aimed that an evaluation of *Chlorella* strains incubation time with cryoprotectant at room temperatures had given on loss of viability during the process.

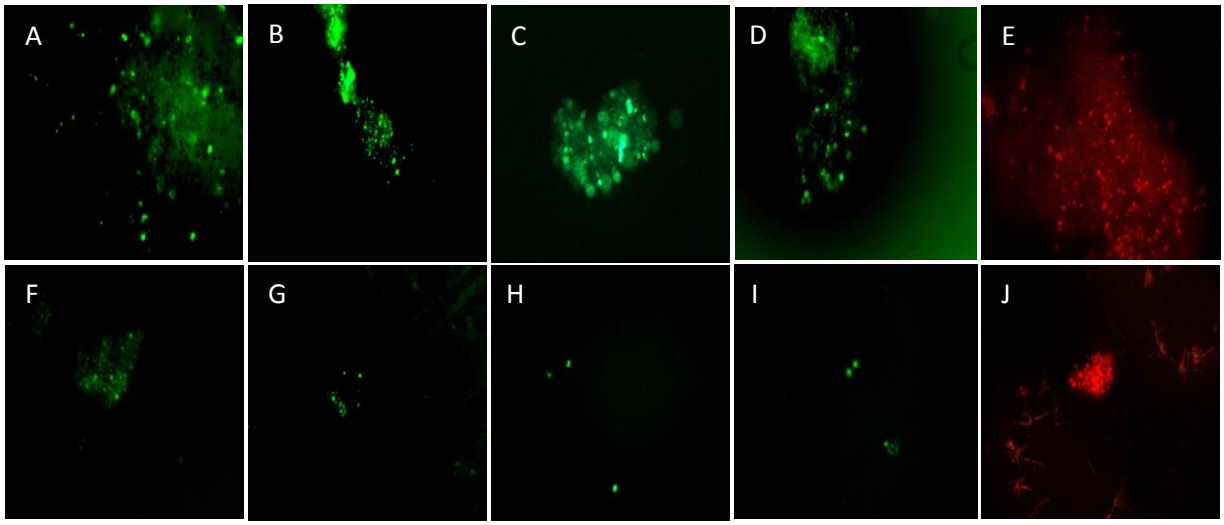

Figure 2. Micrographs of FDA stained microalgae cells. (A-E) *C. saccharophila*, (F-J) *C. zofingiensis.* A. 13% DMSO- day 7, B. 20% DMSO- day 42, C. 13% DMSO- day 94, D. 13% DMSO- day 180, E. 0% DMSO- day 94, F. 13% DMSO- day 7, G. 20% DMSO- day 42, H. 13% DMSO- day 94, I. 13% DMSO- day 180, J. 0% DMSO- day 94.

The validation tests were performed at the optimum conditions to ensure the accuracy of the model in triplicate tests. Optimization of procedure for reply was produced via numerical optimization styles pursuing desirability function.

Optimized results for *C. saccharophila* (at the DMSO concentration of 12.86% at the incubation time of 7.99 min and at cryopreservation time of 95.17 day) and *C. zofingiensis* (at the DMSO concentration of 12.89% at the incubation time of 8.14 min and at cryopreservation time of 93.45 day) were selected and results of the experimental analysis and model predictions were compared. The responses were in good agreement for both strains. For the cultivation of *C. saccharophila*, the experimental response was 0.112 which was closer to the predicted value of 0.102. Under the optimum conditions of *C. zofingiensis*, the value of prediction maximized response was 0.79 while the experimental result was found to be 0.81, indicating the accuracy of the model. The verification studies also indicated that designs suitable test results well.

The results of the work showed that cryopreserved microalgae were stored for long term preservation successfully for periods of up to 3 months. The values obtained in this study showed that the selected value range of parameters at the beginning of the study was correct.

4. Conclusions

In conclusion, we have demonstrated that the viability of *C. saccharophila* and *C. zofingiensis* post thaw of cryopreservation was influenced by parameters such as cryoprotectant concentrations, incubation time and cryopreservation time. In addition, using RSM, the present study was found the cryopreservation conditions for the highest cell viability, seemed to be in keeping with the test values achieved in subsequent validation assay, indicating that RSM may be a useful means for estimating the optimum conditions for test design.

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