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African Journal of Biotechnology

Full Length Research Paper

Encapsulation by complex coacervation of total flavonoids and total phenols of methanol extract of *Anacardium occidentale* L. (Anacardeaceae) obtained by microwave assisted extraction

BETOLOUM Salomon Madjitoloum[1*], MBAIOGAOU Abel[1], MBAIHOUGADOBE Severin[1], TALLA Emmanuel[2], NGASSOUM Martin Benoît[3] and MAHMOUT Yaya[1]

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The study was carried out to produce a microcapsule powder of total flavonoids and total total phenols of methanol extract of the cashew, using a complex coacervation encapsulation method. In the search for optimal conditions for encapsulation, a three level factorial design was set up, while taking into account factors like time and proportions in Arabic gum and gelatin. The kinetic of encapsulation follows a kinetic of 2nd order which gives polynomial equations of the second degree. The conditions found are respectively 45 min, 30% Arabic gum and 70% gelatin, for an encapsulation yield is 84.37%; the encapsulation rate is 77.9% for the total flavonoids and 76.5% for the total phenols. The powder obtained has a doubled concentration in total flavonoids and total phenols than the raw bark powder.

Key words: Encapsulation, complex coacervation, total flavonoids, total phenols, *Anacardium occidentale*.

INTRODUCTION

Humans have always used the products of their environment, especially plants for their medical needs. Plants have several therapeutic properties and their uses for the treatment of diseases in living beings are very old and have always been done empirically (Jeansheng and Cheng, 2018). These plants represent a primary source of medicines and have continued to provide humanity with new remedies to date. Now, research is showing more and more that the active ingredients in herbal medicines are often linked to secondary metabolites. Thus, the African and Malagasy Council for Higher Education (CAMES) program has empowered the Central Africa Network to carry out research activities on medicinal plants used in the treatment of complicated diseases, with a view to producing improved traditional medicines (Ngolo et al., 2018). In the perspective of researching molecules endowed with properties against these diseases, this study was oriented on one of the

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plants with proven pharmacological activities. It is *Anacardium occidentale* (Anacardiaceae).

A. occidentale is an 8-10 m tall tree, native to tropical America and introduced to all tropical countries. Phytochemical studies conducted on the plant reveal the presence of tannins, flavonoids (Annalisa et al., 2017), saponins (Dharamveer et al., 2013) and alkaloids (Alfa et al., 2017). Resorcinolic acid, ascorbic acid, carotenoids (α-carotene, β-carotene and β-cryptoxantin), vitamin C and phenols are also identified in cashew apples (Farid et al., 2014). The work of Madjitoloum et al. (2018b) isolated two flavonoids: quercetin-3-O--D-glucopyranoside and Kempferol-3-O--D-glucopyranoside. The decoction and infusion of the leaves and bark of the trunk are used in pharmacopoeia to treat several diseases namely: High blood pressure, gastrointestinal ailments, ulcers, and sore throat (Jeansheng and Cheng, 2018). In this practice, the effectiveness and quantity of the active ingredient in the solution is not controlled hence the need to extract it.

The performance of extracts in terms of biological activities is linked to extraction techniques. Speaking of extraction methods, apart from the so-called conventional extraction methods such as decoction, infusion, maceration, mechanical agitation and soxhlet, innovative methods of extraction, fractionation and identification of natural products of plant origin have been implemented in order to participate in technological advances faster, more efficient while reducing the quantities of solvent, energy and consumption of samples. Among these innovative extraction methods, microwave assisted extraction has also been implemented. This is also aiming to improve these traditional techniques that are long, tedious, and require large amounts of organic solvents harmful to our environment and health (Madjitoloum et al., 2018a). This technique can be influenced by the parameters such as power, time, solvent polarity, and liquid/solid ratio; its effects can be independent or interactive (Jing et al., 2016). Then, it is necessary to define the best extraction conditions in terms of biological activities and yield. Faced with these difficulties, the use of substances of plant origin is increasingly considered as an alternative or complementary means for poor populations (Raoufou and Kouami, 2013).

In addition to this concern, there is the procedure of formulating a powder by complex coacervation of the active extracts of *A. occidentale*. It should be noted that the quality and the rate of encapsulation of this powder depend on factors such as the time of mixing and the proportions of adjuvants such as gum arabic and gelatin. Encapsulation which is an inclusion technique to confine a substance in a polymeric matrix covered by one or more semi-permeable membranes, whereby the encapsulated compound becomes more stable than the one from which it was isolated. It becomes necessary to solve the shortcomings found in the adsorption technique (Deepak and Sheweta, 2020). In order to minimize the negative aspects, several techniques have been developed to implement different formulations to suit different applications. Nowadays, powder formulation has become one of the most attractive methods for immobilization and protection of active ingredients (Yuksel et al., 2018). To achieve this powder formulation, two main techniques have been developed following the example of adsorption and coacervation encapsulation (Yuksel et al., 2018). Coacervation can be simple or complex: Simple coacervation involves only a single polymer with the addition of strongly hydrophilic agents into the colloidal solution and complex coacervation, two or more polymers are used (Lv et al., 2012). The choice of complex coacervation is the easy release of the active ingredient into the solution.

Our objective is to protect the methanolic extract *of A. occidentale* trunk bark obtained under the optimal conditions of microwave-assisted extraction from adverse effects, minimizing the interactions between the active ingredient and the polymers of the formulation by complex coacervation (Mohammadinejad et al., 2016; Vázquez-González et al., 2021). To grasp this technique, a study on the kinetics and encapsulation parameters needed to be carried out followed by investigating the most influential levels of factors in complex coacervation.

MATERIALS AND METHODS

Plant material

Active ingredient

The active ingredient, called the active principe, in the formula is the methanol extract from the bark of the plant's trunk. This extract is obtained under optimal conditions of microwave assisted extraction at the time of 83 s, at the power of 620 W, at the solvent-matter ratio of 30 mL/g and at 63% water-methanol for which the values total phenols and total flavonoids, are respectively 655.90 mg EGA / 100 g DM and 82.94 mg EQ / 100 g DM of bark (Madjitoloum et al., 2018a). After filtration, the filtrate is concentrated using a rotary evaporator and dried in the jars, then crushed, pulverized and stored for further study.

Auxiliaries

The auxiliaries are adjuvants which have secondary and tertiary functions of the formula. These are gum Arabic and gelatin. Gum Arabic is a highly branched hydrocolloid and a polysaccharide polymer. Its solution has a density of negative charges compared to the acid function (Oumarou et al., 2010). Protein polymer gelatin is used as an encapsulation material thanks to its amphiphilic properties, its ability to interact with different types of molecules, its high molecular weight and the flexibility of its molecular chains. It has a density of positive charges in solution with respect to its amine functions to form ammonium ions (Marta et al., 2020).

Preparation of coacervates

The method used for the preparation of microcapsules is that of Sarunyoo et al. (2018) which has been adapted. The colloidal

Table 1. Matrix of the factorial plan at three levels (27 experiments).

Factor		Factor levels			Responses		
					TF	TP	Tu
Coded values	X	-1	0	+1			
	X_1						
Real values	X_2						
	X_3						

With X1: gum Arabic; X2: gelatin and X3: mixing time; TP: total phenols; TF: total flavonoids and Tu: turbiddiy.

$X = \frac{x_i - x_0}{\Delta xi}$ Hence $X_i = X * \Delta X_i + X_0$

This transformation operation use was adapted from Pillet et al. (2011).

Source: Author

solution is prepared by mixing the solutions of the two polymers with Moulinex to properly emulsify the solution: the gum arabic solution (2% m/v) with that of gelatin (8% m/v) at 40°C. The active principle (0.2 g) is introduced into the colloidal solution (1 mL) and the addition of CH_3CO_2H at 50% v/v is made to adjust the pH of the mixture to pH = 4.5 while stirring for 30 min. Then the addition of CH_2O (37% w/v) 4 mL per 100 mL in the mixture allows crosslinking. Finally, the whole is incubated at 4°C for 30 min. Two phases were observed and after screening the coacervates are recovered and subjected to lyophilization to produce the powder of the microcapsules. The freeze-dryer (Scientz-10ND vacuum Freezer driyer) freezes the coacervate solution at -20°C for 4 h before producing the powder in 48 h.

Quantitative study of flavonoids and total phenols

For quantitative analysis, phytochemical quantification of total flavonoids and total phenols was carried out according to the protocol of Jothi et al. (2013) adapted by Madjitoloum et al. (2018a).

Study of the encapsulation parameters

Effect of time on encapsulation

Time is a parameter which influences the encapsulation of active extracts by complex coacervation. The mixing was carried out for a period of 0 to 120 min in steps of 15 min, the other parameters being constant. The models studied for modeling are as follows.

The kinetic model of order 2: Its equation is of the form: $c(t) = \frac{t}{k_1 + k_2 * t}$ with c (t) the concentration of the extract at time t, t times the extraction in seconds then k_1 and k_2 the speed constants (Tsatsop et al., 2016). The speed constant k_1 makes it possible to have the speed of the extraction B_0 at the initial instant, that is to say at the instant when the solvent comes into contact with the dry matter: $B_0 = \frac{1}{k_1}$ and the constant of speed k_2 allows to have the quantity of extract at equilibrium:

$$c_0 = \frac{1}{k_2}.$$

Gauss model: It obeys the equation: $Y = ae^{-(\frac{t-t0}{4*k})^2}$ where Y is the rate of encapsulation, a is the maximum value of the encapsulation rate, t is the time studied in the encapsulation, and t_0 is the time corresponding to the maximum value of the encapsulation rate and k is the kinetic constant reflecting the influence of the factor studied on the mixture (Zhang et al., 2013). Note that this last model was the one best suited for modeling the encapsulation taking into account all of our different factors in the context of this work.

Influence of the proportion of gum arabic on the encapsulation

The proportion of the gum arabic solution varies from 0 to 100% in 25% steps and the time remains constant (45 min).

Influence of the proportion of gelatin on the encapsulation

We also varied the proportion of the gelatin solution from 0 to 100% in 25% steps, keeping the time constant (45 min).

Encapsulation optimization

At the end of the tests, the experimental field for each of the three factors (time (min), proportion of the gum arabic solution (%) and proportion of the gelatin solution (%)) was chosen. A three-level factorial design is used to find the levels of the most influential factors in complex coacervation (Table 1).

Proposal of a model

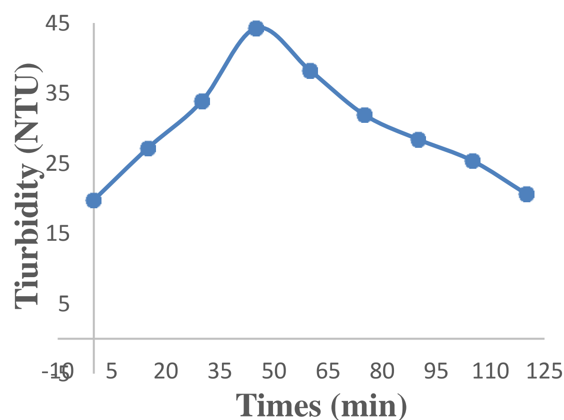
The proposed model has the advantage of properly representing the experimental responses studied in the experimental field of interest and making it possible to obtain an estimate of the value of the studied responses of acceptable quality. The model is as follows:

$$Y_i = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_3X_3^2 + \varepsilon$$

where Y_i are the expected responses, a_0 the constant, a_1, a_2, a_3 the linear coefficients, a_{11}, a_{22}, a_{33} the square coefficients, a_{12}, a_{13}, a_{23} the interaction coefficients, $X_1, X_2, X_3; X_1X_2, X_1X_3, X_2X_3\ et\ X_1^2, X_2^2, X_3^2$ are the levels of the independent variables, and finally being the error.

Validation of models

The performance of the model was measured by comparing the



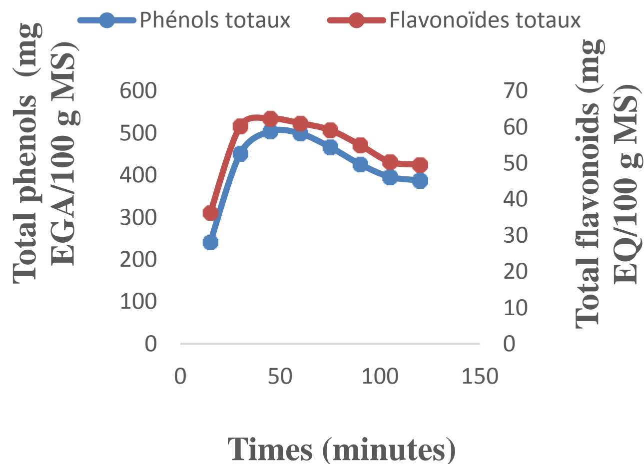


Figure 1. Encapsulation kinetics.
Source: Author

x values of the predicted responses with those observed. In addition to the linear regression coefficient (R^2), other mathematical procedures and tools were used; the Absolute Analysis of Average Deviation (AADM), the bias factor (Bf) and the Accuracy factors Af_1 (Catherine, 2015) and Af_2 (Baranyi et al., 1999) were determined using the following expressions:

$$\text{AADM} = \frac{\sum_{i=1}^{p}\left(\frac{|Y_{iexp} - Y_{ical}|}{Y_{iexp}}\right)}{p}$$

with Y_{iexp} the experimental response and Y_{ical} the response calculated from the model for an experiment i; p being the total number of experiments. $Bf = 10^B$; Bias B is given by the relation:

$$B = \frac{1}{n}\sum log \left(\frac{Y_{théo}}{Y_{Obs}}\right).$$

$Af_1 = 10^{A_1}$, with A_1 and A_2 the accuracy which is determined according to the following relationships:

$$A_1 = \frac{1}{n}\sum_{i=1}^{n}\left|\log\left(\frac{Y_{théo}}{Y_{Obs}}\right)\right|.$$

Thus, a model is considered perfect if the bias factor and the accuracy factors are equal to the unit, and the AADM equal to zero:

$$Bf = Af_1 = Af_2 = 1 \; et \; AADM = 0$$

The graphical representations of the response surfaces of the postulated models were made using the STATGRAPHICS Centurion XV software.

Evaluation of the behaviour of the microcapsule powder

At the end of the encapsulation, a quantitative study of the powder obtained is made. The active ingredient present in microcapsules is characterized by several quantities.

Yield

The most common size of the powders obtained by coacervation is the encapsulation yield which is calculated according to the following formula:

$$\tau = \frac{m_P}{m_{GA} + m_G + m_{PA}} \times 100$$

Where m_P is the mass of the powder, m_{GA} is the mass of the gum arabic, m_G is the mass of the gelatin and m_{PA} is the mass of the active ingredient. It is also the yield of the formulation.

Encapsulation rate

The optimized encapsulation rate of the extract is the content of total phenols or total flavonoids encapsulated on the content of total phenols or total flavonoids introduced expressed as a percentage (%) according to the formula:

$$\tau = \frac{Q_{encapsulée}}{Q_{introduite}} \times 100.$$

RESULTS AND DISCUSSION

Study of the influence of factors on coacervation encapsulation

Effect of time on coacervation encapsulation

The influence of time on complex coacervation is shown in Figure 1. The first part of the curves corresponds to an increasing curve from time t = 0 min to time t = 45 min where the optimum is reached at 44.28 NTU of the turbidity of the mixture and the degree of encapsulation of the total phenols and flavonoids respectively at 503.88 mg EGA/100 g P and 62.32 mg EQ/100 g P with a concavity turned upwards at t = 30 min. In this phase, the

Table 2. Coefficients of kinetic models.

Responses	K_1	K_2	Pseudo kinetics of 2nd order			Gauss model		
			B_0	q_e	R^2	a	k	R^2
Tu	0.05	0.02	19.76	44.38	0.96	44.28	27.7	0.90
TF	0.004	0.002	240.7	503.88	0.97	503.88	106.4	0.93
TP	0.03	0.02	36.22	62.32	0.95	62.32	73.9	0.91

Source: Author

methanol extract of the bark from the trunk of the cashew is being encapsulated by polymers (gum arabic and gelatin). The second part corresponds to a decreasing curve at the end of t = 45 min to t = 120 min with an upward concavity at t = 75 min with a reduction in turbidity up to 20.62 NTU and the rate of encapsulation total phenols and flavonoids at 395.82 mg EGA/100 g P and 49.56 mg EQ/100 g P respectively. This phase corresponds to destruction of the microcapsules. The explanation that one could bring to these results is that at t = 45 min, the equilibrium of encapsulation of the active substance of the matrix and the polymers is reached, this is the isoelectric point. Beyond the point, the mixture mixing apparatus breaks the bonds between the polymers and there is degradation of the microcapsules which dissolve in the mixture. The study terminals chosen are [30; 60 min].

Kinetic modeling of encapsulation by coacervation

Two kinetic models allowed us to describe the rate of encapsulation:

1. Gauss model:

$$Y = a_0 \exp[-(\frac{t-t_0}{4*k})^2]$$

Y is the quantity encapsulated at time t and α_0 is the quantity encapsulated at time t_0.

2. Pseudo kinetics of 2nd order (Tsatsop et al., 2016): $\frac{dq_e}{dt} = k(q_e - qt)^2$ then $q_t = \frac{t}{k_1 + k_2 \times t}$ with $B_0 = \frac{1}{k_1}$ la initial rate of encapsulation, and $q_e = \frac{1}{k_2}$ the maximum amount encapsulated.

We notice that the pseudo kinetics of 2nd order adjust better because the curves contain an increasing part up to a maximum and an almost constant part for flavonoids and total phenols. As for the Gauss model the curve decreases sharply after their maximum for turbidity. This was justified by the values of the constants in Table 2.

Pseudo kinetics of 2nd order has well described the encapsulation with respect to its R^2 which are superior to those of the Gauss model. However, these two models are valid. So in kinetics of 2nd order, the first phase corresponds to the rapid approximation of molecules (k_1) to coacervation and the second corresponds to the slow approximation (k_2) to crosslinking. The adjustment curves of the Gauss models and kinetics order 2 (Figures 2 and 3) show that the rate of encapsulation and the turbidity of the mixture depend on the mixing time.

Effect of gum arabic solution on coacervation encapsulation

Figure 4 shows the turbidity of the mixture, total phenols and flavonoids as a function of the proportion of the gum arabic solution. The mixture is made with a variation of the proportions of the gum arabic solution and the time, are constants.

For the effect of gum arabic, we find that the curves reach their maximum at around 30% which is the equilibrium point then they decrease. The phenomenon of decrease is that the more the gum arabic is increased, the more the density of negative charges increases and the bonds between the polymers break from where there is degradation of the microcapsules: this is dilution. The limits set for the experiment are [20; 40%].

Effect of gelatin solution on coacervation encapsulation

One of the parameters studied during encapsulation is the effect of the proportion of the gelatin solution. This is done in different proportions and the time is kept constant. Figure 5 illustrates the evolution of the turbidity of the mixture and that of the encapsulated total phenols and flavonoids.

Increasing the amount of gelatin increases the turbidity of the mixture and the content of total phenols and total flavonoids. This could be explained by the gradient of encapsulation of the microcapsules between the polymer and the gelatin matrix which is high when the percentage used is large. The application bounds for the experiment plan adopted are [50; 70%]. Thus, the summary of the preliminary tests is presented in Table 3.

The specifications which will allow us to define a compromise zone are defined with turbidity greater than

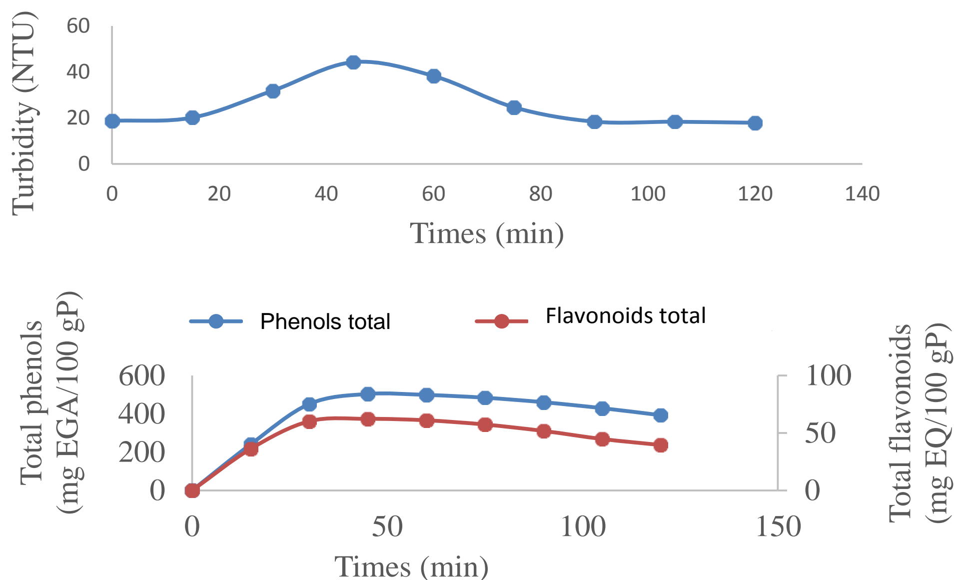


Figure 2. Gauss model.
Source: Author

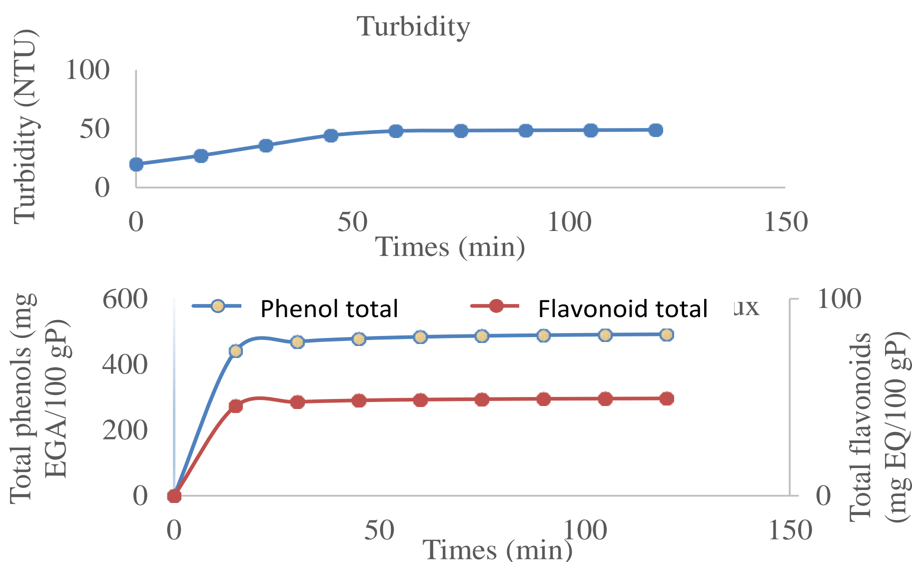


Figure 3. Kinetics model of second order.
Source: Author

or equal to the value of 44.28 NTU; the content of total phenols greater than or equal to 503.88 mg EGA/100 g P and the total flavonoid content greater than or equal to 62.32 mg EQ/100 g P.

The experiment plan

At the end of the experiment plan, the statistical analysis allowed us to have the validation indicators of the models designed in Table 4.

For a model to be validated, R^2 must be adjusted ≥ 80%; Bias factor and accuracy factor ϵ [0.75 and 1.25]; and 0 ≤ AADM ≤ 0.3. We have three models which are turbidity model (Y_T), total phenol model (Y_{TP}) and total flavonoid model (Y_{TF}). All three models are valid because their validation indicators are in the standards. The equations of these models are as follows:

Y_T (NTU) = $-74.4 + 0.9X_1 + 0.4X_2 + 4.2X_3 - 0.004X_1X_2 +$

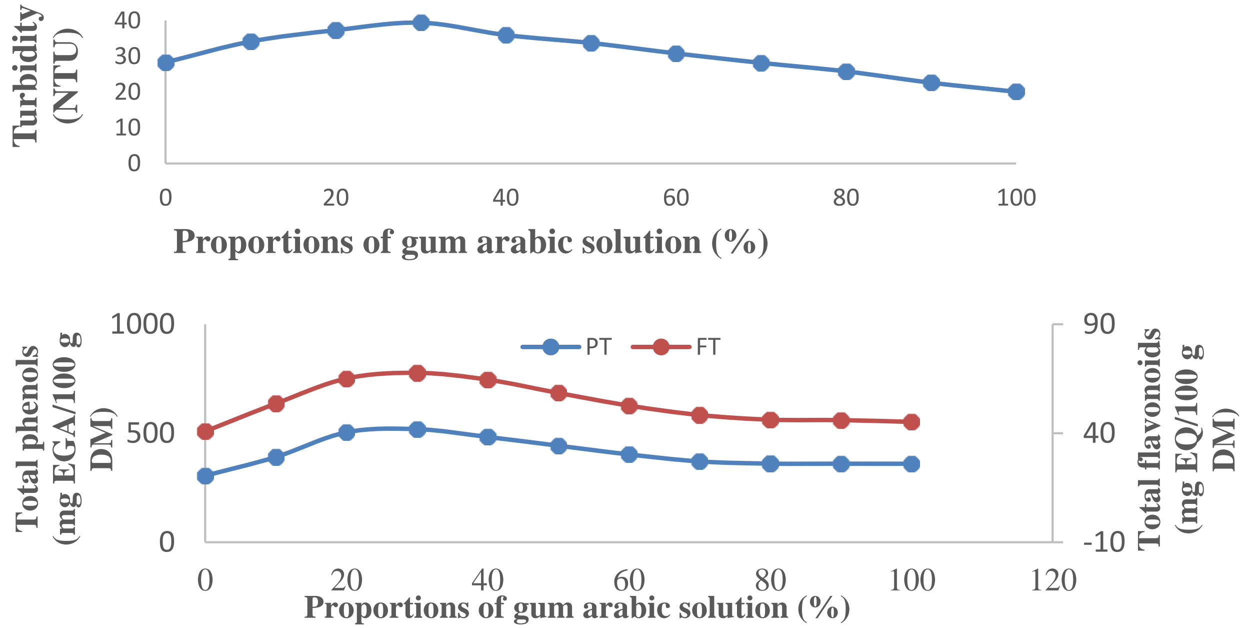


Figure 4. Effect of gum arabic solution on the mixture.
Source: Author

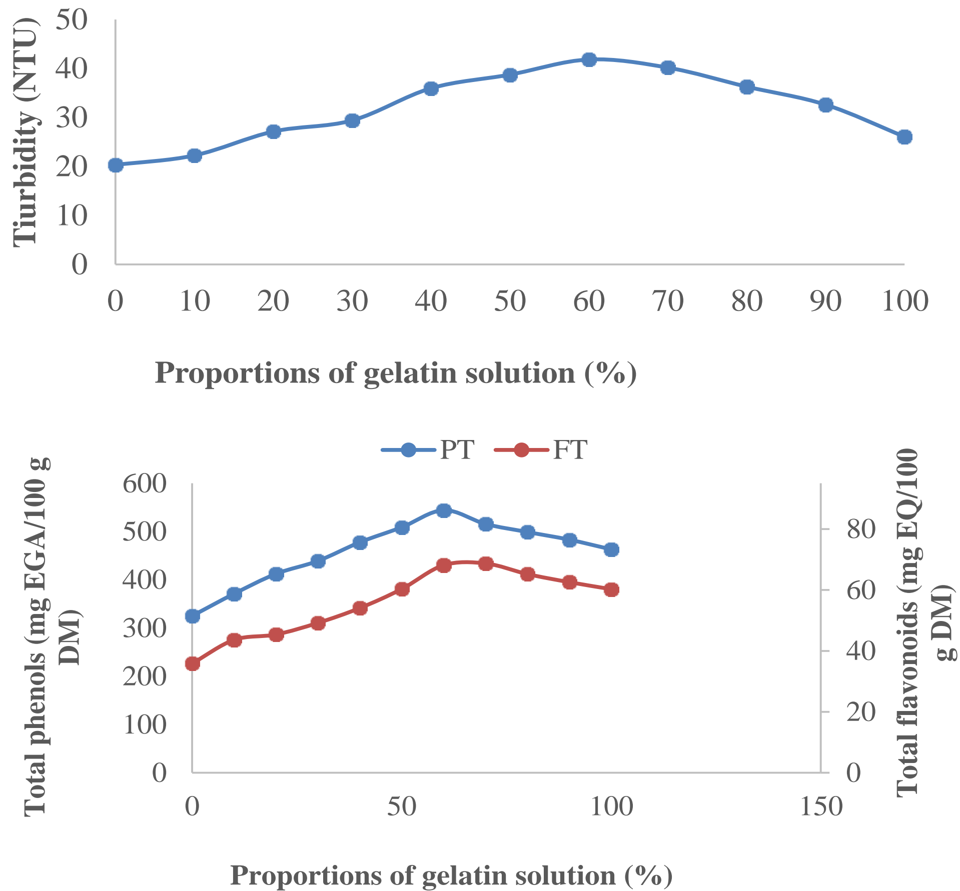


Figure 5. Effect of the gelatin solution on the mixture.
Source: Author

Table 3. Values of the low and high levels for the four factors chosen.

Factors	Low level	Center	High level
Times (s)	30	45	60
Gum arabic (%)	20	30	40
Gelatine (%)	50	60	70

Source: Author

Table 4. Validation of models.

Validation indicator	Model Y_T	Model Y_{TP}	Model Y_{TF}
R^2	99.52	99.63	98.14
Adjusted R^2	99.27	99.43	97.15
AADM	0.01	0.02	0.01
Bias factor	0.894	0.747	0.804
Accuracy factor	1.136	1.003	1.074

Source: Author

$0.004X_1X_3 - 0.01X_2X_3 - 0.01X_1^2 + 0.003X_2^2 - 0.04X_3^2$

Y_{TP} (mg EAG/100 gP) = $-763.5 + 11.7X_1 + 2.4X_2 + 46.6X_3 - 0.03X_1X_2 - 0.004X_1X_3 - 0.1X_2X_3 - 0.2X_1^2 + 0.04X_2^2 - 0.46X_3^2$

Y_{TF} (mg EQ/100 gP) = $-166.6 + 0.5X_1 + 2.4X_2 + 6.9X_3 - 0.002X_1X_2 + 0.01X_1X_3 - 0.03X_2X_3 - 0.02X_1^2 - 0.01X_2^2 - 0.1X_3^2$

The factor coefficients are shown in Table 5. All the factors X_1, X_2 and X_3 have a positive and significant influence on the encapsulation. While all interactions and quadratic effects have insignificant influence. Figure 6 presents the contribution of the factors of the models. It confirms that the direct effects contribute to the encapsulation and especially X_3 which contributes strongly, but its quadratic effect contributes negatively. This confirms that the longer the agitation takes place, the more the microcapsules are not broken by the shocks of the agitation.

Encapsulation optimization

The results of the optimization of the encapsulation by complex coacervation are reported in Table 6. The optimum values of responses such as turbidity, total phenols and total flavonoids were measured in two trials and then compared to the values calculated by the equations of the models found. Let's remember that the higher the values of turbidity, total phenols and total flavonoids, the greater the encapsulation yield and these are the best responses. At each optimal condition, for the responses, experimental tests were carried out and the results obtained are recorded in Table 6. The experimental results are almost similar to the calculated results, which is why the design of these experiments is validated.

In addition, a multi-response optimization was performed. Indeed, the mixture is obtained from the multi-response optimization of the microcapsules. The combination of the different factors is shown in Table 7. Under these conditions, we represent the combination of factors for optimizing the encapsulation of microcapsules by coacervation of the models of turbidity, total phenols and total flavonoids in Table 7.

Evaluation of the powder obtained

Formula yield obtained

The mixture is made according to the experimental plan, by adding in 2 L of distilled water, 40 g of gum arabic, 160 g of gelatin and 40 g of the active Principe (methanol extract from the bark of the trunk of the cashew). After lyophilization, we obtained 202.48 g of powder from the microcapsules. The yield is calculated according to the following formula:

$$\tau = \frac{m_P}{m_{GA} + m_G + m_{PA}} \times 100$$

m_P = mass of the powder, m_{GA}= mass of the gum arabic, m_G = mass of the gelatin and m_{PA} = mass of the active Principe.

So we have:

$$\tau = \frac{202,48}{40+160+40} \times 100 = 84,37\%.$$

The optimized extract encapsulation rate is the quantity of active compounds encapsulated over the quantity of

Table 5. Coefficients of the factors of the models.

Factor	$Y_{Turbidity}$	$Y_{Total\ phenols}$	$Y_{Total\ flavonoids}$
X_1	0.9	11.7	0.5
X_2	0.4	2.4	2.4
X_3	4.2	46.6	6.9
X_1X_2	-0.004	-0.03	-0.002
X_1X_3	0.004	-0.004	0.01
X_2X_3	-0.01	-0.1	-0.03
X_1^2	-0.01	-0.2	-0.02
X_2^2	0.003	0.04	-0.01
X_3^2	-0.04	-0.05	-0.1

X_1: Gum arabic; X_2: gelatin and X_3: mixing time.
Source: Author

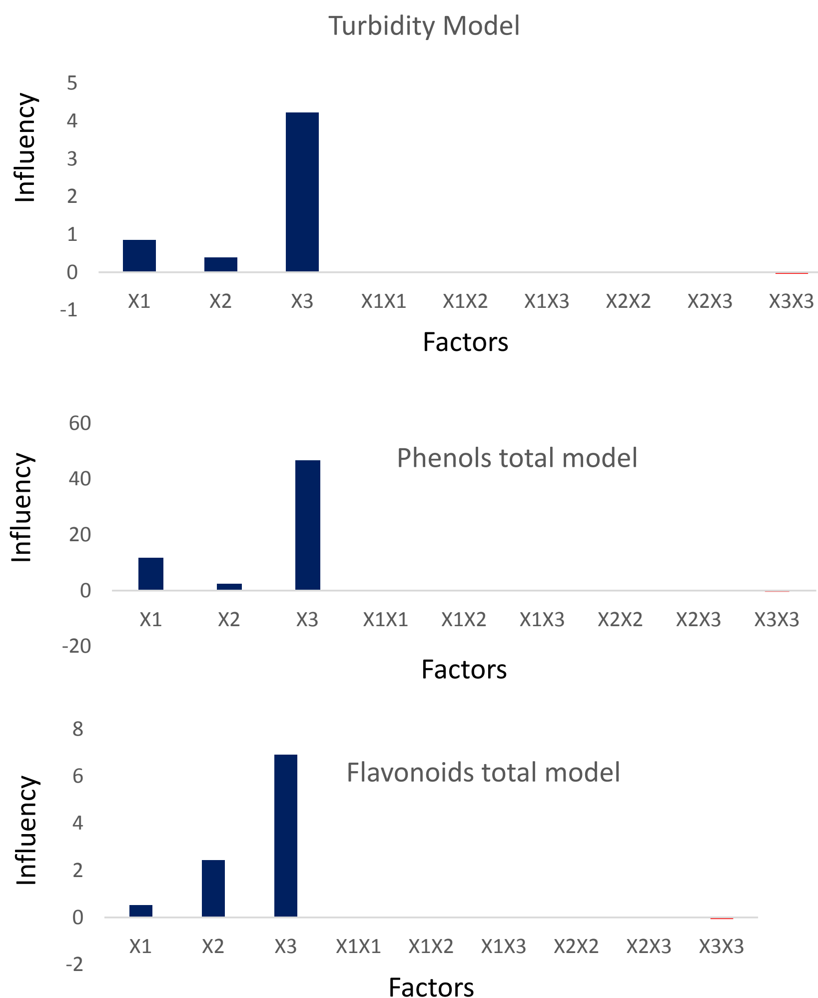


Figure 6. Contribution of model factors.
Source Author

active compounds introduced, all multiplied by 100, that is the content of total phenols and total flavonoids encapsulated on the content of total phenols and total flavonoids introduced expressed as a percentage (%)

Table 6. Optimization results of the encapsulation by complex.

Factor actual values		$Y_{Turbidity}$ (NTU)			Y_{PT} (mg EGA/100 g P)			Y_{FT} (mg EQ/100 g P)		
		Calculated	Test 1	Test 2	Calculated	Test 1	Test 2	Calculated	Test 1	Test 2
X_1 (%)	30									
X_2 (%)	70	42.61	42.84	43.15	492.94	503.88	506.13	62.32	61.50	64.93
X_3 (min)	45									

X_1: proportion of the gum arabic solution; X_2: proportion of the gelatin solution; X_3: mixing time; $Y_{Turbidity}$: turbidity response; Y_{PT}: response of total phenols and YFT: response of total flavonoids.
Source: Author

Table 7. Combination of factors for multi-response optimization of microcapsule encapsulation models.

Factor	Level		Turbidity optimum		PT Optimum		FT Optimum	
	Low	High	Coded value	Real value	Coded value	Real value	Coded value	Real value
X_1 (%)	20	40	0.999878	39.999	0.59284	35.9284	-0.99999	20.0001
X_2 (%)	60	80	-0.98351	60.1649	-1.0	60	0.49506	74.9506
X_3 (min)	30	60	1.0	60	1.0	60	1.0	60

X1: proportion of the gum arabic solution; X2: proportion of the gelatin solution and X3: mixing time. So for the implementation of our formula, we will take the following conditions: 40% of the gum arabic solution, 60% of the gelatin solution and the mixing time is 60 min.
Source: Author

Table 8. Comparison of the quantity of the encapsulated principle with that in the bark.

Extraction technique	Bark powder	Microcapsule powder obtained
Yield (%)	39.71	84.37
TF (mg EQ/100 g P)	262.11	505.01
TP (mg EGA/100 g P)	32.23	63.22

Source: Author

according to the formula:

$$\tau = \frac{Q_{encapsulée}}{Q_{introduite}} \times 100.$$

Then

$$\tau_{FT} = \frac{TF_{encapsulée}}{TF_{introduite}} \times 100 = \frac{49,25}{63,22} \times 100 = 77,90\%,$$

and

$$\tau_{PT} = \frac{TP_{encapsulée}}{TP_{introduite}} \times 100 = \frac{386,38}{505,01} \times 100 = 76.51\%.$$

These values are acceptable because the complex coacervation method encapsulates between 70 and 90%. It is the best chemical method of encapsulation. These results corroborate those of Annalisa et al. (2017).

So the amount of the active ingredient in the powder obtained and the bark powder of the plant used in this study can be compared. Table 8 gives the results of the comparison.

The results show that the powder obtained contains twice as much of the active principle as the powder of the bark of the trunk of the cashew. So the study microcapsule powder is rich and can be valued.

Conclusion

This study has shown and confirmed previous studies which attest that the rate of encapsulation of the active compounds depends on the proportion of gum arabic, the proportion of gelatin and also on the mixing time. The kinetics of encapsulation follows kinetics of order 2 which gives polynomial equations of the second degree. The best conditions for encapsulation by complex coacervation are as follows: 40% of the gum arabic solution, 60% of the gelatin solution and the mixing time is 60 min. They give the values of total phenols and total flavonoids of 689.82 mg EAG / 100 g DM and 88.64 mg EQ/100 g DM, respectively. The evaluation of the powder gives us a yield of the powder formulation of 84.37% and the encapsulation rate of 77.9% for the total flavonoids

and 76.5% for the total phenols. Finally, the powder obtained is twice as rich in active principle as the plant material used.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of temperature on lipid accumulation in three green microalgae species

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The objective of this work was to analyze the growth of *Scenedesmus acutus*, *Nannochloropsis oculata* and *Chlorella vulgaris* at different temperatures (25, 30 and 38°C) in order to identify changes in lipid content, accumulation of neutral lipids and fatty acids profile. According to the results obtained, the temperature of 30°C does not affect the growth of the microalgae *S. acutus*, *N. oculata* and *C. vulgaris*; however, there is a greater amount of total lipids in *S. acutus* at 38°C, while for *N. oculata* and *C. vulgaris*, temperature variation does not affect the accumulation of total lipids. From the data obtained from the fatty acid profile, we observed a greater accumulation of palmitic acid followed by oleic acid. According to the cetane number, any temperature condition of any evaluated microalgae culture can be used for biodiesel production. The results suggest that a change in temperature during the growth of microalgae could be applied to enhance lipid production and to obtain fatty acids suitable for biodiesel production.

Key words: *Chlorella vulgaris*, *Nannochloropsis oculata*, *Scenedesmus acutus*, fatty acids, lipids, temperature.

INTRODUCTION

Humanity confronts two problems in the energy area: The decrease in oil reserves and pollution caused by the burning of fossil fuels. Due to this, viable energy alternatives have been pursued to replace the use of oil, which must be renewable, sustainable, and come from environmentally friendly sources (Banerjee et al., 2018; Zhang et al., 2022). It has been estimated that the production of biodiesel is the only technology capable of substituting the consumption of fuels derived from petroleum (Chisti and Yan, 2011). In addition, it offers several environmental advantages such as the reduction of greenhouse gas emissions up to 70 to 90% compared to conventional diesel (Ajikumar et al., 2008; Timilsina and Mevel, 2013).

Biodiesel is a liquid biofuel composed of alkyl esters of short-chain alcohols, such as ethanol and methanol, with long-chain fatty acids obtained from renewable biomass, like vegetable oils, animal fats and microalgae oils (Robles-Medina et al., 2009). Among the main advantages of biodiesel is that during its combustion it produces fewer harmful emissions of sulfides, aromatic hydrocarbons and soot particles (Balat and Balat, 2010),

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it also has lubricating properties that reduce engine wear, and it is a safe product for transport and handling due to its high flash point (150°C) and low volatility (Demirbas, 2009).

Microalgae-based biofuels can be used as a substitute for petroleum fuels. The biofuels derived microalgae can be processed by using thermochemical and biochemical conversion. After the oil extraction from algal cells, the lipids can be converted by a chemically transesterified into biodiesel. Other microalgae biofuels such as bioethanol and biomethane can be produced by fermentation of the biomass under anaerobic conditions. Microalgae also have potential in the production of gaseous biofuel such as biohydrogen (Zhang et al., 2022; Banerjee et al., 2018).

The use of microalgae for biodiesel production is an advantageous alternative due to their lipid content, ability to assimilate CO_2 as a carbon source and their rapid biomass generation compared to plants (Sharma et al., 2012). Lipids in microalgae are composed of polar and neutral lipids. Neutral lipids are composed of triacylglycerides (TAGs), which, once extracted, can be easily converted into biodiesel through transesterification reactions (Sharma et al., 2012; Sato et al., 2014). Lipid production and accumulation in microalgae depends mainly on the species and its genetic constitution, but it is also affected by various physical and chemical conditions of the culture, such as: Growth phase, nutrient availability, salinity, periods of light, intensity, temperature, and pH. Under these abiotic stresses, the microalgae constantly adjust their cellular mechanisms to cope with them. The accumulation of stress metabolites, such as lipids, is directly related to the changes occurring in their metabolic pathways; under these conditions, the yields are higher because microalgae accumulate more neutral lipids as a mechanism to protect cells against oxidative damage (Paliwal et al., 2017; Poh et al., 2020).

It has been established that an increase in temperature has a positive effect on photosynthesis, cell division, lipid production, and fatty acids formation. High temperature promotes the uptake and fixation of CO_2 by microalgae; however, extremely high temperature inhibits the respiratory metabolism of microalgae (Tripathi et al., 2002; Barten et al., 2021). Due to heat stress, highly thermal sensitive enzymes which control the synthesis and accumulation of lipids may be affected; in addition, proteins involved in photosynthetic processes may be modified (Converti et al., 2009; Xin et al., 2011; Ras et al., 2013).

To the researchers' knowledge, there have been no studies reported about the comparison of these 3 species of microalgae (*S. acutus*, *N. oculata* and *C. vulgaris*) at the temperatures of 25, 30 and 38°C and it effects in the cell growth, total and neutral lipid production, including the prediction of cetane number based on its fatty acid composition. Therefore, in the present study, the association between the growth of 3 species of microalgae at different temperatures and the accumulation of lipids and its effect on the fatty acid profile was probed. The cetane number prediction was also assessed.

MATERIALS AND METHODS

Microorganisms, culture media and aeration system

The microalgae *Scenedesmus acutus* UTEX 72, *Nannochloropsis oculata* CCAP 849/7 and *Chlorella vulgaris* OW-01 were obtained from the Autonomous University of Aguascalientes. The identification of these strains was performed via 18S rRNA gene amplification followed by sequencing. Cells were maintained in Bold Basal Medium (BBM) agar plates at 25°C under a photoperiod of 16/8 h light-dark. A single colony was picked-up to the sterilized test tube containing 5 mL of growth medium, the scaling up was made in BBM on a rotary shaker at 120 rpm and used as pre-inoculum for each microalga specie. Carbon supply, in the form of CO_2, was provided by an aeration system (air diffusion) that was designed based on transparent 2 mm radius flexible PVC hoses connected to an air pump of 2.2 $L.min^{-1}$. The air flow in the nine experiments was controlled by flow regulating valves of 1⁄4 in.

Strains growth at different temperatures and growth kinetics

The pre-inoculum was maintained at a temperature of 25°C with continuous stirring and photoperiods of 16 h light / 8 h dark until an optical density (OD) of 0.2 was reached at a wavelength of 750 nm. The readings were recorded using the GloMax®-Multi Microplate Reader spectrometer (Promega Corporation, Madison, WI, USA).

Once the desired OD was reached, nine 1000 mL flasks containing 500 mL each of BBM were inoculated. The cultures (by triplicate) were maintained with photoperiod and aeration, the latter to supply CO_2 as a carbon source and maintain the system homogeneous. Cultures were grown at three different temperatures: 25, 30 and 38 °C. Every 24 h, 2 samples of 1 mL were taken until the cultures reached stationary phase, which were used for neutral lipid analysis, optical density recording to monitor growth and to perform cell counting. Upon reaching stationary phase, the biomasses were recovered by centrifugation at 2,000 x g for 20 min and stored at -20°C for use in lipid quantification. Each experiment consisted of triplicate flasks.

Cell counting in Neubauer chamber

A 0.1 mm deep hematocytometer with Neubauer's ruler was used for cell counting as described by Galarza et al. (2019).

Quantitative identification of total and neutral lipids

The final samples obtained at the end of the kinetics were processed based on previously described protocols (Folch et al., 1957; Bligh and Dyer, 1959) to quantify total lipids. For the analysis of neutral lipid content, the samples obtained during the growth kinetics were analyzed by triplicate as described before (Bertozzini et al., 2011).

Identification of the fatty acid profile

The free fatty acid profile was carried out using a methodology

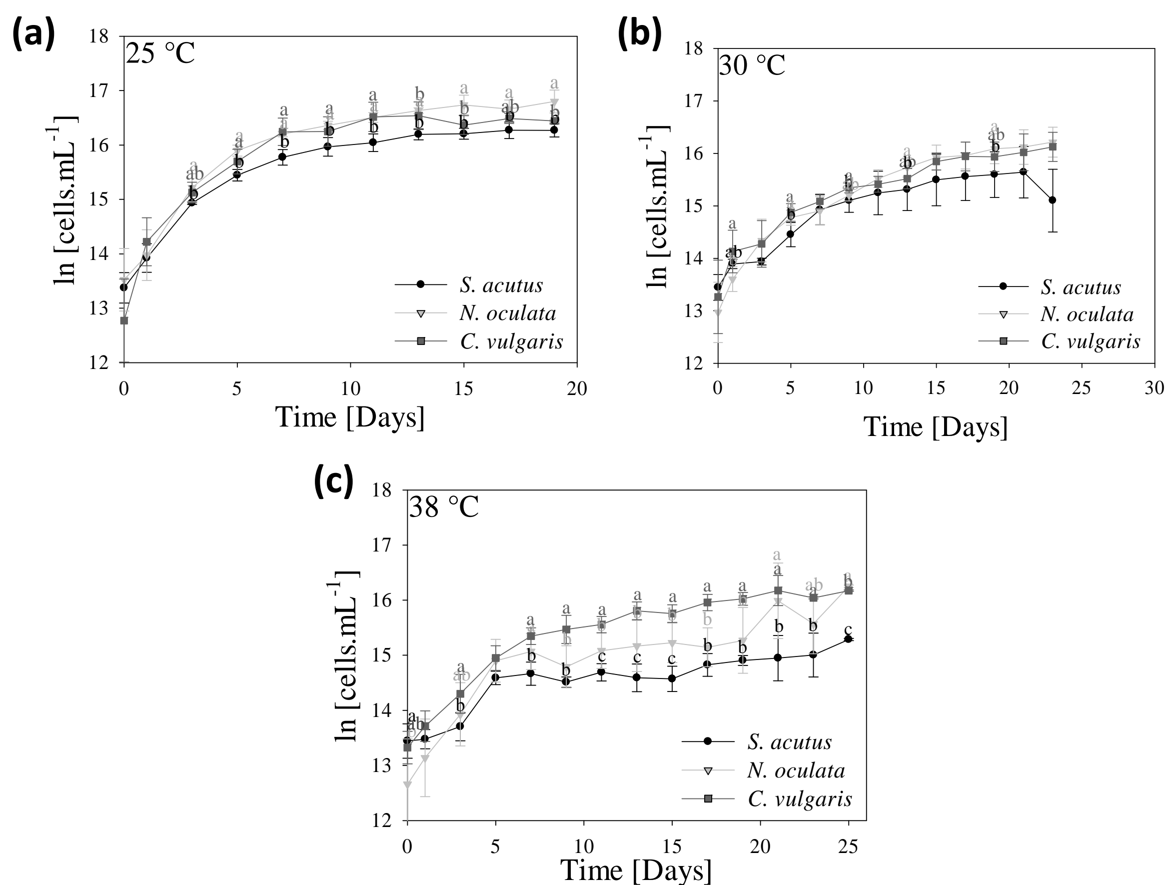

Figure 1. Growth kinetics in natural logarithm of number of cells per milliliter for *N. oculata*, *S. acutus* and *C. vulgaris* strains incubated at (a) 25°C, (b) 30°C and (c) 38°C. Points not sharing a letter are statistically different (ANOVA p<0.05, Fisher's test). Points containing no letter indicate that their difference is not statistically significant among the three strains.
Source: Authors

described by Flores Ruedas et al. (2020) using an Agilent 7890 gas chromatograph (Agilent Technologies, Sta. Clara Cal., USA), which is equipped with a flame ionization detector (GC-FID). The analysis was made in duplicate and the mean and standard deviation were reported. An analysis of variance (ANOVA, $p < 0.05$), using Fisher's test, was made to detect differences between the fatty acids obtained by the different strains.

Cetane number prediction

The cetane number (CN) was evaluate with the model as described by Piloto-Rodríguez et al. (2013) using the fatty acid profiles described previously.

RESULTS AND DISCUSSION

Strains growth kinetics and the specific growth rate

To determine the effect of temperature on the growth of the microalgae *S. acutus*, *N. oculata* and *C. vulgaris* growth kinetics were obtained. As can be seen in Figure 1a and b, the microalga *N. oculata* exhibited apparently higher growth at 2 temperature conditions (25 and 30°C), followed by *C. vulgaris* and *S. acutus*, while for the third condition (38°C) the microalga *C. vulgaris* showed the highest growth (Figure 1c).

The specific growth rate μ [d^{-1}] was calculated and a comparison was made between the three strains. According to Table 1, there is no significant difference between the three microalgae subjected to the three temperature conditions, and when the comparison was made by species at different temperatures, the same result was found, indicating that this temperature range does not affect the growth of these microalgae.

Staehr and Birkeland (2006) reported for *S. acutus* at 25°C a value of μ=0.57 d^{-1}, this difference may be due to the growth conditions used by these authors, who included the use of a modified O_2 medium in a final volume of 2 L, and an evaluation period of growth of 14 days. The photoperiod evaluated by them was the same

Table 1. Specific growth rate μ [d^{-1}] for the 3 strains subjected at different temperatures.

Temperature [°C]	*S. acutus*, μ [d^{-1}]	*N. oculata*, μ [d^{-1}]	*C. vulgaris*, μ [d^{-1}]
25	0.11± 0.02[a]	0.14±0.03[a]	0.12±0.03[a]
30	0.12±0.06[a]	0.14 ±0.08[a]	0.14±0.06[a]
38	0.07±0.06[a]	0.13 ±0.05[a]	0.14±0.06[a]

The means that do not share a letter in the same row are statistically different (ANOVA p<0.05, Fisher's test, n=3).
Source: Authors

Table 2. Percentage of total lipids [g lipids/ g biomass] in the 3 strains evaluated at different temperatures.

Temperature (°C)	*S. acutus*	*N. oculata*	*C. vulgaris*
25	2.52% ± 1.58[b]	7.46%± 7.24[a]	5.44% ± 3.58[a]
30	16.89% ± 7.37[ab]	4.50% ± 2.05[a]	11.21% ± 5.37[a]
38	21.72% ± 14.41[a]	15.68% ± 14.57[a]	4.10% ± 1.92[a]

The means that do not share a letter in the same column are statistically different (ANOVA p<0.05, Fisher's test, n=3).
Source: Authors

as the used in this work (16 h of light and 8 h of darkness). Wei et al. (2015) reported for *N. oculata* at 25, 30 and 35°C, similar values of μ; the conditions under which they obtained these values included the use of 60 L photobioreactors with f/2 medium for a growth period of 10 days, after which they make the total lipids extraction.

In the same way, Converti et al. (2009) reported for *C. vulgaris* at 25 and 30°C similar values of μ as the ones found in this work, while for *N. oculata* at 25°C the value of μ=0.07 was two times lower than that reported by us. The growth conditions reported involved the use of BBM for the culture of *C. vulgaris* and f/2 medium for *N. oculata* during a 14-day culture period, followed by ultrasonication and Folch's method for lipid extraction.

Extraction of total lipids

According to Table 2, *S. acutus* accumulates the highest amount of lipids (21.72%) at the temperature of 38°C. At the same temperature, *N. oculata* accumulate 15.68%, although statistically there is no significant difference (p<0.05) with respect to the other two temperature conditions. On the other hand, *C. vulgaris* at 30°C accumulates 11.21% of lipid, however, there is no significant difference statistically with the data obtained at the other two temperatures.

El-sheekh et al. (2017) reported for *S. acutus,* at temperature of 25°C, a percentage of total lipids 3.82 times higher than that reported in this work and at 30°C only 1.78 times lower. Different factors affect the lipid accumulation, one of the main is the culture media, and other variable is the duration of the photoperiod. El-sheekh et al. (2017) conducted their experiments for 14 h light and 10 h dark, this change can cause a greater accumulation of lipids at 25°C since it increases the time in which the microalgae are photosynthetically active. Likewise, there are differences in the lipid extraction procedure which can cause differences in the content of total lipids of the microalgae. Their experiments also included the use of KC culture medium for 22 days.

Converti et al. (2009) described for *N. oculata* a percentage of total lipids of 13.89% at 25°C which is 1.86 times higher than that reported in this work; at the same temperature, for *C. vulgaris,* they reported a 14.71% of total lipids while at 30°C it was only 5.9%, which is 1.90 times lower than that reported by us. They also quantified the lipids at 38°C, obtaining a 11.3% which is 2.76 times higher in relation to this work

Neutral lipids content

The neutral lipids present in the microalgae cultures were quantified by a fluorometric method described as described before (Bertozzini et al., 2011) and the results are presented in Table 3. With the data obtained, we identified the day in which the highest content of neutral lipids was accumulated. At 25°C, among the three microalgae, *S. acutus* presents the highest accumulation of neutral lipids on day 19 (3.02 μg/mL), however, it does not show significant difference with respect to the lipids accumulated on days 23 and 21. At 30°C, *S. acutus* was the strain that accumulated the most neutral lipids, with

Table 3. Neutral lipid content in the 3 strains evaluated at different temperatures.

Day	25°C			30°C			38°C		
	S. acutus (µg/mL)	*N. oculata* (µg/mL)	*C. vulgaris* (µg/mL)	*S. acutus* (µg/mL)	*N. oculata* (µg/mL)	*C. vulgaris* (µg/mL)	*S. acutus* (µg/mL)	*N. oculata* (µg/mL)	*C. vulgaris* (µg/mL)
0	2.28^{f} ± 0.07	2.34^{g} ± 0.10	2.53^{d} ± 0.10	2.26^{f} ± 0.04	2.37^{abc} ± 0.07	2.47^{def} ± 0.14	2.27^{bc} ± 0.05	2.21^{fg} ± 0.05	2.23^{bcd} ± 0.04
1	2.29^{f} ± 0.08	2.55^{bcde} ± 0.15	2.33^{b} ± 0.01	2.34^{ef} ± 0.06	2.20^{fg} ± 0.07	2.68^{ab} ± 0.10	2.31^{abc} ± 0.06	2.19^{g} ± 0.03	2.22^{bcd} ± 0.05
3	2.41^{ef} ± 0.11	2.34^{g} ± 0.06	2.44^{bcd} ± 0.12	2.33^{ef} ± 0.05	2.28^{cdef} ± 0.06	2.28^{g} ± 0.11	2.40^{a} ± 0.04	2.20^{fg} ± 0.04	2.16^{d} ± 0.09
5	2.45^{e} ± 0.06	2.36^{fg} ± 0.06	2.37^{cd} ± 0.09	2.51^{cd} ± 0.06	2.16^{g} ± 0.05	2.28^{g} ± 0.07	2.24^{c} ± 0.23	2.21^{fg} ± 0.05	2.15^{d} ± 0.05
7	2.57^{cde} ± 0.02	2.45^{efg} ±0.08	2.36^{cd} ± 0.10	2.59^{bc} ± 0.13	2.20^{efg} ± 0.05	2.52^{cdef} ± 0.04	2.22^{c} ± 0.07	2.18^{g} ± 0.03	2.17^{d} ± 0.08
9	2.52^{de} ± 0.03	2.43^{efg} ± 0.08	2.66^{a} ± 0.12	2.63^{bc} ± 0.09	2.43^{ab} ± 0.07	2.41^{f} ± 0.04	2.37^{ab} ± 0.07	2.44^{a} ± 0.09	2.22^{cd} ± 0.04
11	2.73^{b} ± 0.27	2.52^{cde} ± 0.08	2.62^{a} ± 0.08	2.33^{ef} ± 0.10	2.25^{defg} ± 0.05	2.42^{ef} ± 0.08	2.29^{abc} ± 0.05	2.19^{g} ± 0.04	2.28^{abc} ± 0.05
13	2.63^{bcd} ± 0.18	2.43^{efg} ± 0.09	2.46^{bcd} ± 0.06	2.29^{ef} ± 0.06	2.32^{bcd} ± 0.15	2.41^{f} ± 0.02	2.27^{bc} ± 0.09	2.36^{b} ± 0.05	2.31^{abc} ± 0.08
15	2.67^{bcd} ± 0.11	2.47^{defg} ± 0.06	2.53^{ab} ± 0.09	2.40^{de} ± 0.20	2.33^{bcd} ± 0.15	2.55^{cd} ± 0.05	2.31^{abc} ± 0.06	2.23^{efg} ± 0.04	2.38^{a} ± 0.11
17	2.69^{bc} ± 0.03	2.43^{efg} ± 0.07	2.54^{ab} ± 0.16	2.71^{b} ± 0.13	2.29^{cdef} ± 0.03	2.54^{cd} ± 0.03	2.34^{abc} ± 0.08	2.33^{bc} ± 0.02	2.32^{ab} ± 0.09
19	3.02^{a} ± 0.17	2.65^{bc} ± 0.17	2.47^{bc} ± 0.15	2.40^{de} ± 0.11	2.31^{cde} ± 0.04	2.58^{bc} ± 0.07	2.25^{bc} ± 0.08	2.26^{def} ± 0.03	2.39^{a} ± 0.12
21	2.94^{a} ± 0.11	2.60^{bcd} ± 0.15	NR	2.84^{a} ± 0.13	2.34^{bcd} ± 0.07	2.73^{a} ± 0.18	2.25^{bc} ± 0.14	2.30^{bcd} ± 0.07	2.33^{ab} ± 0.03
23	2.98^{a} ± 0.08	2.51^{bcdef} ± 0.10	NR	2.32^{ef} ± 0.03	2.30^{cdef} ± 0.11	2.76^{a} ± 0.10	NR	2.29^{cde} ± 0.03	2.36^{a} ± 0.04
25	NR	2.68^{b} ± 0.19	NR	NR	2.33^{bcd} ± 0.12	2.53^{cde} ± 0.04	NR	NR	2.40^{a} ± 0.15
27	NR	3.02^{a} ± 0.09	NR	NR	2.37^{abc} ± 0.11	2.58^{bc} ± 0.04	NR	NR	NR
29	NR	NR	NR	NR	2.46^{a} ± 0.07	2.45^{def} ± 0.11	NR	NR	NR

Not Recorded (NR). The means that do not share a letter in the same column are statistically different (ANOVA $p<0.05$, Fisher's test. n=3).
Source: Authors

2.84 µg/mL on day 21, being the highest amount recorded among the three microalgae. For a temperature of 38°C, *N. oculata* reached a concentration of 2.44 µg/mL on day nine, which was the highest among the three microalgae studied.

Fatty acid profiles

Fatty acid profiles were made to identify the fatty acids (FA) produced under each temperature condition for each microalga strain. It has been reported that the FA composition affects the quantity and quality of the synthesized biodiesel since the longer and more saturated the fatty acid carbon chains are, the higher the number of cetanes (CN), which is related to the ignition delay time and combustion quality (Piloto-Rodríguez et al., 2013). The FA were classified into four categories: saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and Others (which included the fatty acids that weren't identified) (Figure 2).

SFA are the most abundant in the 3 microalgae evaluated at different temperatures, followed by MUFA and finally PUFA. Figure 3a shows that C16:0, C18:0 and C18:1 are the fatty acids that accumulate in greater amount in the 3 strains of microalgae incubated at 25°C; in particular, for *N. oculata,* the fatty acid with the highest percentage was C18:1, followed by C18:0 and C16:1. On the other hand, for *C. vulgaris* the following distribution was found: C16:0, C18:0 and C14:0, this indicates a different fatty acid distribution depending on the microalgae strain even when grown at the same temperature.

At 30°C, is observed that C16:0 is still the highest percentage found, followed by C18:0, and

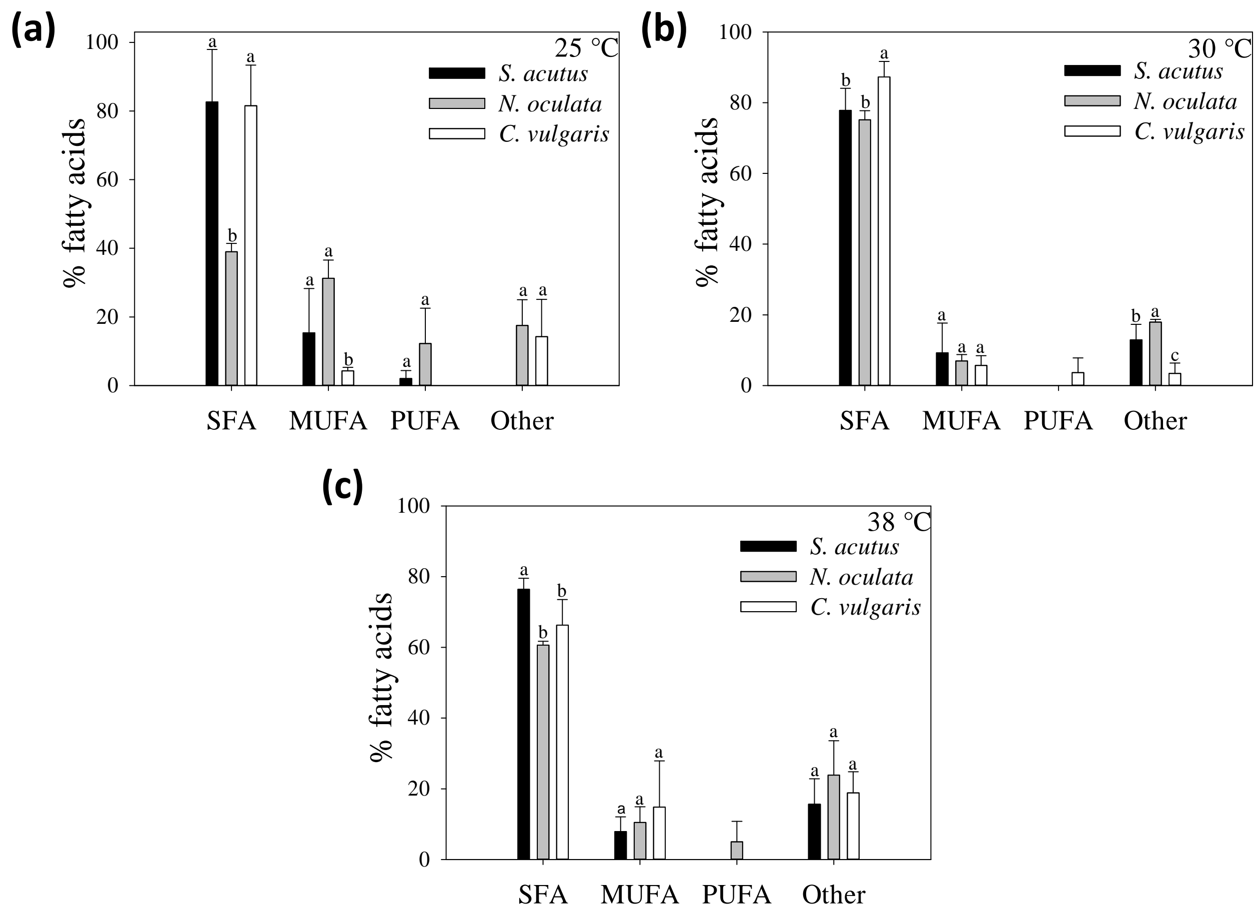

Figure 2. Classification of the fatty acid composition of the microalgae *N. oculata*, *S. acutus* and *C. vulgaris* incubated at (a) 25°C, (b) 30°C and (**c)** 38°C, into 4 categories. SFA, MUFA, PUFA, Other (fatty acids not identified in the chromatogram). Grouped bars not sharing a letter are statistically different (ANOVA p<0.05, Fisher's test).
Source: Authors

is observed that C16:1 begins to accumulate (Figure 3b), a decrease in the percentage of C18:1, C18:2 and C18:3 was also observed. Figure 3c shows, at 38°C, a decrease in C16:0 and C18:1 for *N. oculata* and *C. vulgaris* with respect to the temperature of 25°C. At this temperature, the main FAs present in a greater proportion in the 3 microalgae are C16:0, C18:0 and C16:1.

El-sheekh et al. (2017) reported for *S. acutus* much lower levels of SFA compared to those obtained in this work at temperatures of 25, 30 and 38°C. A similar study was carried out with *N. oculata* (Wei et al., 2015), in which the authors reported higher values of SFA compared to those obtained in this study at 25, 30 and 35°C.

For *C. vulgaris* at 25°C, Converti et al. (2009) reported SFA values of 71.00 %, which is 1.15 times lower as reported in this work, but their values of MUFA and PUFA were higher. Similar behavior was observed for SFA at 38°C.

Cetane number

Cetane number, which indicates ignition delay characteristics, is considered the most significant fuel property of biodiesel (Lin and Wu, 2022). Using the model for the prediction of the cetane number (CN) and the fatty acid percentage data obtained from the fatty acid profile (Figure 3), the CN corresponding to each of the nine experiments was obtained. The results are reported in Table 4, where the data obtained were compared with the CN required by the ASTM D6751 standard of the United States of America and the EN 14214 of Europe, giving as a result that the three different temperatures evaluated in *S. acutus*, *N. oculata* and *C. vulgaris* are in premise candidates for obtain biodiesel because their CN is major that the minimum required in both international standards. Accordingly, it is proposed that efforts should be focused on evaluating the feasibility of using *C. vulgaris* at 30°C, *S. acutus* at 25°C, 30°C or 38°C, and *C.*

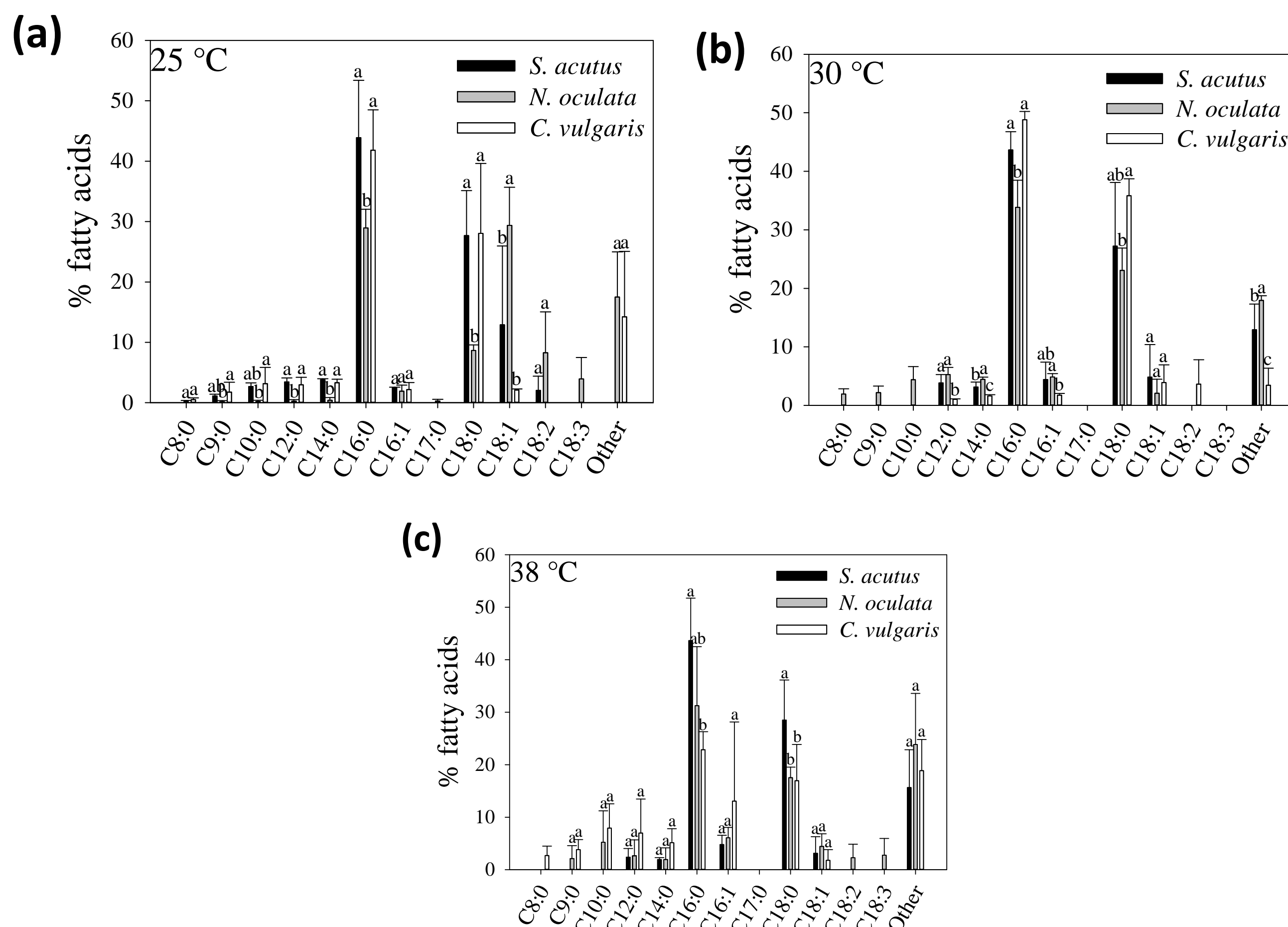

Figure 3. Fatty acid profile for *N. oculata*, *S. acutus* and *C. vulgaris* strains incubated at (a) 25°C, (b) 30°C and (**c)** 38°C. Grouped bars not sharing a letter are statistically different (ANOVA p<0.05, Fisher's test)**.**
Source: Authors

Table 4. Cetane number of the microalgae *S. acutus*, *N. oculata* and *C. vulgaris* evaluated at different temperatures.

Microalgae	Experimental temperature (°C)		
	25	30	38
S. acutus	68.84^{a} ± 3.91	67.90^{ab} ± 2.04	67.72^{ab} ± 0.74
N. oculata	57.02^{e} ± 0.90	65.26^{bc} ± 1.57	61.15^{d} ± 1.05
C. vulgaris	67.83^{ab} ± 4.66	70.72^{a} ± 1.51	61.90^{cd} ± 1.28

Different letters in column show significant differences (ANOVA p<0.05, Fisher's test).
Source: Authors

vulgaris at 25°C as possible platforms for biodiesel production.

Conclusion

This study compares the effect of different temperatures (25, 30 and 38°C) on the growth, lipid accumulation and fatty acid profile in 3 species of microalgae. The data obtained for the specific rates indicate that the growth of microalgae is not affected by the temperatures of 30 and 38°C. *S. acutus* accumulates a higher amount of total lipids at 38°C. *N. oculata* and *C. vulgaris* show no significant differences in total lipid accumulation at the

different temperatures analyzed in this study. Of the total lipids extracted, there is a greater accumulation of palmitic acid (C16:0) followed by oleic acid (C18:1) for the 3 microalgae, which are be suitable for biodiesel production. These results suggest that a change in temperature during the growth of microalgae could be applied to enhance lipid production and to obtain fatty acids suitable for biodiesel production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Screening for phosphate deficiency tolerance and expression of phosphate uptake genes in Nigerian local rice landraces

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Twenty-one Nigerian rice landraces were screened for tolerance to phosphate deficiency in nutrient medium using selected morphological indices from which Phosphate Deficiency Tolerance Index (PDTI) was evaluated. All landraces were analyzed for the presence of four *phosphate uptake 1* (*Pup-1*) gene-linked markers while relative expression levels of two *Pup-1* genes were evaluated in selected landraces under zero, normal and excess phosphate. PDTI analysis grouped the landraces into negative, low positive or high positive PDTI categories depending on whether performance at zero P was better than normal P, comparable to normal P, or less than normal P, respectively. However, irrespective of PDTI grouping, each landrace had at least one *Pup-1* gene marker. Under zero P, *Phillipine* landrace showed no superior expression of *OsPupK05-1* and *OsPupK04-1* genes despite belonging to negative PDTI group. Thus, PDTI-based characterization of the landraces was not completely consonant with the presence or expression levels of the *Pup-1* genes, suggesting a possible influence of other P deficiency tolerant genes. However, with a combination of negative PDTI, superior performance in root and shoot traits under zero P, and possession of at least 3 *Pup 1* genes, *Dantala Mass*, *Ankulyan*, and *Variety 44* may be regarded as P tolerant landraces.

Key words: *Oryza sativa*, phosphate deficiency tolerance index, RT-PCR, *Pup* genes.

INTRODUCTION

Phosphorus (P) is one of the essential macronutrients required for plant growth. Its deficiency in soil affects about 50% of rice-cultivated areas worldwide (Pariasca-Tanaka et al., 2014). Together with drought and salinity, they constitute the major abiotic stresses confronting rice production in the growing areas (GRiSP, 2013; Uyoh et al., 2019; Umego et al., 2020). Phosphorus is usually added to the soil in the form of fertilizer for enhanced yield. However, many peasant farmers in sub-Saharan Africa including Nigeria cannot afford this extra cost. Concerns are also expressed that fertilizer usage is not sustainable (Cordell et al., 2009).

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Furthermore, if applied in excess it could be washed into the water bodies leading to environmental pollution and eutrophication. Thus, the use of cultivars with good yield in both P- deficient and P- rich soils will help ameliorate these problems and improve food security. One way of achieving this would be through marker-assisted introgression of Phosphorus Starvation Tolerance-1 gene (*PSTOL1*) in rice varieties with high yield potential but which perform poorly under phosphate deficient conditions (Pariasca-Tanaka et al., 2014). This protein kinase-encoding gene was identified within the *Phosphorus uptake 1* (*Pup-1*) QTL, located on chromosome 12 of rice (*Oryza sativa*) and has been reported to confer tolerance to phosphate deficiency by enhancing phosphate uptake (Gamuyao et al., 2012). However, genetic instability of the *Pup-1* loci was earlier reported by Heuer et al. (2009), who attributed it to the presence of transposons and truncated elements. This was later corroborated by Chin et al. (2011) when they found that many of the developed markers for these loci were not informative when tested in a wider range of rice germplasm. There is need to authenticate these reports using local rice varieties grown by peasant farmers in Nigeria. The present study was aimed at screening twenty-one local rice cultivars in Nigeria for phosphate deficiency tolerance using morphological indices and validating the result obtained with phosphate uptake 1 (*Pup1*)-linked markers. The levels of *Pup1* gene expression under normal, excess and phosphate-deficient (zero) conditions were also determined using reverse transcription-polymerase chain reaction (RT-PCR) to further ascertain the role of this gene in phosphate tolerance in rice.

MATERIALS AND METHODS

Morphological studies

Seed collection and germination

Twenty-one (21) rice land races were obtained from local rice farmers in Benue and Cross River States, Nigeria (Supplementary Table 1). The rice seeds were soaked in water for 24 h to break seed dormancy and thereafter incubated in Petri dishes containing wet filter paper for 7 days for germination.

Preparation of nutrient solution

The modified Hoagland's solution described by Hoagland and Arnon (1950) was used for the present study with slight modification. The main adjustment made was addition of dipotassium hydrogen phosphate (K_2HPO_4) in different concentrations in place of monopotassium dihydrogen phosphate (KH_2PO_4) (Supplementary Table 2).

Transplanting and treatment application

Three (3) seedlings per landrace were transplanted into 50 ml labeled Falcon tubes containing water and kept for two days for acclimatization. The Falcon tubes were wrapped with foil paper to protect them from direct sunlight. After two days, water was drained from the labeled Falcon tubes and replaced with the modified Hoagland solutions containing 0.00 mM, 0.4 mM and 0.8 mM K_2HPO_4 (zero phosphate, normal phosphate and excess phosphate, respectively). The experiment was a 21 X 3 factorial, laid out using the Completely Randomized Design. At 6 weeks of age, the seedlings were transplanted to more spacious Polyvinyl Chloride (PVC) pipes with the same treatment solutions continued (Supplementary Figure 1). In all cases, the treatment solution was changed every four days to prevent a build-up of ions released by the plants.

Data collection and analysis of morphological parameters

At ten weeks after planting, data were collected in triplicates on plant height, number of tillers, root length, shoot and root dry mass in all the 21 landraces at the 3 phosphate concentrations. The means from these were used in estimating Phosphorus Deficiency Tolerance Index (PDTI) or Phosphorus stress factor for each landrace in all the traits using the formula: Trait under sufficient Pi - Trait under deficient Pi / Trait under sufficient Pi, that is (Trait under Pi^N - trait under Pi^-/ trait under Pi^N) (Chankaew et al., 2019; Irfan et al., 2020).

Molecular studies

DNA extraction and amplification of Pup markers

DNA was extracted from the twenty-one rice landraces using modified cetyltrimethylammonium bromide (CTAB) method as described by Uyoh et al. (2019). Four (4) gene-based *Pup* markers selected from the improved set of *Pup-1* markers developed by Chin et al. (2011) based on stable and conserved protein-coded genes verified through gene expression and sequencing data, were used in this study. The reaction was constituted in a total of 50 µL made up of 40.75 µL distilled water, 2.5 µL 10X Ex Taq buffer (1X), 2.5 µL $MgCl_2$ (1.5 mM), 1 µL deoxynucleoside triphosphate (dNTPs) (200 µM), 1 µL forward primer (0.2 µM), 1 µL reverse primer (0.2 µM), 0.25 µL Ex Taq polymerase enzyme (1.25 units), and 1 µL DNA (100 ng/µL). The polymerase chain reaction (PCR) conditions were as follows: Initial denaturation at 95°C for 30 s, 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 55 s, extension at 68°C for 1 min, followed by a final extension at 72°C for 5 min and holding at 4°C. The amplified products were separated by electrophoresis in 1.3% agarose gel at 100 V for 30 min, stained with ethidium bromide and visualized under UV trans-illuminator.

RNA extraction and cDNA synthesis

To assess gene expression level and responsiveness to P, reverse transcriptase-PCR (RT-PCR) analyses were done using root and shoot from rice samples germinated from the same seed stock used previously for the morphological analyses. They were grown in Falcon tubes for 2 weeks with (Pi^N) or without P (Pi^-) as described previously. RNA was extracted from the root and shoot of selected samples (Phillipine, Ayange and Obasanjo) using the E.Z.N.A.® Total RNA kit (Omega Bio-tek), according to the manufacturer's protocol manual. The landraces were selected randomly from those that showed PCR amplicons for all genes tested. The final RNA was eluted with 40 µL diethyl pyrocarbonate (DEPC) water and stored at -80°C. cDNA was synthesized using LunaScript™ RT SuperMix kit (New England BioLabs Inc.) according to the manual instructions. 5 µL of the synthesized cDNA was used for PCR analysis to detect the base pairs of the phosphate uptake gene

using the primers *OsPupK04-1* and *OsPupK05*-1. Ubiquitin gene was used as internal control to check the quality of the cDNA using the primers OsUbq_F and OsUbq_R (Supplementary Table 3). The reaction was constituted in a total of 50 µL volume containing 5 µL of 10X Ex Taq buffer (1x), 1 µL dNTPs (200 µM), 1 µL of 10 µM forward primer (0.2 µM), 1 µL of 10 µM reverse primer (0.2 µM), 0.25 µL of Ex Taq polymerase enzyme (1.25 units), 5 µL of cDNA, and 36.75 µL of nuclease-free water. PCR amplification was performed as follows: Initial denaturation set at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 1 min, extension at 72°C for 1 min, final extension at 72°C for 7 min. After amplification, 10 µL of the PCR products were resolved on 1% agarose gel stained with ethidium bromide.\

RESULTS

Morphological studies and phosphate deficiency tolerance indices (PDTI)

The mean responses of the 21 rice landraces in terms of shoot length, tiller number, root length, wet and dry biomass under Pi^+, Pi^N, Pi^- conditions, along with the calculated PDTI values are presented in Table 1. The mean shoot length ranged from 20.3 cm in Togo to 51.33 cm in OC for those treated with Pi^N; it ranged from 19.5 cm in Togo to 49.43 cm in Election 3 for those given deficient P (Pi^-) and 17.71 cm in Variety 1.4 to 56.33 cm in Election 3 for those given excess P (Pi^+). The mean tiller number ranged from 2.00 in Variety 1.4 to 5.33 in Sedi 1, Election 2 and Dantala Mass under Pi^N; it ranged from 1.00 in variety 1.4 to 3.67 in Sedi1 and Achancha for those treated with Pi^-; and from 1.33 in Variety 1.4 to 5.03 in Sidi 3 for those given Pi^+. Wet biomass ranged from 0.76 g in Togo to 3.99 g in Election 3 for those treated with Pi^N; those treated with Pi^- gave a range from 0.69 g in variety 45 to 2.37 g in Election 3 and for those treated with Pi^+, it ranged from 0.46 g in Variety 1.4 to 2.91 g in Obasanjo. Dry biomass ranged from 0.25 g in Varieties 1.4 and 45 to 0.59 g in Ayange for those treated with Pi^N; it ranged from 0.22g in Variety 1.4 and Election 2 to 0.38 g in Variety 44 for those treated with Pi^-; for those treated with Pi^+ it ranged from 0.22 g in Togo to 0.49 g in Election 3. About 90% of the landraces showed increased root length in Pi^- condition compared with the values for Pi^N and Pi^+. Based on the mean PDTI values obtained, the 21 landraces can be seen to fall into 3 groups (Table 2): (a) those with negative PDTI values ranging from -0.29 to -2.18, indicating that their mean performance under Pi^- was better than that under Pi^N; (b) those with low positive values (≤ 1.50) signifying that their mean performance under Pi^N and Pi^- were quite similar and (c) those with high positive values (>1.5) indicating their mean performance under Pi^N was much higher than at Pi^-.

Validation of Pup-1 linked markers in the rice landraces

Four *Pup-1* linked markers were validated on the 21 rice landraces (Figure 1). The presence of any of the PCR products in a land race was taken as an indication that the land race possessed the *Pup-1* gene (Table 3). At least, one of the four *Pup-1* genes was confirmed to be present in each of the 21 landraces, but none of the genes was present in all samples.

Expression of Pup genes in the rice landraces

Expression of 2 phosphate uptake genes (*OsPupKO4-1 and OsPupKO5-1*) under zero (Pi-), normal (Pi^N) and excess (Pi^+) phosphate levels was tested in 3 out of the 21 rice land races using RT-PCR. The 3 land races were selected randomly from those that showed PCR products for all the 4 genes tested. Expression profiles of the 2 genes and the control (Ubiquitin gene) are given in Figure 2a, b and c. The highest expression of *OsPupKO5-1* gene was seen in the shoot of *Ayange* under normal and excess phosphate conditions (Figure 2aii). The gene was also strongly expressed in the root of this landrace under excess phosphate but poorly expressed under normal and zero phosphate levels (Figure 2ai). It was poorly expressed in the shoots of *Philippine* landrace but showed slightly better expression in the roots. For *Obasanjo* landrace, there was no expression of this gene in the shoots, but it was strongly expressed in the roots under low and normal phosphate levels.

DISCUSSION

The 21 rice landraces evaluated in this study revealed different responses under Pi^+, Pi^N and Pi^- conditions. Tiller number, wet and dry biomass of shoot and root were generally higher when the plants were raised under sufficient P than when raised under deficient P, except in a few (5) landraces. On the other hand, all the landraces except Togo showed increased root length to different levels under deficient P condition, an adaptation for increased nutrient uptake under stressful situations. Wang et al. (2015) reported that different crops show divergent morphological and physiological responses to low P availability, including specific traits of root morphology and root exudation which enhance their P-uptake capacity under low-P conditions. Nirubana et al. (2020) also observed a wide range of varied responses to P deficiency for all the traits they studied in the 30 rice genotypes from India. They reported increased mean performance of root length and enzyme activity under P-condition compared to P+ condition. Vejchasarn et al. (2016) observed an increase in root hair length and density and a reduction in tiller number and shoot length under low P availability in all the 15 rice accessions they studied.

The banding pattern of the four *Pup* markers used in the present study suggests that these genes, especially

Table 1. Mean morphological features and phosphate deficiency tolerance indices (PDTI) at 8 weeks after planting for 21 rice landraces treated with 3 phosphate concentrations.

Landrace	Phosphate level and PDTI	Shoot length (cm)	Root length (cm)	Number of tillers	Wet biomass (g)	Dry biomass (g)	Mean PDTI
Achancha	P^N	30.90	8.00	4.67	1.07	0.29	0.11
	P^-	29.83	8.83	3.67	0.75	0.26	
	P^+	30.67	6.33	4.00	0.93	0.28	
	PDTI	0.03	-0.1	0.21	0.30	0.10	
Sedi 1	P^N	41.50	7.17	5.33	2.77	0.31	0.15
	P^-	31.83	9.17	3.67	2.18	0.23	
	P^+	33.00	7.67	4.67	2.27	0.24	
	PDTI	0.23	-0.28	0.31	0.21	0.26	
Election 3	P^N	55.00	10.10	2.33	3.99	0.45	0.17
	P^-	49.43	12.60	1.67	2.37	0.30	
	P^+	56.33	9.00	1.33	2.60	0.48	
	PDTI	0.10	-0.25	0.28	0.41	0.33	
Ayange	P^N	44.87	7.30	2.67	3.52	0.59	0.17
	P^-	35.50	11.90	2.33	1.19	0.31	
	P^+	42.03	8.50	2.33	1.87	0.42	
	PDTI	0.21	-0.63	0.13	0.66	0.47	
Ankulyan`	P^N	28.70	8.43	3.33	1.51	0.33	-0.71
	P^-	32.77	11.63	3.50	1.54	0.37	
	P^+	26.67	7.0	2.33	1.34	0.28	
	PDTI	-0.14	-0.38	-0.05	-0.02	-0.12	
Faro15	P^N	47.30	6.90	2.67	3.59	0.52	0.15
	P^-	36.67	11.07	2.33	1.89	0.24	
	P^+	47.33	9.83	2.50	2.37	0.28	
	PDTI	0.22	-0.60	0.13	0.47	0.54	
OC	P^N	51.33	6.33	5.00	2.58	0.43	0.14
	P^-	43.17	10.70	3.33	1.12	0.29	
	P^+	47.33	9.83	5.33	2.37	0.29	
	PDTI	0.16	-0.69	0.33	0.57	0.33	
Zomuje	P^N	46.83	8.33	4.67	2.03	0.30	2.47
	P^-	44.17	11.17	3.00	1.24	0.24	
	P^+	47.83	7.33	5.00	2.35	0.25	
	PDTI	0.06	-0.34	0.36	0.39	0.20	
Dantala mass	P^N	39.00	4.70	3.33	2.03	0.27	-2.18
	P^-	46.83	10.53	5.33	2.23	0.28	
	P^+	44.83	5.00	4.33	2.05	0.33	
	PDTI	-0.20	-1.24	-0.6	-0.1	-0.04	
Bnarda Sipi	P^N	43.43	6.70	4.00	2.80	0.31	0.04
	P^-	38.67	10.67	3.33	1.83	0.26	
	P^+	45.33	6.97	4.67	2.84	0.30	
	PDTI	0.11	-0.59	0.17	0.35	0.16	

Table 1. Cont;d

Gadanwaye	P^N	55.83	5.0	4.33	2.47	0.37	
	P^-	37.83	11.07	3.0	2.34	0.35	
	P^+	56.07	7.33	4.67	2.92	0.31	
	PDTI	0.32	-1.2	0.31	0.05	0.05	
Sipi3	P^N	41.83	6.83	4.33	1.81	0.28	0.14
	P^-	36.00	10.67	2.67	0.83	0.23	
	P^+	43.00	10.67	5.03	1.85	0.33	
	PDTI	0.14	-0.56	0.38	0.54	0.18	
Variety1.4	P^N	25.07	8.43	2.67	0.93	0.25	0.13
	P^-	26.83	10.77	1.00	0.72	0.22	
	P^+	17.73	9.33	1.33	0.46	0.26	
	PDTI	-0.07	-0.28	0.63	0.23	0.12	
Variety44	P^N	39.90	9.83	2.00	1.03	0.31	-0.29
	P^-	43.67	10.52	2.67	1.77	0.38	
	P^+	31.3	8.93	2.00	0.71	0.28	
	PDTI	-0.09	-0.07	-0.34	-0.72	-0.23	
Sipi2	P^N	52.2	5.60	2.33	3.63	0.56	0.19
	P^-	35.53	11.53	1.33	0.88	0.28	
	P^+	38.9	3.93	1.67	1.10	0.25	
	PDTI	0.32	-1.06	0.43	0.76	0.50	
Togo	P^N	20.3	12.10	2.33	0.76	0.29	0.12
	P^-	19.5	10.83	1.33	0.74	0.29	
	P^+	33.9	6.67	2.67	1.22	0.22	
	PDTI	0.04	0.10	0.43	0.03	0.0	
Election2	P^N	31.07	5.67	5.33	1.81	0.34	0.20
	P^-	29.50	6.83	3.67	0.95	0.22	
	P^+	30.67	5.67	5.00	1.42	0.23	
	PDTI	0.05	-0.2	0.31	0.48	0.35	
Phillipine	P^N	33.17	5.33	4.17	1.76	0.29	-1.03
	P^-	27.67	14.83	3.33	1.36	0.24	
	P^+	33.53	6.67	4.33	1.75	0.30	
	PDTI	0.17	-1.8	0.20	0.23	0.17	
Obasanjo	P^N	47.50	5.33	5.00	2.19	0.39	0.33
	P^-	34.00	10.00	2.33	1.20	0.23	
	P^+	45.33	7.17	5.00	2.91	0.28	
	PDTI	0.28	-0.88	0.53	0.45	0.41	
Sedi2	P^N	48.50	7.50	4.67	1.76	0.31	0.22
	P^-	31.67	10.17	3.00	0.85	0.24	
	P^+	33.80	7.67	4.33	0.88	0.23	
	PDTI	0.35	-0.36	0.36	0.52	0.23	
Variety45	P^N	35.77	5.9	3.67	3.31	0.29	1.79
	P^-	22.83	10.53	2.33	0.69	0.25	
	P^+	32.33	5.83	2.33	1.96	0.23	
	PDTI	0.36	-0.79	0.37	0.79	0.50	

Source:Author

Table 2. Mean phosphate deficiency tolerance index (PDTI) grouping of 21 rice land races at 8 weeks after planting.

Negative PDTI (*)	Low positive PDTI ≤1.50 (**)	High positive PDTI >1.50 (***)
Ankulyan *Dantala mass* *Gadanwaye* *Variety 44* *Phillipine*	*Achancha* *Sedi 1* *Election 3* *Ayange* *Faro15* *OC* *Bnarda Supi* *Supi 3* *Variety 1.4* *Supi 2* *Togo* *Election 2* *Obasanjo* *Sedi 2*	*Zomoje* *Variety 45*

*Landraces performed better under deficient P conditions at least in some traits (Good P deficiency tolerance); **Landraces performed similarly under both deficient and sufficient P conditions (reduced P deficiency tolerance); ***Landraces performed better under sufficient P conditions (weak P deficiency tolerance). In each landrace, PDTI was evaluated individually for each morphological trait using the formular: (Trait under sufficient Pi – Trait under deficient Pi)/ (Trait under sufficient Pi) following Chankaew et al. (2019). Mean PDTI for all the traits was used as a basis for categorization in Table 1.
Source: Author

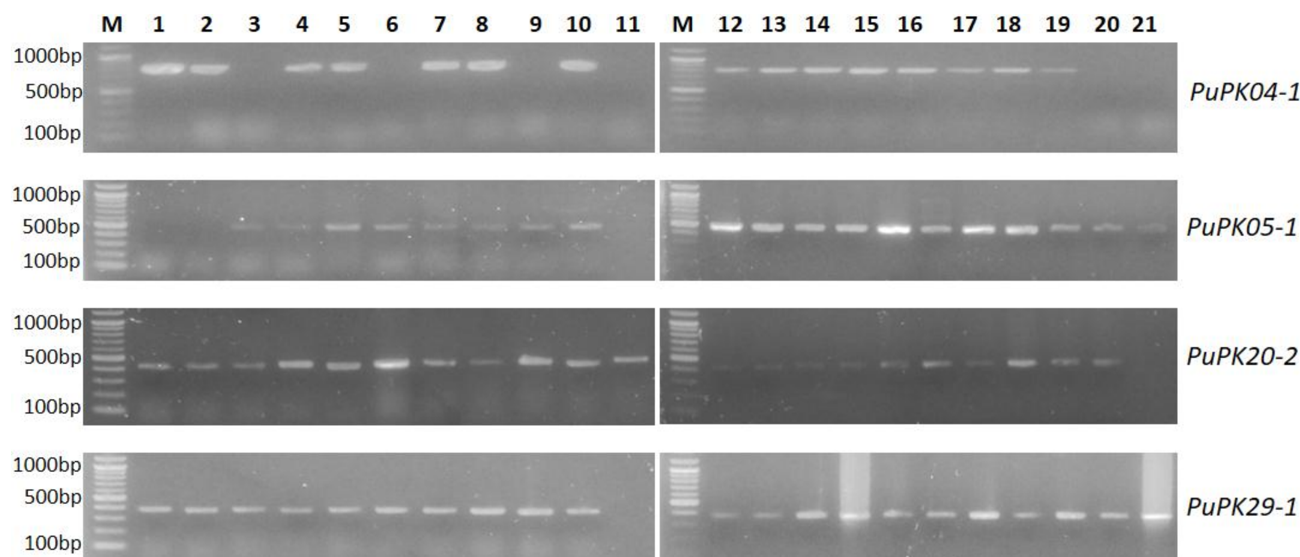

Figure 1. PCR amplification of *PupK04-1, PupK05-1, PupK20-2 and PupK29-1* in 21 rice landraces. 1=Achancha; 2=Sedi1, 3=Election 3, 4=Ayange, 5= Ankulyan,` 6=Faro15, 7=OC, 8= Zomoje, 9= Dantala max, 10 = Bnarda Supi, 11=Gadanwaye, 12= Supi3, 13=Variety1.4, 14= Variety44, 15=Supi2, 16=Togo, 17=Election2, 18=Phillipine, 19=Obasanjo, 20 = Sedi2, 21= Variety45, M= 100 bp DNA marker
Source: Author

those of *OsPup*K29-1 and *OsPup*K20-2 which showed up in over 95% of the landraces are reasonably conserved in the 21 upland rice landraces. Previous reports also indicated that *Pup-1* gene family was highly conserved in stress-adapted rice accessions (Chin et al., 2010, 2011). Heuer et al. (2009) added that *Pup-1* locus increases P uptake under adverse conditions rather than increasing internal P-use efficiency. Chankaew et al. (2019) however reported that the presence or absence of the tolerant allele at the *Pup*-1 locus only showed a slight relationship

Table 3. Summary of the genotyping results[1] of 21 rice landraces with 4 *Pup-1* linked markers.

Landraces	OsPupK04-1	OsPupK05-1	OsPupK20-2	OsPupK29-1
Achancha	1	0	1	1
Sedi 1	1	0	1	1
Election 3	0	1	1	1
Ayange	1	1	1	1
Ankulyan	1	1	1	1
Faro15	0	1	1	1
OC	1	1	1	1
Zomoje	1	1	1	1
Dantala mass	0	1	1	1
Bnarda Supi	1	1	1	1
Gadanwaye	0	0	1	0
Supi 3	1	1	1	1
Variety 1.4	1	1	1	1
Variety 44	1	1	1	1
Supi 2	1	1	1	1
Togo	1	1	1	1
Election 2	1	1	1	1
Phillipine	1	1	1	1
Obasanjo	1	1	1	1
Sedi 2	0	1	1	1
Variety 45	0	1	0	1

[1]Expected PCR product of *Pup-1* linked markers present (1), absent (0)
Source: Author

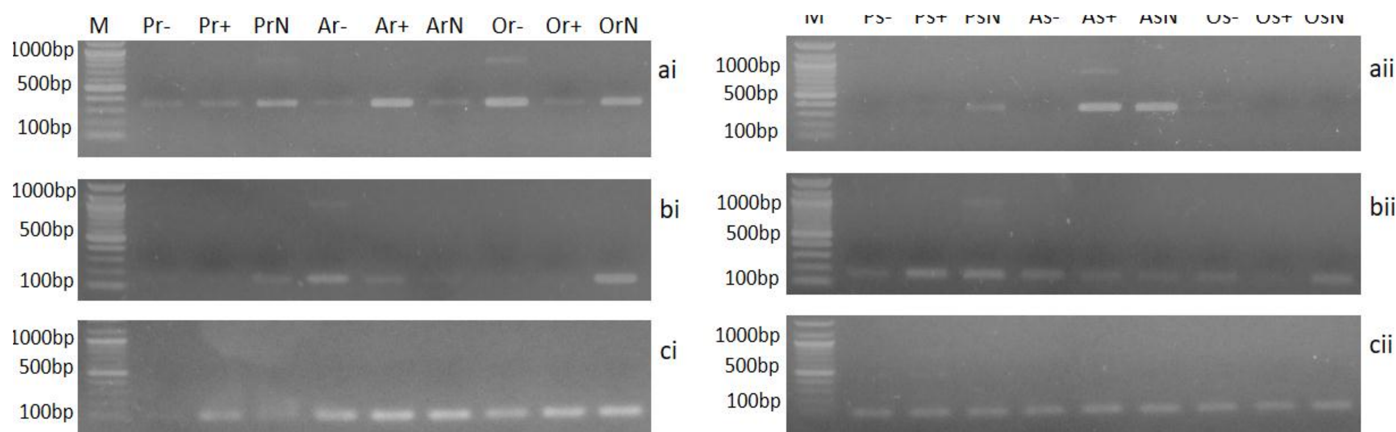

Figure 2. RT-PCR analysis of *OsPupK05-1*(ai and aii), *OsPup04-1* (bi and bii) and *OsUbiqPCR* (ci and cii) genes in the root (r) and shoot (s) of 3 rice landraces (P = *Phillipine*, A = *Ayange*, O = *Obasanjo*) under low (-), normal (N) and excess (+) phosphate conditions.
Source: Author

with the tolerance indices in Thai indigenous upland rice germplasm. Moreover, some lines expressed high tolerance without the *Pup*-1- linked gene product. These lines, they suggested, may carry unknown P-deficient tolerance genes. Sarkar et al. (2011) reported that haplotyping of *Pup*-1-K42 markers showed the expected 918 bp amplification in 9 out of the 31 genotypes studied, but among them, only 3 genotypes showed higher P-uptake and dry-matter weight in P-limiting condition. Thus, the gene may be present but not expressed. This differential expression of the *Pup-1* genes was also observed in the present study. Even though at least one of the genes was present in all the 21 landraces, their expression levels varied with the landrace and the

phosphate concentration in the medium. The highest expression of *OsPupK05-1* gene was seen in the root and shoot of *Ayange* under excess (Pi^+) and normal phosphate (Pi^N) rather than under Pi^- when it is most needed. This perhaps resulted in the better performance of this land race under Pi^N compared to that at deficient P. Moreover, *OsPupK04-1* gene was weakly expressed in the root and shoot of this landrace under deficient P. *OsPupK05-1* gene was well expressed in the root of *Obasanjo* under normal (Pi^N) and deficient (Pi^-) levels which also explains the enhanced performance of Obasanjo seen under Pi^N compared to Pi^- (the medium had zero P). It was also observed that Phillipine had the longest root under low P relative to the length under normal P, as reflected in the PDTI value of -1.8 for this trait. However, this did not reflect in the overall plant biomass for this landrace as all the other traits performed better under normal P level. Furthermore, *OsPupK04-1* gene was well expressed in the shoot of Philippine under excess and normal phosphate which explains the better performance of this land race under Pi^N compared with Pi^-. The average tolerance index for this landrace was, however, negative because of the very long root produced under Pi^-. Since this *OsPupKO4-1* gene was not expressed under Pi^- condition in this landrace, the possibility of another phosphate uptake gene operating under this stress condition cannot be ruled out.

The present report showed that only 3 out of the 21 landraces (Variety 44, Ankulyan and Dantala Mass) had higher dry-matter weight in P-limiting conditions which supports the report by Chankaew (2019) that the presence of the tolerant allele at the *Pup* locus does not have significant correlation with the tolerance indices. The choice of *OsPupK04-1* and *OsPupK05-1* for gene expression analysis in the present study was based on previous report of their ubiquitous expression in both roots and shoots of P- and P+ rice accessions by Chin et al. (2011) unlike *OsPup20* which is restricted to roots with higher transcript abundance under P deficient conditions. Chin et al. (2011) observed that most of the *Pup* markers were not informative when tested in a wider range of rice germplasm and that *OsPup-K29* was unstable. This marker, however, gave very consistent bands in 95% of the landraces used in the present study even though some were not expressed.

Conclusion

The study indicated that although low phosphate concentration causes adverse effects on the growth and productivity of these rice plants, the presence and adequate expression of phosphate deficiency tolerance genes in such plants improves adaptation to the stress by producing much longer roots in search of phosphate in the growth medium. Three genotypes namely Dantala Mass, Ankulyan, and Variety 44 may be regarded as tolerant to phosphate deficiency as they each had mean negative PDTI value with better performance in desirable root and shoot traits under zero phosphate, in addition to possessing at least 3 of the 4 *Pup 1* genes used. Also, the landraces that had good biomass with PDTI values close to zero (which means they performed well in both P-deficient and P-sufficient media) such as Achancha and which invariably also contained *the Pup-1* gene(s), may be further explored along with the other three for possible inclusion in the breeding of this crop for P deficiency tolerance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. Rice *(Oryza sativa)* landraces used for the experiment and their sources.

Code	Local name	Source	Coordinates
BN-001	Achancha	Agasha, Benue State	7°58'0''N8°54'0''E
BN-002	Sedi one	Makurdi, Benue State	7.732°N8.5391°E
BN-003	Election 3	Agasha, Benue State	7°58'0''N8°54'0''E
BN-004	Ayange	Gbajimba, Benue State	7°49'0''N8°51'0''E
BN-005	OC	Makurdi, Benue State	7.732°N8.5391°E
BN-006	Zomuje	Gbajimba, Benue State	7°49'0''N8°51'0''E
BN-007	Dantala	Makurdi, Benue State	7.732°N8.5391°E
BN-008	Election 2	Agasha, Benue State	7°58'0''N8°54'0''E
BN-009	Mass	Agasha, Benue State	7°58'0''N8°54'0''E
BN-010	Bnarda Sipi	Naka, Benue State	7°34'59.9''N8°12.264'E
BN-011	Sipi 2 (Faro 44)	Naka, Benue State	7°34'59.9''N8°12.264'E
BN-012	Sipi 3 (Faro 44)	Naka, Benue State	7°34'59.9''N8°12.264'E
BN-013	Variety 45	Agasha, Benue State	7°58'0''N8°54'0''E
BN-014	Togo	Agasha, Benue State	7°58'0''N8°54'0''E
BN-015	Variety 1.4	Naka, Benue State	7°34'59.9''N8°12.264'E
BN-016	Variety 44	Naka, Benue State	7°34'59.9''N8°12.264'E
BN-017	Obasanjo	Makurdi, Benue State	7.732°N8.5391°E
BN-018	Election 3	Makurdi, Benue State	7.732°N8.5391°E
CR-001	Philippine	Adim, Cross River State	5°44' 0" N8°2' 0" E
FARO 15	-	Adim, Cross River State	5°44' 0" N8°2' 0" E

Supplementary Table 2. Modified Hoagland solution used in the study.

S/N	Components	Concentration (mM)	Molecular wt. (g/mol)	Stock (g/L)	Stock (500 mL)	Working vol. (mL)
	Macronutrients					
1	NH_4NO_3	5.6	80.04	448.224	224.1	1
	K_2HPO_4	0.4 (P^N)	174.18	69.672	34.84	1
2	K_2HPO_4	0.8 (Pi^+)	174.18	139.34	69.67	
	K_2HPO_4	0.0 (Pi^-)	-	-	-	
3	$MgSO_4.7H_2O$	0.8	246.48	197.184	98.592	1
	K_2SO_4	0.8	174.25	139.4	69.7	
4	$FeSO_4.7H_2O$	0.18	278.0	50.04	25.02	1
	$Na_2EDTA.2H_2O$	0.18	374.2	67.36	33.68	
5	$CaCl_2.2H_2O$	1.6	147.0	235.2	117.6	1
6	KNO_3	0.8	101.10	80.88	40.44	1
	Micronutrients					
	H_3BO_3	0.023	61.811	1.422	0.711	
	$MnCl_2.4H_20$	0.0045	197.84	0.890	0.445	
7	$CuSO_4.5H_2O$	0.0003	249.61	0.075	0.037	1
	$ZnCl_2$	0.0015	136.29	0.204	0.102	
	$Na_2MoO_4.2H_2O$	0.0001	241.93	0.024	0.012	

Supplementary Table 3. Oligonucleotides used in the study.

Primer name	Sequence 5'-3'	Application
OsPUpK04-1 F	GGGATATCAAGCTTGTGGTG	PCR, RT-PCR
OsPUpK04-1 R	GAATGCTGTTTCGCTTATGG	
OsPUpK05-1 F	AGTACAGTCCGGCGTCATAC	PCR, RT-PCR
OsPUpK05-1 R	CCGAGATCTGGTCCTCAATA	
OsPUpK20-2 F	CTGGACTTGACCCCAATGTA	PCR
OsPUpK20-2 R	TCTGATGGAGTGTTCGGAGT	
OsPUpK29-1 F	CCAATGCATCCAATTCTTGT	PCR
OsPUpK29-1 R	ATGAGCCCAGATTACGAATG	
*OsUbq*_F	GCCCAAGAAGAAGATCAAGAAC	RT-PCR
*OsUbq*_R	AGATAACAACGGAAGCATAAAAGTC	

Supplementary Figure 1. Rice seedlings at P-, P+ and P^N respectively, in each of the PVC pipes at 7 weeks after planting.

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Cordycepin production by the potential fungal strains *Cordyceps militaris* BCC 2819 and *Cordyceps cicadae* BCC 19788 in submerged culture during batch and Fed-batch fermentation

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Cordycepin is one of the most important bioactive compounds; the low productivity and long production cycle of cordycepin are barriers to its commercialization. The optimal media for cordycepin production by *Cordyceps militaris* BCC 2819 and *Cordyceps cicadae* BCC 19788, which are potent cordycepin-producing-fungal strains, were determined through statistical experiments. Six nutrients including glucose, adenine, glycine, alanine, casein hydrolysate and vitamin solution were found to influence the cordycepin production by *C. militaris* BCC 2819, while the same factors were found for the cordycepin production by *C. cicadae* BCC 19788 except glucose that was replaced by ammonium sulfate. The highest cordycepin production of 1,176.69 ± 263.33 mg/L was obtained by *C. militaris* BCC 2819 using the central composite design. The highest cordycepin production of 4,259.63 ± 224.20 mg/L was obtained by *C. cicadae* BCC 19788 using the central composite design. Cordycepin production in 5-L fed-batch fermentation by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using optimized medium reached maximum production levels of 3,112.50 and 3,587.10 mg/L, respectively, accounted for more than 1.2-fold compared to those in batch fermentation. Furthermore, the highest levels of the bioactive compounds; exopolysaccharide, adenosine and mannitol produced by *C. militaris* BCC 2819 were 43.90 ± 2.51 g/L, 2,897.40 ± 382.47 mg/L and 5,981.10 ± 254.72 mg/L, respectively. The highest levels of the bioactive compounds; exopolysaccharide, adenosine and mannitol produced by *C. cicadae* BCC 19788 were 38.10 ± 2.84 g/L, 3,78520 ± 165.70 mg/L and 6,100.20 ± 191.14 mg/L, respectively. These results demonstrated the new isolates produced high amounts of bioactive compounds, especially cordycepin. Interestingly, this process can be applied for cordycepin production for future applications or scale-up studies.

Key words: Batch fermentation, cordycepin, *Cordyceps militaris*, fed-batch fermentation, *Cordyceps cicadae*, optimization.

INTRODUCTION

Cordycepin (3'-deoxyadenosine), a nucleoside analog, was first isolated from *Cordyceps militaris* (Cunningham et al., 1950). Cordycepin has been used as a medicinal agent due to its anticancer (Yoshikawa et al., 2008), antifungal (Sugar and McCaffrey, 1998), antiviral (Hashimoto and Simizu, 1976), anti-inflammatory, antioxidant (Tuli et al., 2013) and immunological regulation activities (De Silva et al., 2012). Hence, cordycepin has received attention for its potential applications as a functional food and healthcare product. It has been produced by chemical synthesis (Kwon et al., 2003) and microbial fermentation using *C. militaris* (Cunningham et al., 1950) or *Aspergillus nidulans* (Kaczka et al., 1964). However, it is difficult to purify cordycepin produced through chemical synthesis, leading to high production costs. Thus, cordycepin production through microbial fermentation is an interesting avenue.

Microbial fermentation is a cost-effective process for the production of cordycepin on an industrial scale. However, in the present study, cordycepin production through microbial fermentation still needs to be optimized due to its low productivity and long production cycle time upto 40 days (Lim et al., 2012). The production of cordycepin via solid-state fermentation (Wen et al., 2008b) and submerged fermentation (Kang et al., 2012; Wen et al., 2009) has been often used for the cordycepin production, in which solid-state cultivation of fungi on various insect pupae and larvae has been used for commercial purposes (Zhang et al., 2011). Various solid substrates such as rice, oat and wheat were used as solid substrate for cordycepin production (Chen et al., 2010). Moreover, physical condition such as pH, temperature and light condition also affected the cordycepin production using solid state fermentation (Chen et al., 2010; Lim et al., 2012). However, the drawback of solid-state fermentation is that it takes a long time for complete fruiting body development, and it is difficult to achieve commercial scale production (Chen et al., 2010). The consistency of cordycepin composition in the fruiting bodies obtained is one of the key problems for fruiting body production in solid-state fermentation. Presently, liquid static culture (Kang et al., 2014) and submerged fermentation is an alternative process for cordycepin production. Liquid culture is considered as a better culture procedure for industrial purposes because of the shorter cultivation time and the higher cordycepin production yield (Kunhorm et al., 2019). Furthermore, in addition to optimizing the fermentation process, Improving cordycepin-producing fungal strains should also receive attention. Previously, mutant strains that produce higher cordycepin levels than wild-type strains were generated by various technologies, such as ion-beam irradiation (Das et al., 2008) and space mutation treatment (Wen et al., 2008a). The components of the culture medium also affect the cordycepin yield. Some previous research showed that glucose and yeast extract were components that improved cordycepin production in *C. militaris* (Mao et al., 2005; Das et al., 2010). Specific amino acids, precursors, and inducers are also required for secondary metabolite production by microorganisms, including fungi (Oh et al., 2019). Other studies have used different culture components and additives (Wen et al., 2009; Mao and Tu, 2005; Masuda et al., 2007; Das et al., 2009) for cordycepin production in liquid culture. These studies revealed that the most effective components for cordycepin production depend on the fungal strain. Thus, the nutrition requirements and physical conditions, including seed culture preparation, for cordycepin production by microorganisms are of interest. Our previous preliminary study found two fungal strains that produce cordycepin at high levels among those 10 candidate strains chosen (data not shown), *C. militaris* BCC 2819 and *C. cicadae* BCC 19788, and these strains can be used as potential fungal cell factories for high-titer cordycepin production. In this study, the effects of medium components (carbon source, nitrogen source, amino acid and precursors) on cordycepin production were elucidated through Plackett-Burman design. Then, the optimal cordycepin production medium for both fungal strains was determined by central composite design. Validation of the optimized medium was performed in batch fermentation in a 5-L fermenter. Finally, fed-batch fermentation was used to improve cordycepin production and the production of other important bioactive compounds that are effectively synergistic with the mechanism of cordycepin production in animal bodies as functional food supplements.

MATERIALS AND METHODS

Microorganism

C. militaris BCC 2819 and *C. cicadae* BCC 19788 were obtained from Thailand Bioresource Research Center (TBRC). The stock cultures were stored on potato dextrose agar (PDA) slant. The cultures were incubated at 25°C for 5 to 7 days and then supplemented with 20% glycerol. The stock slants were stored at 4°C.

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Table 1. Plackett-Burman design for cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788.

Treatment	Factor										
	A	B	C	D	E	F	G	H	J	K	L
1	40	10	5	5	2	5	10	2	2	4	2
2	20	10	15	2	2	5	20	2	2	1	4
3	40	5	15	5	1	5	20	5	2	1	2
4	20	10	5	5	2	2	20	5	5	1	2
5	20	5	15	2	2	5	10	5	5	4	2
6	20	5	5	5	1	5	20	2	5	4	4
7	40	5	5	2	2	2	20	5	2	4	4
8	40	10	5	2	1	5	10	5	5	1	4
9	40	10	15	2	1	2	20	2	5	4	2
10	20	10	15	5	1	2	10	5	2	4	4
11	40	5	15	5	2	2	10	2	5	1	4
12	20	5	5	2	1	2	10	2	2	1	2
13	30	7.5	10	3.5	1.5	3.5	15	3.5	3.5	2.5	3
14	20	5	15	2	1	2	20	5	5	1	4
15	40	5	5	5	1	2	10	5	5	4	2
16	20	10	5	2	2	2	10	2	5	4	4
17	40	5	15	2	1	5	10	2	2	4	4
18	40	10	5	5	1	2	20	2	2	1	4
19	40	10	15	2	2	2	10	5	2	1	2
20	20	10	15	5	1	5	10	2	5	1	2
21	20	5	15	5	2	2	20	2	2	4	2
22	20	5	5	5	2	5	10	5	2	1	4
23	40	5	5	2	2	5	20	2	5	1	2
24	20	10	5	2	1	5	20	5	2	4	2
25	40	10	15	5	2	5	20	5	5	4	4
26	30	7.5	10	3.5	1.5	3.5	15	3.5	3.5	2.5	3

A =Glucose (g/L), B =peptone (g/L), C =yeast extract (g/L), D =$NH_4(_2SO_4)$ (g/L), E =adenine (g/L), F =glutamine (g/L), G = glycine (g/L), H =alanine (g/L), J =casein hydrolysate (g/L), K =vitamin solution (mL/L), and L =trace solution (mL/L).
Source: Author

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788

The seed culture was prepared in 1,000 mL Erlenmeyer flasks containing 200 mL of potato dextrose broth (PDB) and incubated on a rotary shaker at 200 rpm at 25°C for 5 to 7 days. The culture was blended and transferred by portioned to 50 mL of production medium with 5% v/v.

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using Plackett-Burman (PB) design

The PB design (Plackett and Burman, 1946) was used to identify the factors that influenced cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. A total of 11 factors, that is, glucose, glycine, alanine, glutamate, adenine, casein hydrolysate, peptone, yeast extract, (NH4)2SO4, trace solution (consisting of 14.3 g/L $ZnSO_4.7H_2O$, 2.5 g/L $CuSO_4$, 0.5 g/L $NiCl_2.6H_2O$, 6 g/L $MnCl_2$ and 13.8 g/L $FeSO_4.7H_2O$), and vitamin solution (Blackmore) (Vitamin complex consisted of 75 mg vitamin B1 (thiamine hydrochloride), 10 mg vitamin B2 (riboflavin), 50 mg nicotinamide, 25 mg calcium pantothenate, 10 mg vitamin B6 (pyridoxine hydrochloride), 25 mcg vitamin B12 (cyanocobalamin), 15 mcg biotin, 500 mg vitamin C (derived from ascorbic acid 260 mg and calcium ascorbate 290.5 mg), 10 mg choline bitartrate, 10 mg inositol, 10 mg zinc amino acid chelate (zinc 2 mg), 175 mg calcium phosphate, and 75 mg magnesium phosphate), were evaluated. The base medium component consisted of 0.5 g/L $MgSO_4$, 0.5 g/L KH_2PO_4 and 0.5 g/L K_2HPO_4. The PB experimental design with center points (26 treatments) for cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 is shown in Table 1. The center points were run to evaluate the curvature and the linearity of the variables. For each condition, the cultures were shaken at 200 rpm on an orbital shaker at 25°C for 11 days. The experiments were performed in triplicate.

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using central composite design (CCD)

After screening the influencing factors by PB design, the optimal

value of each influencing factors was optimized using CCD (Box and Wilson, 1992) to enhance cordycepin production. The factor name and actual level of the factors for *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 for each experimental design (47 treatments) are shown in Tables 2 and 3, respectively. The cultures were shaken at 200 rpm on an orbital shaker at 25°C for 11 days. The experiments were performed in triplicate. The adequacy of the quadratic model was determined using the coefficient of determination (R^2) and analysis of variance (ANOVA). Design Expert software version 10.0 (State-Ease, US) was used to draw contour plots to explain the influences of the variables on cordycepin yield and predict the best conditions for cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. The production in potato dextrose broth (PDB) was used as a control.

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in batch fermentation

After optimization in shake flasks, cordycepin was produced in 5-L stirred tank fermenters (BIOSTAT B Plus, Sartorius stedim, Germany). A total of 10% seed culture was inoculated into 3 L of optimal medium and cultivated at 25°C with agitating at 400 rpm at an aeration rate of 1 vvm. Fermentation finished after glucose was depleted. Samples were taken to measure the cell mass, exopolysaccharide and active compounds (cordycepin, adenosine and mannitol).

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in fed-batch fermentation with an exponential feed rate

For fed-batch fermentation, the initial culture was grown in 1.5 L for the batch process. After the culture reached the exponential phase, 2 L of feeding medium was added to the culture according to the feeding profile using the following model (Chongchittapiban et al., 2016):

$$F_0 = \frac{\mu_{set}}{S_0 Y_{X/S}} X_{10} V_0 e^{\mu_{set}(t-t_0)}$$

where μ_{set} = 0.02 h^{-1}, S_0= 150 g. L^{-1}, X= 36.3 g. L^{-1}, and $Y_{X/S}$= 0.67 g.g^{-1} for *C. militaris* BCC 2819, and μ_{set} = 0.02 h^{-1}, S_0= 100 g. L^{-1}, X= 35.36 g. L^{-1}, and $Y_{X/S}$= 0.39 g.g^{-1} for *C. cicadae* BCC 19788.

The feeding medium for *C. militaris* BCC 2819 consisted of glucose 150 g/L, adenine 4.41 g/L, glycine 26.4 g/L, alanine 10 g/L, glutamine 2 g/L, casein hydrolysate 5 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 ml/L and vitamin solution 4 ml/L. The feeding medium for *C. cicadae* BCC 19788 consisted of glucose 100 g/L, adenine 5 g/L, glycine 30 g/L, alanine 5 g/L, glutamine 2 g/L, casein hydrolysate 10 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 5.68 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 mL/L and vitamin solution 6 mL/L.

Dried cell weight and exopolysaccharide quantification

The culture was filtered through filter paper (Whatman), and the mycelium and supernatant were separated. The mycelium was rinsed and lyophilized to obtain the dry cell weight. The supernatant was added to cold 95% ethanol 4 times to precipitate exopolysaccharide. The exopolysaccharide was lyophilized to obtain the dry weight.

Cordycepin and adenosine extraction and quantification

Cordycepin and adenosine were produced inside the cell and secreted into the medium. After filtration, the mycelium was ground with liquid nitrogen to obtain a fine powder. The mycelium powder was extracted with water at 80°C for 3 h to obtain cordycepin and adenosine. The cordycepin and adenosine concentrations were determined by HPLC equipped with a UV detector (Waters 2489 UV/Visible detector, Waters, Massachusetts, US) and reversed-phase column (Xterra MS C18 column, Waters, Massachusetts, US). Methanol (8%) was used as the mobile phase. The flow rate was 0.5 mL/min, and the column temperature was 25°C. The total cordycepin and adenosine contents were measured by quantification of each compound from the supernatant and extracted solution of mycelium and comparing the values to standard curves (10 - 100 mg/L).

Mannitol extraction and quantification

Mannitol was extracted from dried mycelium of both fungal strains. The dried mycelium was ground with liquid nitrogen to obtain a fine powder. The mycelium powder was extracted with 0.1 M phosphate buffer pH 7. The mannitol concentration was determined by HPLC with an RI detector (RI 501, Shodex, Yokohama, Japan) and an ion exchange column (Aminex HPX-87H column, Biorad, California, US). Sulfuric acid (0.025 M) was used as the mobile phase. The flow rate was 0.5 mL/min, and the column temperature was 65°C. The content of mannitol was compared with a standard curve of mannitol ranging from 0.0625 to 20 g/L.

RESULTS

The influential factors of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using Plackett-Burman design

The data listed in Tables 4 and 5 show the variation in dry cell weight, exopolysaccharide and active compounds produced by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788, respectively, from 27 treatments. The dry cell weights and exopolysaccharides produced by *C. militaris* BCC 2819 were 6.59 ± 0.91 - 25.54 ± 1.11 g/L to 0.72 ± 0.17 - 16.60 ± 1.61 g/L, respectively. Additional bioactive compounds such as cordycepin, adenosine and mannitol were produced in the ranges of 64.17 ± 0.46 - 960.14 ± 263.33 L, 49.59 ± 2.21 - 632.51 ± 229.30, and 0 - 224.46± 19.44 mg/L, respectively. The highest cordycepin concentration of 960.14 ± 263.33 mg/L produced by *C. militaris* BCC 2819 was obtained with medium containing glucose 40 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 2 g/L, adenine 1 g/L, glutamine 2 g/L, glycine 20 g/L, alanine 2 g/L, casein hydrolysate 5 g/L, vitamin solution 4 mL/L, and trace solution 2 mL/L.

Table 2. Central composite design for cordycepin production by *C. militaris* BCC 2819.

Treatment	Factor					
	A	B	C	D	E	F
1	60	5	30	10	5	4
2	60	5	30	10	5	4
3	60	5	30	5	15	4
4	60	5	30	5	15	4
5	60	5	20	10	5	6
6	60	5	20	10	5	6
7	60	2	30	5	15	6
8	60	2	30	5	15	6
9	40	5	20	10	15	6
10	40	5	20	10	15	6
11	60	2	30	10	5	6
12	60	2	30	10	5	6
13	40	5	30	5	5	4
14	40	5	30	5	5	4
15	60	5	20	5	15	6
16	60	5	20	5	15	6
17	60	2	20	10	5	4
18	60	2	20	10	5	4
19	40	2	30	5	5	6
20	40	2	30	5	5	6
21	40	5	20	5	5	6
22	40	5	20	5	5	6
23	60	2	20	5	15	4
24	60	2	20	5	15	4
25	40	2	20	10	15	4
26	40	2	20	10	15	4
27	40	2	30	10	15	6
28	40	2	30	10	15	6
29	40	5	30	10	15	4
30	40	5	30	10	15	4
31	40	2	20	5	5	4
32	40	2	20	5	5	4
33	34.35	3.5	25	7.5	10	5
34	65.65	3.5	25	7.5	10	5
35	50	1.15	25	7.5	10	5
36	50	5.85	25	7.5	10	5
37	50	3.5	17.18	7.5	10	5
38	50	3.5	32.83	7.5	10	5
39	50	3.5	25	3.59	10	5
40	50	3.5	25	11.41	10	5
41	50	3.5	25	7.5	2.18	5
42	50	3.5	25	7.5	17.83	5
43	50	3.5	25	7.5	10	3.44
44	50	3.5	25	7.5	10	6.57
45	50	3.5	25	7.5	10	5
46	50	3.5	25	7.5	10	5
47	50	3.5	25	7.5	10	5

A =Glucose (g/L), B = adenine (g/L), C =glycine (g/L), D =alanine (g/L), E = casein hydrolysate (g/L), and F = vitamin solution (mL/L).
Source: Author

Table 3. Central composite design of cordycepin production by *C. cicadae* BCC 19788.

Treatment	Factor					
	A	B	C	D	E	F
1	15	5	30	15	5	4
2	15	5	30	15	5	4
3	15	5	30	5	10	4
4	15	5	30	5	10	4
5	15	5	20	15	5	6
6	15	5	20	15	5	6
7	15	2	30	5	10	6
8	15	2	30	5	10	6
9	5	5	20	15	10	6
10	5	5	20	15	10	6
11	15	2	30	15	5	6
12	15	2	30	15	5	6
13	5	5	30	5	5	4
14	5	5	30	5	5	4
15	15	5	20	5	10	6
16	15	5	20	5	10	6
17	15	2	20	15	5	4
18	15	2	20	15	5	4
19	5	2	30	5	5	6
20	5	2	30	5	5	6
21	5	5	20	5	5	6
22	5	5	20	5	5	6
23	15	2	20	5	10	4
24	15	2	20	5	10	4
25	5	2	20	15	10	4
26	5	2	20	15	10	4
27	5	2	30	15	10	6
28	5	2	30	15	10	6
29	5	5	30	15	10	4
30	5	5	30	15	10	4
31	5	2	20	5	5	4
32	5	2	20	5	5	4
33	2.18	3.5	25	10	7.5	5
34	17.83	3.5	25	10	7.5	5
35	10	1.15	25	10	7.5	5
36	10	5.85	25	10	7.5	5
37	10	3.5	17.18	10	7.5	5
38	10	3.5	32.83	10	7.5	5
39	10	3.5	25	2.18	7.5	5
40	10	3.5	25	17.83	7.5	5
41	10	3.5	25	10	3.59	5
42	10	3.5	25	10	11.41	5
43	10	3.5	25	10	7.5	3.44
44	10	3.5	25	10	7.5	6.57
45	10	3.5	25	10	7.5	5
46	10	3.5	25	10	7.5	5
47	10	3.5	25	10	7.5	5

A =$NH_4(_2SO_4)$ (g/L), B = adenine (g/L), C = glycine (g/L), D = alanine (g/L), E = casein hydrolysate (g/L), and F = vitamin solution (mL/L).
Source: Author

Table 4 . Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C .militaris* BCC 2819 using the Plackett-Burman design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	436.77 ± 306.17	367.28 ± 222.77	40.92 ± 16.44	17.78 ± 2.47	4.49 ± 1.61
2	126.28 ± 69.39	129.28 ± 62.90	1.13 ± 1.00	11.15 ± 0.24	4.65 ± 2.42
3	586.97 ± 190.50	166.84 ± 59.62	86.69 ± 112.87	22.34 ± 2.61	5.85 ± 2.59
4	81.34 ± 40.82	131.33 ± 15.23	13.48 ± 22.11	10.57 ± 1.33	16.60 ± 1.61
5	84.69 ± 24.64	60.88 ± 14.06	0.86 ± 1.49	9.11 ± 0.44	5.99 ± 0.46
6	64.17 ± 0.46	52.37 ± 13.69	8.05 ± 9.23	9.39 ± 1.15	4.36 ± 1.24
7	515.50 ± 280.89	282.13 ± 57.11	4.69 ± 4.70	19.26 ± 2.84	7.61 ± 3.12
8	664.96 ± 158.23	170.27 ± 149.92	78.81 ± 36.73	19.24 ± 1.07	3.85 ± 0.00
9	960.14 ± 263.33	145.69 ± 23.46	104.10 ± 32.58	25.54 ± 1.11	7.41 ± 2.33
10	85.65 ± 35.78	49.59 ± 2.21	1.25 ± 1.09	12.09 ± 1.55	6.91 ± 0.57
11	581.58 ± 441.30	632.51 ± 229.31	224.46 ± 19.45	22.49 ± 3.44	7.04 ± 2.43
12	57.39 ± 13.70	64.43 ± 10.46	0.83 ± 1.44	8.56 ± 0.13	2.49 ± 0.61
13	135.94 ± 4.87	185.96 ± 29.09	0.00 ± 0.00	13.28 ± 1.42	5.48 ± 0.47
14	119.82 ± 63.93	123.27 ± 70.40	1.77 ± 3.06	12.51 ± 1.17	7.87 ± 2.34
15	350.06 ± 74.83	321.04 ± 98.62	57.00 ± 22.75	16.93 ± 1.80	3.13 ± 0.35
16	81.21 ± 50.64	70.04 ± 41.85	0.00 ± 0.00	10.50 ± 0.50	7.79 ± 2.09
17	316.86 ± 63.98	520.90 ± 140.13	21.18 ± 31.11	21.10 ± 0.49	4.95 ± 0.54
18	306.68 ± 39.00	351.16 ± 42.13	20.29 ± 26.57	17.01 ± 1.12	7.85 ± 1.80
19	225.20 ± 115.07	386.23 ± 177.87	23.78 ± 26.19	19.61 ± 5.93	8.20 ± 1.56
20	77.25 ± 27.85	65.62 ± 12.19	11.33 ± 8.27	10.73 ± 2.05	9.97 ± 2.95
21	90.20 ± 19.60	86.07 ± 37.07	0.04 ± 0.06	10.13 ± 0.58	16.45 ± 1.62
22	70.50 ± 27.81	118.61 ± 63.97	0.00 ± 0.00	9.51 ± 2.38	7.32 ± 1.24
23	524.86 ± 286.77	317.25 ± 131.11	180.51 ± 13.16	19.78 ± 3.52	4.86 ± 1.11
24	53.91 ± 11.11	51.56 ± 12.01	0.00 ± 0.00	11.01 ± 2.17	6.07 ± 0.88
25	615.90 ± 402.84	493.67 ± 193.76	95.59 ± 3.02	25.38 ± 2.23	8.10 ± 1.13
26	158.89 ± 150.74	157.50 ± 122.90	3.75 ± 5.30	14.43 ± 1.78	5.29 ± 0.04
Control (PDB)	69.57 ± 12.30	107.28 ± 66.31	24.31 ± 26.97	6.59 ± 0.91	0.72 ± 0.17

Source: Author

The dry cell weights and exopolysaccharides produced by *C. cicadae* BCC 19788 were in the ranges of 4.68 ±0.58 - 32.09 ±1.64 g/L and 1.59 ± 0.60 - 27.57 ± 3.18 g/L, respectively.

Cordycepin, adenosine and mannitol were produced in the ranges of 39.47 ± 10.05 – 3,833.39 ± 194.99, 198.24 ± 59.22 – 5,354.24 ± 1,650.152, and 8.53 ± 7.89 - 639.57 ± 209.87 mg/L, respectively.

The highest cordycepin concentration of 3,833.39 ± 194.99 mg/L produced by *C. cicadae* BCC 19788 was obtained with medium containing glucose 20 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 5 g/L, adenine 1 g/L, glutamine 2 g/L, glycine 10 g/L, alanine 5 g/L, casein hydrolysate 2 g/L, vitamin solution 4 mL/L, and trace solution 4 mL/L.

The factors that influenced cordycepin production were chosen based on the *p* value of ANOVA analyses (Tables S1 and S2) and the generated models were significant for both fungal strains of each factor and the interaction with the other factors (data not shown).

The factors that influenced cordycepin production by *C. militaris* BCC 2819 were glucose, adenine, glycine, alanine, casein hydrolysate and vitamin solution. However, $(NH_4)_2SO_4$, adenine, glycine, alanine, casein hydrolysate and vitamin solution showed a significant effect on cordycepin production by *C. cicadae* BCC 19788. These influential factors were then used in a CCD experiment to determine the optimal level of each factor for cordycepin production by both fungal strains.

Optimization of cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 using Central Composite Design (CCD)

The CCD was used to optimize the cordycepin production

Table 5. Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C. cicadae* BCC 19788 using the Plackett-Burman design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	2652.97 ± 936.86	3048.95 ± 851.66	565.64 ± 35.35	24.69 ± 2.46	9.67 ± 2.13
2	2651.36 ± 1126.69	2169.15 ± 998.25	177.24 ± 9.02	14.82 ± 4.43	27.57 ± 3.18
3	3587.73 ± 928.72	5290.52 ± 1348.47	579.16 ± 79.51	31.01 ± 0.92	15.15 ± 3.84
4	3225.67 ± 644.45	1287.97 ± 376.51	17.67 ± 2.97	10.24 ± 1.18	18.81 ± 3.21
5	3359.56 ± 509.26	2589.56 ± 951.67	172.21 ± 22.02	14.94 ± 1.04	15.40 ± 2.36
6	3374.81 ± 463.38	1216.68 ± 416.04	140.69 ± 27.02	12.26 ± 1.56	18.35 ± 0.79
7	2874.20 ± 1350.61	3707.89 ± 922.61	436.66 ± 9.21	24.65 ± 0.67	12.76 ± 1.58
8	2087.15 ± 693.91	3461.37 ± 448.92	15.37 ± 7.78	30.43 ± 2.49	6.88 ± 4.91
9	2117.58 ± 1068.58	4270.68 ± 846.81	420.73 ± 88.68	30.17 ± 0.56	10.84 ± 3.34
10	3833.39 ± 194.99	2113.78 ± 443.83	208.21 ± 36.23	17.71 ± 1.07	13.07 ± 1.74
11	2817.55 ± 1288.25	5354.24 ± 1650.15	639.57 ± 209.87	31.79 ± 4.39	11.10 ± 5.81
12	2778.59 ± 171.64	1016.35 ± 159.04	140.73 ± 10.46	9.19 ± 0.56	10.20 ± 1.46
13	2286.70 ± 712.64	3124.42 ± 1150.89	257.37 ± 45.96	21.73 ± 2.76	12.45 ± 1.95
14	1832.28 ± 1153.75	1374.16 ± 760.75	280.80 ± 49.78	14.52 ± 3.34	21.32 ± 1.47
15	2727.57 ± 1308.77	3072.36 ± 2247.79	55.27 ± 33.76	26.29 ± 1.94	5.61 ± 1.69
16	2782.55 ± 538.44	900.40 ± 286.80	135.56 ± 80.41	10.32 ± 0.75	9.01 ± 3.01
17	1483.35 ± 388.88	2942.04 ± 635.95	443.06 ± 12.53	29.52 ± 0.88	6.45 ± 1.02
18	1718.32 ± 868.76	2278.82 ± 1074.74	603.00 ± 17.34	27.76 ± 1.16	6.39 ± 3.56
19	1343.27 ± 150.73	2838.69 ± 1027.41	489.73 ± 110.39	32.09 ± 1.64	6.41 ± 3.00
20	3711.14 ± 460.30	2404.74 ± 412.39	238.67 ± 15.99	19.57 ± 0.54	12.64 ± 0.37
21	2457.43 ± 352.56	1397.20 ± 231.25	19.15 ± 8.78	12.57 ± 2.42	16.51 ± 0.89
22	3095.82 ± 83.73	1144.86 ± 780.28	104.23 ± 5.13	8.89 ± 1.41	13.73 ± 1.37
23	2379.85 ± 71.15	2769.87 ± 1353.30	469.55 ± 184.74	26.00 ± 2.12	7.13 ± 4.99
24	2674.23 ± 229.71	1308.58 ± 296.71	8.53 ± 7.89	12.27 ± 1.31	15.98 ± 3.95
25	2356.82 ± 768.36	4667.31 ± 1961.86	420.65 ± 86.74	30.48 ± 3.51	20.22 ± 6.74
26	2185.49 ± 147.99	2391.68 ± 264.87	458.55 ± 45.37	17.44 ± 0.33	13.13 ± 2.23
Control (PDB)	39.47 ± 10.05	198.24 ± 59.22	154.54 ± 59.93	4.68 ± 0.58	1.59 ± 0.60

Source: Author

medium for *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. The data listed in Tables 6 and 7 show the variation in dry cell weight, exopolysaccharide, cordycepin, adenosine, and mannitol produced by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788, respectively. The dry cell weights and exopolysaccharides produced by *C. militaris* BCC 2819 were in the ranges of 6.88 ± 0.91 - 25.91 ± 1.11 and 0.84 ± 0.31 - 13.22 ±1.61 g/L, respectively. Cordycepin, adenosine and mannitol were produced in the ranges of 71.25 ± 4.06 – 1,176.69 ± 263.33, 13.34 ± 2.21 – 1,273.52 ± 229.30, and 0.16 ± 0.05 – 1,376.92 ± 122.84 mg/L, respectively. The dry cell weight and exopolysaccharide produced by *C. cicadae* BCC 19788 were in the ranges of 2.84 ± 0.58 - 19.67 ± 1.64 and 4.96 ± 1.52 - 42.4 ± 2.52 g/L, respectively. Cordycepin, adenosine and mannitol were produced in the ranges of 42.58 ± 12.57- 4,259.63 ± 224.20, 206.84 ± 114.52- 6,646.58 ± 242.24, and 147.51 ± 17.08– 1,758.81 ± 43.21 mg/L, respectively.

The results from both strains were analyzed and are shown in Tables S3 and S4, in which the generated models of both fungal strains were significant without lack of fits. The cordycepin production model of *C. militaris* BCC 2819 is shown in Table S3. The *p* value ($p \leq 0.001$) for the model and for the lack of fit ($p = 0.21$) demonstrated that the experimental data fit well with the model. The model showed a determination coefficient value (R^2) of 0.94 for cordycepin production, indicating that the model could explain up to 94% of the observed variation in the response. The equation that correlated the six factors and cordycepin production level by *C. militaris* BCC 2819 was as follows:

Y = 547.71 + 167.50A + 45.09B - 29.98C + 82.35D - 129.50E + 32.74F - 32.90AB + 11.93AC - 78.86AD - 0.98AE + 30.33AF + 20.38 BC + 66.73BD - 51.46BE -

Table 6. Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C .militaris* BCC 2819 using the central composite design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	756.99 ± 79.48	356.72 ± 25.84	207.74 ± 19.97	22.94 ± 4.57	3.60 ± 1.25
2	1176.69 ± 263.33	611.25 ± 97.48	222.61 ± 23.54	23.67 ± 1.37	6.82 ± 2.14
3	625.20 ± 141.41	428.52 ± 68.41	158.75 ± 14.02	19.05 ± 3.91	0.00 ± 0.00
4	574.11 ± 135.85	358.74 ± 105.77	125.90 ± 20.14	18.49 ± 1.46	0.00 ± 0.00
5	458.92 ± 229.46	343.10 ± 62.14	78.52 ± 15.24	19.74 ± 1.06	5.04 ± 1.54
6	537.70 ± 268.85	334.62 ± 48.25	318.04 ± 58.14	21.14 ± 3.28	4.96 ± 0.94
7	467.03 ± 26.62	177.25 ± 14.28	38.80 ± 3.25	20.79 ± 0.90	4.58 ± 1.17
8	445.06 ± 25.37	200.79 ± 9.74	210.13 ± 4.85	21.16 ± 0.40	5.46 ± 1.08
9	261.75 ± 104.70	124.97 ± 16.72	24.21 ± 3.47	14.63 ± 0.60	6.58 ± 2.04
10	306.51 ± 78.16	203.35 ± 25.47	30.18 ± 4.96	16.00 ± 3.47	6.32 ± 1.85
11	735.77 ± 272.23	714.27 ± 85.46	262.67 ± 5.41	19.92 ± 1.08	4.50 ± 1.74
12	682.88 ± 187.79	300.01 ± 87.54	226.12 ± 15.24	22.52 ± 4.31	1.74 ± 0.94
13	218.20 ± 40.37	94.13 ± 37.14	9.71 ± 1.05	13.40 ± 0.70	5.04 ± 1.06
14	317.47 ± 65.24	201.70 ± 74.57	30.62 ± 10.54	15.11 ± 0.56	8.94 ± 2.54
15	406.25 ± 117.81	185.14 ± 41.35	261.11 ± 39.57	20.15 ± 0.81	3.70 ± 1.21
16	506.84 ± 146.98	434.29 ± 47.58	227.36 ± 58.14	20.87 ± 1.48	4.68 ± 1.06
17	232.93 ± 67.55	91.76 ± 25.25	6.62 ± 2.41	20.51 ± 3.04	3.98 ± 1.24
18	388.70 ± 112.72	280.31 ± 36.85	4.76 ± 3.54	18.60 ± 1.72	5.40 ± 2.24
19	123.16 ± 61.58	26.13 ± 8.48	9.11 ± 4.12	11.40 ± 1.04	4.74 ± 0.93
20	136.23 ± 32.08	71.43 ± 4.14	1.07 ± 1.01	12.98 ± 1.14	4.34 ± 1.34
21	117.00 ± 27.55	47.28 ± 10.47	0.16 ± 0.05	11.77 ± 2.96	2.66 ± 0.57
22	223.29 ± 52.58	99.33 ± 13.74	4.84 ± 1.24	13.71 ± 0.11	1.98 ± 0.47
23	573.34 ± 135.02	433.56 ± 140.80	148.87 ± 15.24	18.74 ± 2.41	1.62 ± 0.62
24	428.68 ± 234.34	147.83 ± 7.85	178.43 ± 14.15	21.64 ± 5.93	3.94 ± 1.27
25	161.71 ± 70.86	55.03 ± 9.20	4.54 ± 2.17	13.75 ± 2.60	7.88 ± 2.07
26	151.85 ± 45.92	59.36 ± 7.11	55.03 ± 9.87	14.18 ± 1.18	3.50 ± 1.34
27	150.63 ± 35.32	53.87 ± 4.73	12.30 ± 5.57	13.53 ± 1.29	7.74 ± 2.54
28	202.36 ± 71.11	90.59 ± 8.49	65.88 ± 13.53	14.13 ± 0.26	13.06 ± 2.67
29	255.50 ± 36.66	105.04 ± 15.47	381.76 ± 24.95	13.72 ± 3.00	11.20 ± 2.37
30	435.91 ± 183.08	324.81 ± 35.47	198.92 ± 57.84	14.95 ± 0.68	9.30 ± 1.93
31	129.52 ± 47.99	13.34 ± 2.21	5.77 ± 2.41	11.34 ± 1.83	2.56 ± 0.85
32	138.36 ± 32.58	50.22 ± 18.44	5.18 ± 3.14	13.15 ± 2.59	4.70 ± 1.32
33	190.81 ± 81.10	78.94 ± 17.08	18.67 ± 3.41	11.79 ± 0.43	8.48 ± 1.86
34	715.11 ± 157.32	403.00 ± 35.85	333.38 ± 24.74	23.94 ± 1.02	3.02 ± 1.28
35	213.34 ± 47.47	114.52 ± 21.00	2.97 ± 1.65	17.43 ± 1.95	3.36 ± 0.68
36	354.48 ± 78.87	336.57 ± 23.32	12.38 ± 4.58	17.64 ± 1.39	13.22 ± 1.61
37	247.73 ± 55.12	111.78 ± 16.25	6.65 ± 2.87	18.44 ± 1.24	1.78 ± 0.47
38	153.89 ± 34.24	59.69 ± 11.02	9.19 ± 4.24	17.08 ± 3.70	5.24 ± 1.26
39	655.23 ± 177.62	645.30 ± 58.25	302.01 ± 58.74	21.15 ± 1.81	11.78 ± 2.59
40	912.99 ± 184.11	607.97 ± 85.24	574.97 ± 124.02	23.53 ± 3.08	12.66 ± 3.07
41	895.54 ± 217.92	1273.52 ± 229.30	392.92 ± 102.14	18.59 ±1.37	9.16 ± 2.54
42	490.18 ± 174.02	207.58 ± 20.19	1376.92 ± 122.84	25.91 ± 1.11	7.64 ± 2.38
43	472.17 ± 33.05	213.68 ± 6.88	315.95 ± 95.89	22.07 ± 2.07	8.44 ± 3.41
44	574.64 ± 137.32	313.82 ± 60.14	298.50 ± 85.47	20.67 ± 1.52	8.40 ± 2.97
45	552.91 ± 185.57	240.66 ± 22.54	495.10 ± 58.47	19.01 ± 3.36	8.60 ± 2.45
46	502.21 ± 64.78	309.07 ± 15.47	464.39 ± 72.47	20.19 ± 1.42	8.50 ± 2.27
47	430.21 ± 55.50	242.16 ± 19.85	452.59 ± 63.25	19.78 ± 5.34	7.20 ± 2.64
Control (PDB)	71.25 ± 4.06	102.48 ± 8.71	25.47 ± 12.02	6.88 ± 0.91	0.84 ± 0.31

Source: Author

Table 7. Cordycepin, adenosine, mannitol, dry cell weight and exopolysaccharide produced by *C. cicadae* BCC 19788 using the central composite design.

Treatment	Cordycepin (mg/L)	Adenosine (mg/L)	Mannitol (mg/L)	Dry cell weight (g/L)	Exopolysaccharide (g/L)
1	1832.85 ± 433.23	1659.27 ± 412.29	433.23 ± 68.27	11.10 ± 0.99	28.48 ± 2.35
2	2853.09 ± 287.97	2931.86 ± 332.23	287.97 ± 24.59	12.27 ± 1.37	29.66 ± 1.78
3	1285.89 ± 333.19	1094.25 ± 187.42	333.19 ± 20.83	13.75 ± 1.57	29.74 ± 2.30
4	1881.09 ± 324.88	2087.04 ± 411.23	324.88 ± 110.52	14.29 ± 0.85	34.68 ± 2.34
5	2853.00 ± 291.80	2608.88 ± 76.18	291.80 ± 38.90	11.59 ± 0.53	24.54 ± 3.24
6	3558.90 ± 316.13	3073.18 ± 295.04	316.13 ± 22.48	8.97 ± 0.25	29.36 ± 3.00
7	2409.86 ± 277.35	3513.97 ± 312.41	277.35 ± 38.72	17.36 ± 2.16	26.92 ± 8.02
8	2253.22 ± 483.41	2510.15 ± 226.89	483.41 ± 59.46	17.21 ± 3.20	35.30 ± 4.20
9	2563.85 ± 303.88	2491.70 ± 204.43	303.88 ± 27.08	12.97 ± 3.74	31.88 ± 1.29
10	2908.25 ± 307.81	2899.04 ± 187.42	307.81 ± 110.52	13.57 ± 1.10	27.50 ± 1.78
11	3648.77 ± 316.05	3088.36 ± 341.82	316.05 ± 9.59	8.80 ± 1.01	36.80 ± 4.33
12	3653.10 ± 355.99	3452.58 ± 293.88	355.99 ± 11.83	8.26 ± 4.11	29.22 ± 4.72
13	4131.80 ± 284.24	3078.85 ± 170.99	284.24 ± 57.62	9.78 ± 1.90	26.54 ± 1.86
14	4259.63 ± 224.20	6646.58 ± 242.24	180.87 ± 65 .88	10.87 ± 3.74	32.24 ± 5.62
15	2517.58 ± 343.73	2999.62 ± 147.18	343.73 ± 12.82	8.50 ± 3.33	24.02 ± 5.81
16	1537.28 ± 320.19	1512.47 ± 216.35	320.19 ± 25.98	9.47 ± 2.81	28.22 ± 2.50
17	1522.70 ± 289.18	1718.21 ± 515.60	289.18 ± 9.58	16.15 ± 4.83	30.20 ± 3.35
18	2033.61 ± 330.80	2290.91 ± 102.80	333.80 ± 16.50	17.10 ± 6.79	27.82 ± 7.58
19	1799.21 ± 340.81	1889.28 ± 222.59	340.81 ± 29.56	13.05 ± 5.35	33.32 ± 6.54
20	1908.30 ± 222.49	2066.72 ± 317.04	222.49 ± 31.42	12.76 ± 5.59	34.96 ± 6.07
21	1508.98 ± 318.86	1668.58 ± 394.18	318.86 ± 78.52	9.99 ± 0.95	27.14 ± 5.92
22	2532.24 ± 517.79	3981.52 ± 353.75	517.79 ± 48.53	9.09 ± 1.98	25.34 ± 2.31
23	3938.12 ± 233.04	3732.03 ± 276.92	233.04 ± 79.42	13.94 ± 7.37	29.46 ± 6.10
24	1784.90 ± 76.18	1786.33 ± 239.94	76.18 ± 6.41	19.67 ± 1.64	29.94 ± 1.14
25	2604.81 ± 392.75	2372.19 ± 340.94	392.75 ± 10.51	16.15 ± 1.51	24.36 ± 3.82
26	2368.54 ± 167.92	3018.80 ± 248.50	167.92 ± 6.85	15.77 ± 6.64	22.78 ± 2.09
27	1142.12 ± 335.29	1587.53 ± 414.00	335.29 ± 48.71	12.98 ± 3.24	34.06 ± 3.12
28	3064.54 ± 307.98	2516.98 ± 305.87	307.98 ± 42.11	15.31 ± 1.43	29.40 ± 4.53
29	2829.10 ± 267.60	2654.60 ± 359.41	267.60 ± 132.04	10.14 ± 1.27	32.66 ± 6.22
30	2865.42 ± 293.23	2490.21 ± 234.41	293.23 ±86.47	10.73 ± 0.75	33.58 ± 3.36
31	3101.15 ± 492.29	2645.72 ± 267.62	492.29 ± 78.52	13.30 ± 4.22	23.34 ± 5.38
32	1296.25 ± 362.00	1410.11 ± 320.81	362.00 ± 72.15	14.75 ± 0.79	23.88 ± 4.25
33	2180.30 ± 1758.81	2406.75 ± 189.10	1758.81 ± 43.21	11.51 ± 1.83	29.38 ± 2.82
34	2267.07 ± 310.97	2525.89 ± 222.58	310.97 ± 31.52	11.02 ± 2.03	31.72 ± 4.73
35	1612.73 ± 414.89	1158.02 ± 206.29	414.89 ± 46.18	13.01 ± 3.44	18.36 ± 5.36
36	2331.27 ± 243.41	2533.30 ± 362.00	243.41 ± 41.20	10.47 ± 5.43	29.76 ± 1.12
37	2211.01 ± 1154.35	2080.00 ± 226.97	1154.35 ± 78.25	10. ± 0.07	25.24 ± 1.02
38	1916.76 ± 344.95	2159.65 ± 162.23	344.95 ± 57.43	12.11 ± 0.34	42.40 ± 2.52
39	1474.06 ± 306.82	1309.83 ± 210.97	306.82 ± 27.13	10.38 ± 3.94	32.02 ± 11.20
40	3253.62 ± 394.43	2637.07 ± 324.60	394.43 ± 45.40	10.31 ± 2.65	26.44 ± 2.39
41	863.43 ± 295.05	1394.57 ± 176.92	295.05 ± 48.16	8.14 ± 2.54	27.90 ± 7.21
42	1412.47 ± 399.66	1858.89 ± 308.91	399.66 ± 26.98	12.71 ± 0.74	35.22 ± 15.24
43	1973.13 ± 468.89	2236.15 ± 158.24	468.89 ± 96.58	11.90 ± 2.25	29.62 ± 3.19
44	3236.59 ± 253.05	3264.85 ± 256.19	253.05 ± 83.23	12.41 ± 6.22	27.80 ± 4.10
45	1706.80 ± 326.75	1948.98 ± 344.18	326.75 ± 79.20	11.24 ± 3.22	28.16 ± 9.41
46	3123.80 ± 261.24	2795.53 ± 498.95	261.24 ± 67.58	11.64 ± 1.50	29.42 ± 7.19
47	213.71 ± 111.27	2252.84 ± 259.56	311.27 ± 100.11	10.99± 2.35	31.04 ± 9.21
Control (PDB)	42.58 ± 12.57	206.84 ± 114.52	147.51 ± 17.08	2.84 ± 0.58	4.96 ± 1.52

Source: Author

$63.73BF + 84.77CD - 86.29 CE + 19.13 CF + 27.86 DE - 26.35DF + 33.85EF - 47.75A^2 - 166.77B^2 - 150.69C^2 + 87.44D^2 + 50.19E^2 - 18.99F^2$

where Y is the concentration of cordycepin (g/L), A is the concentration of glucose (g/L), B is the concentration of adenine (g/L), C is the concentration of glycine (g/L), D is the concentration of alanine (g/L), E is the concentration of casein hydrolysate (g/L), and F is the concentration of vitamin solution (mL/L).

According to the model using CCD, the optimal medium for cordycepin production by *C. militaris* BCC 2819 consisted of glucose 60 g/L, adenine 4.41 g/L, glycine 6.4 g/L, alanine 10 g/L, glutamine 2 g/L, casein hydrolysate 5 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 2 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 mL/L and vitamin solution 4 mL/L.

The cordycepin production model of *C. cicadae* BCC 19788 is shown in Table S4. The *p* value ($p = 0.0245$) for the model and for the lack of fit ($p = 0.74$) demonstrated that the experimental data fit well with the model. The model showed a determination coefficient value (R^2) of 0.78 for cordycepin production, indicating that the model could explain up to 78% of the observed variation in the response.

The equation that correlated the six factors and cordycepin production level by *C. cicadae* BCC 19788 was as follows:

$$Y = 94.2120 - 16.494A - 40.175B + 46.319C - 39.10D + 448.46E + 70.16F + 240.50AB + 187.57AC + 516.02AD - 317.20AE + 37.71AF + 107.16BC + 6.21BD - 203.31BE + 636.80BF - 277.48CD + 178.76CE -457.05CF - 230.83DE + 314.59DF + 29.42EF + 163.67A^2 - 384.31B^2 + 134.30C^2 + 159.93D^2 + 185.92E^2 + 26.17F^2$$

where Y is the concentration of cordycepin (g/L), A is the concentration of $(NH_4)_2SO_4$ (g/L), B is the concentration of adenine (g/L), C is the concentration of glycine (g/L), D is the concentration of alanine (g/L), E is the concentration of casein hydrolysate (g/L), and F is the concentration of vitamin solution (mL/L).

According to the model using CCD, the optimal medium for cordycepin production by *C. cicadae* BCC 19788 consists of glucose 20 g/L, adenine 5 g/L, glycine 30 g/L, alanine 5 g/L, glutamine 2 g/L, casein hydrolysate 10 g/L, peptone 10 g/L, yeast extract 15 g/L, $(NH_4)_2SO_4$ 5.68 g/L, $MgSO_4$ 0.5 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, trace element 2 mL/L and vitamin solution 6 mL/L.

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in batch fermenters

To evaluate the optimal medium for cordycepin production obtained by CCD experiment, batch fermentation was performed with optimal medium determined from previous experiments at a working volume of 3 L in a 5-L fermenter. The highest cordycepin production of 2,598.44 ± 57.16 and 2,998.44 ±20.11 mg/L was produced by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 at 120 and 72 h, respectively, as shown in Figure 2A and B.

For other bioactive compounds, *C. militaris* BCC 2819 *and C. cicadae* BCC 19788 produced the highest adenosine and mannitol contents of 3,158.58 ± 287.45 and 4,580.35 ± 287.14 mg/L, respectively. Moreover, the highest cell mass and exopolysaccharide content produced by *C. militaris* BCC 2819 were 56.08 ± 1.29 and 42.18 ± 1.40 g/L, respectively (Figure 1A). The highest cell mass and exopolysaccharide content produced by *C. cicadae* BCC 19788 were 45.23 ± 3.41 and 44.08 ± 3.55 g/L, respectively (Figure 1B). This result demonstrated that the optimal medium from the CCD experiment could be used at a larger production scale of 5-L fermenter.

Cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 in fed-batch fermenters with an exponential feed rate

In this experiment, fed-batch fermentation was performed to increase the concentration of cordycepin by both fungal strains. The highest cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 reached 3,112.50 ± 712.00 and 3,587.10 ± 247.70 mg/L, respectively, as shown in Figure 4. For the other bioactive compounds (adenosine and mannitol), *C. militaris* BCC 2819 produced 2,897.40 ± 382.47 and 5,981.10 ± 254.72 mg/L, and *C. cicadae* BCC 19788 produced 3,78520 ± 165.70 and 6,100.20 ± 191.14 mg/L, respectively, as shown in Figure 4A and B. Furthermore, the highest cell mass and exopolysaccharide content of *C. militaris* BCC 2819 reached 66.35 ± 1.91 and 43.90 ± 2.51 g/L, while those of *C. cicadae* BCC 19788 reached 63.81 ± 1.03 and 38.10 ± 2.84 g/L, respectively, as shown in Figure 3A and B. This result indicated that fed-batch fermentation could be used to improve the production in term of concentration of cordycepin and the other bioactive compounds by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788.

DISCUSSION

In this study, the two potential candidate strains of *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 were selected to develop cordycepin production processes in submerged fermentation based on our prelimary screening data (data not shown). The media compositions were screened and selected using statistical experiments to favor and improve higher production of cordycepin.

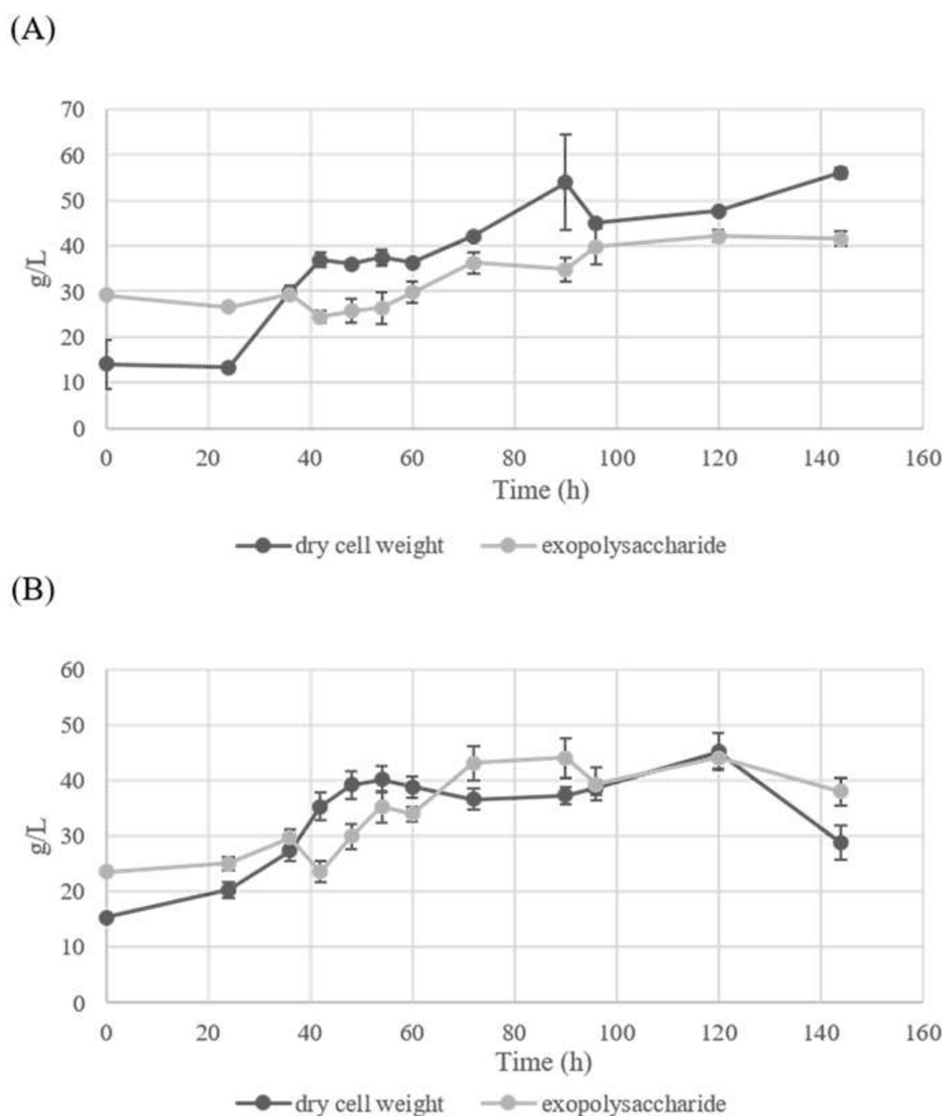
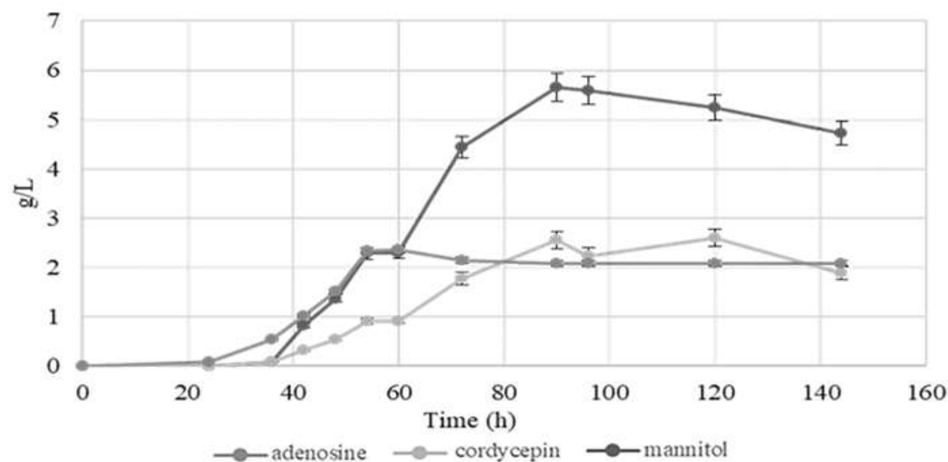


Figure 1. Dry cell weight and exopolysaccharide content of *C .militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in batch fermentation.
Source: Author

The results showed that glucose, adenine, glycine, alanine, casein hydrolysate and vitamin solution favored the cordycepin production of *C. militaris* BCC 2819, while $(NH_4)_2SO_4$ affected the cordycepin production of *C. cicadae* BCC 19788 instead of glucose. These results were similar to the report of Mao et al. (2005) in which they demonstrated that glucose was the most suitable carbon source for cordycepin production of *C. militaris* (Mao et al., 2005) and Lee et al. (2019) reported that casein hydrolysate was the most beneficial nitrogen source for cordycepin production of *C. militaris* KYL05. Das et al. (2010) and Mao et al. (2005) demonstrated the improvement of cordycepin production of *C. militaris* using yeast extract and peptone in the medium compositions and obviously improved cordycepin production. Moreover, glycine, glutamine and alanine were reported as additives for the improvement of cordycepin production (Das et al., 2009; Wen et al., 2016). Furthermore, adenosine was also used as an additive for cordycepin production as shown in Vikas et al. (2020) report.

In this study, a significant improvement in cordycepin production was achieved by formulating the optimal medium for submerged fermentation. The highest cordycepin production by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 reached 3,112.50 and 3,587.10 mg/L

(A)



(B)

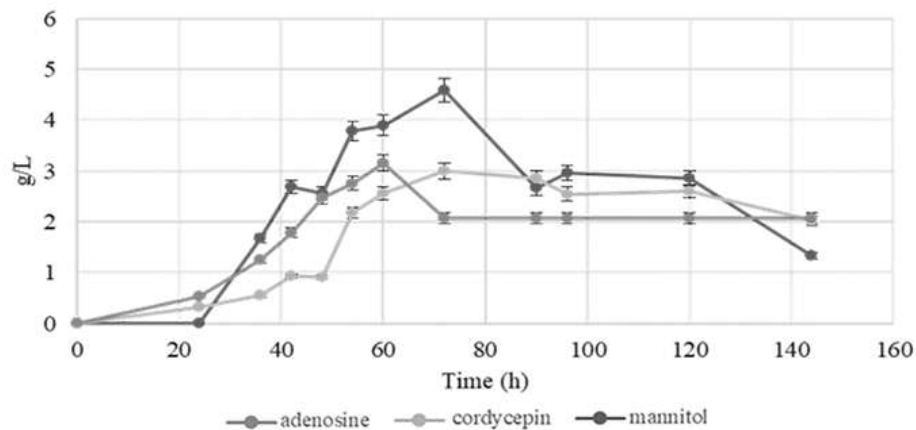


Figure 2. Adenosine, cordycepin and mannitol production by *C. militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in batch fermentation
Source: Author

in the 5-L fed-batch fermenter, respectively. Compared to other studies, Das et al. (2010) reported that a mutant *C. militaris* strain generated by ion beam irradiation produced 6,840 mg/L cordycepin in a 100-mL culture. Tang et al. (2018) reported a production of 5,290 mg/L cordycepin via two-step culture of *C. militaris* in a 100-mL culture. However, on a larger scale, Mao and Zhong (2004) reported that *C. militaris* produced 201.1 mg/L cordycepin by using two-stage dissolved oxygen control in a 5-L fermenter. Moreover, Mao and Zhong (2006) reported the production of 346.1 mg/L cordycepin in a 3.5-L fermenter through fed-batch fermentation with $NH4^+$ feeding. In a previous report, the yield of cordycepin decreased in the scale up step to the fermenter. In our study, the cordycepin yield slightly decreased after performing the validation in the fermenter. The present study reported the highest cordycepin production in fermenters by submerged fermentation using the new isolates *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 as fugal cell factories.

In addition to cordycepin, additional bioactive compounds, including adenosine, mannitol and exopolysaccharide, were produced by *Cordyceps*. The pharmacological effects of adenosine have been reported. Adenosine can be used as a cardioprotective and therapeutic agent for chronic heart failure (Kitakaze and Holi, 2000), and it could also inhibit the release of neurotransmitters in the central nervous system (Ribeiro, 1995). Polysaccharides are considered to possess anti-inflammatory, antioxidant (Wen et al., 2013), antitumor (Zhang et al., 2007), antimetastatic, immunomodulatory, hypoglycemic activity (Kiho et al., 1996), steroidogenic, and hyperlipidemia. Mannitol (cordycepic acid) has diuretic, anti-tussive and anti-free

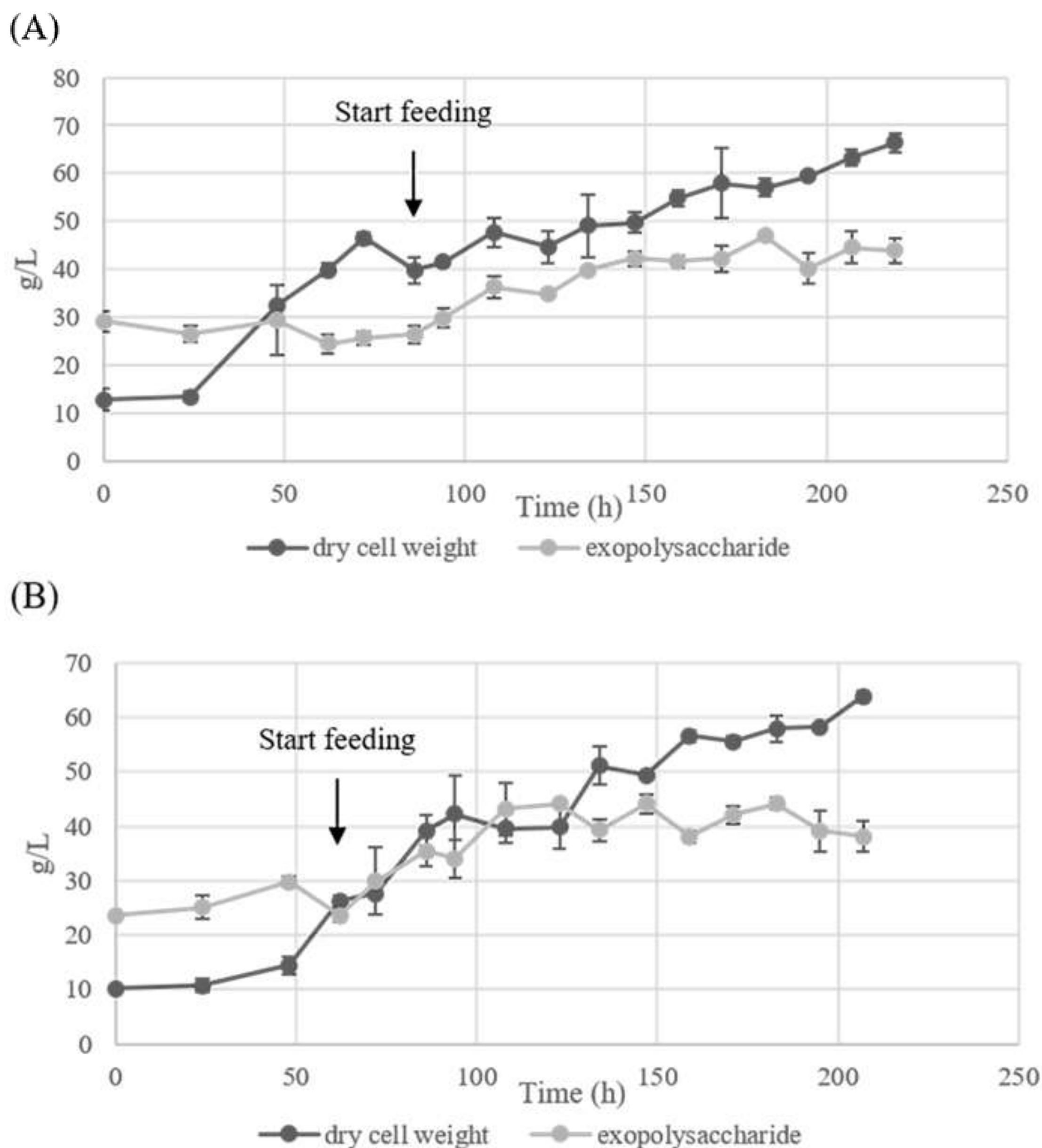


Figure 3. Dry cell weight and exopolysaccharide content of *C .militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in fed-batch fermentation.
Source: Author

radical activities (Li et al., 2006). In this study, the highest yield of cordycepin was produced, and both fungal strains also produced adenosine, mannitol and exopolysaccharide at high yield in optimal medium with fermentation processes. These results demonstrated the optimal cordycepin production media, fermentation processes and cordycepin-producing strains with high potential for applications in cordycepin production on pilot and industrial scales.

Conclusion

The present study successfully optimized medium for cordycepin production, a potential bioactive compound in cordyceps, along with the production of other bioactive compounds of *C. militaris* BCC 2819 and *C. cicadae* BCC 19788. These two fungal cell factories are new high cordycepin-producing fungal isolates that were isolated from natural resources. Compared with the unoptimized

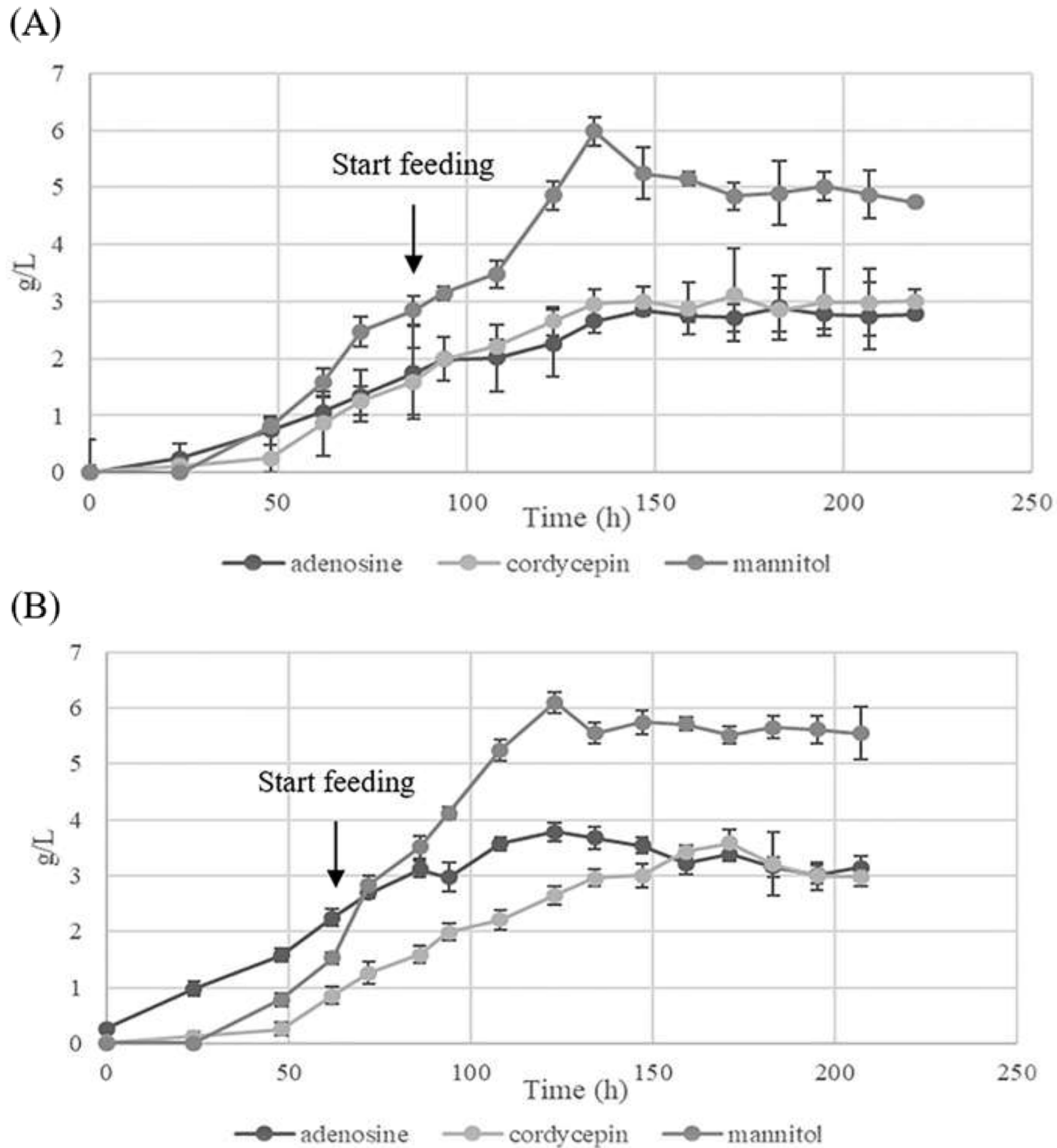


Figure 4. Adenosine, cordycepin and mannitol production by *C .militaris* BCC 2819 (A) and *C. cicadae* BCC 19788 (B) cultivated in fed-batch fermentation.
Source: Author

conditions, cordycepin production in fed-batch fermentation increased up to 45-fold in *C. militaris* BCC 2819 and up to 90-fold in *C. cicadae* BCC 19788. Moreover, other bioactive compounds, including adenosine, mannitol and exopolysaccharide, were also produced at high yields. This result showed that the optimized medium and processes developed for the production of cordycepin and other bioactive compounds by *C. militaris* BCC 2819 and *C. cicadae* BCC 19788 will be new approaches for commercial-scale production that replace the conventional process of solid-state fermentation. This process can also be used on an industrial scale, potentially with a shortened cultivation period for higher productivity and lower production costs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY

Table S1. ANOVA of cordycepin production by *C .militaris* BCC 2819 using the Plackett-Burman design.

Source	Sum ofsquares	df	Meansquare	FValue	p-value (Prob > F)	
Block	0.16	2	0.079			
Model	2.86	24	0.12	3.30	0.0021	significant
A-Glucose	2.06	1	2.06	57.02	< 0.0001	
B-Peptone	0.026	1	0.026	0.72	0.4054	
C-Yeast extract	0.078	1	0.078	2.14	0.1557	
D-$(NH_4)_2SO_4$	0.032	1	0.032	0.89	0.3543	
E-Adenine	0.048	1	0.048	1.34	0.2587	
F-Glutamine	0.023	1	0.023	0.65	0.4291	
G-Glycine	0.098	1	0.098	2.71	0.1122	
H-Alanine	0.045	1	0.045	1.26	0.2730	
J-Casein hydrolysate	0.19	1	0.19	5.13	0.0324	
K-Vitamin solution	0.049	1	0.049	1.35	0.2563	
L-Trace element	0.026	1	0.026	0.71	0.4061	
AB	0.022	1	0.022	0.61	0.4430	
AC	0.035	1	0.035	0.96	0.3375	
AD	0.033	1	0.033	0.90	0.3507	
AE	0.051	1	0.051	1.42	0.2451	
AF	0.054	1	0.054	1.48	0.2348	
AG	0.039	1	0.039	1.09	0.3069	
AH	0.087	1	0.087	2.41	0.1332	
AJ	0.25	1	0.25	7.00	0.0139	
AK	0.081	1	0.081	2.24	0.1468	
AL	0.051	1	0.051	1.42	0.2441	
BC	0.028	1	0.028	0.79	0.3836	
ABC	0.063	1	0.063	1.74	0.1988	
ABCD	0.074	1	0.074	2.04	0.1651	
Residual	0.90	25	0.036			
Lack of Fit	0.89	24	0.037	3.52	0.4013	not significant
Pure Error	0.011	1	0.011			
Cor Total	3.92	51				
R-Squared	0.76					

Source: Author

Table S2. ANOVA of cordycepin production by *I .cicadae BCC 19788* using the Plackett-Burman design.

Source	**Sum of squares**	**df**	**Mean square**	**FValue**	**p-Value (Prob > F)**	
Block	3.39	2	1.70			
Model	31.82	24	1.33	2.45	0.0150	Significant
A-Glucose	3.75	1	3.75	6.94	0.0143	
B-Peptone	0.56	1	0.56	1.03	0.3198	
C-Yeast extract	0.65	1	0.65	1.20	0.2835	
D-$(NH_4)_2SO_4$	0.79	1	0.79	1.46	0.2383	
E-Adenine	0.63	1	0.63	1.17	0.2896	
F-Glutamine	0.21	1	0.21	0.40	0.5347	
G-Glycine	0.45	1	0.45	0.84	0.3691	
H-Alanine	2.50	1	2.50	4.61	0.0416	
J-Casein hydrolysate	0.43	1	0.43	0.80	0.3787	
K-Vitamin solution	0.72	1	0.72	1.33	0.2589	
L-Trace element	1.70	1	1.70	3.14	0.0887	
AB	8.11	1	8.11	14.99	0.0007	
AC	0.21	1	0.21	0.39	0.5400	
AD	0.94	1	0.94	1.74	0.1986	
AE	0.87	1	0.87	1.61	0.2164	
AF	0.79	1	0.79	1.46	0.2379	
AG	0.55	1	0.55	1.02	0.3216	
AH	0.68	1	0.68	1.26	0.2726	
AJ	0.021	1	0.021	0.040	0.8438	
AK	6.577E-003	1	6.577E-003	0.012	0.9131	
AL	1.01	1	1.01	1.86	0.1844	
BC	0.93	1	0.93	1.72	0.2011	
ABC	0.92	1	0.92	1.70	0.2037	
ABCD	1.07	1	1.07	1.97	0.1725	
Residual	13.53	25	0.54			
Lack of Fit	13.13	24	0.55	1.38	0.5975	Not significant
Pure Error	0.40	1	0.40			
Cor Total	48.74	51				
R-Squared	0.70					

Source: Author

Table S3. ANOVA of cordycepin production by *C .militaris* BCC 2819 using the central composite design.

Source	Sum of squares	df	Mean square	FValue	p-Value (Prob > F)	
Model	2.793E+006	27	1.035E+005	11.43	< 0.0001	significant
A-Glucose	1.374E+005	1	1.374E+005	15.18	0.0010	
B-Adenine	9961.64	1	9961.64	1.10	0.3074	
C-Glycine	4403.04	1	4403.04	0.49	0.4940	
D-Alanine	33218.73	1	33218.73	3.67	0.0706	
E-Casein	82156.05	1	82156.05	9.07	0.0072	
F-Vitamin solution	5249.98	1	5249.98	0.58	0.4557	
AB	34638.50	1	34638.50	3.83	0.0653	
AC	4550.85	1	4550.85	0.50	0.4870	
AD	26422.47	1	26422.47	2.92	0.1039	
AE	4.07	1	4.07	4.490E-004	0.9833	
AF	29445.34	1	29445.34	3.25	0.0872	
BC	1763.84	1	1763.84	0.19	0.6639	
BD	1.425E+005	1	1.425E+005	15.74	0.0008	
BE	84730.73	1	84730.73	9.36	0.0065	
BF	17264.07	1	17264.07	1.91	0.1834	
CD	2.300E+005	1	2.300E+005	25.40	< 0.0001	
CE	2.383E+005	1	2.383E+005	26.32	< 0.0001	
CF	1554.97	1	1554.97	0.17	0.6832	
DE	3298.20	1	3298.20	0.36	0.5533	
DF	22209.97	1	22209.97	2.45	0.1338	
EF	36676.00	1	36676.00	4.05	0.0585	
A^2	30418.42	1	30418.42	3.36	0.0825	
B^2	1.819E+005	1	1.819E+005	20.09	0.0003	
C^2	3.029E+005	1	3.029E+005	33.46	< 0.0001	
D^2	1.020E+005	1	1.020E+005	11.26	0.0033	
E^2	33597.28	1	33597.28	3.71	0.0692	
F^2	4812.06	1	4812.06	0.53	0.4749	
Residual	1.720E+005	19	9054.35			
Lack of Fit	14596.77	1	14596.77	1.67	0.2127	not significant
Pure Error	1.574E+005	18	8746.44			
Cor Total	2.966E+006	46				
R-Squared	0.94					

Source: Author

Table S4. ANOVA of cordycepin production by *I .cicadae* BCC 19788 using the central composite design.

Source	Sum of squares	df	Mean square	FValue	p-Value (Prob > F)	
Model	2.782E+007	27	1.031E+006	2.43	0.0245	significant
A)-NH4(2SO4	1.196E+006	1	1.196E+006	2.82	0.1095	
B-Adenine	1.507E+005	1	1.507E+005	0.36	0.5582	
C-Glycine	4.999E+005	1	4.999E+005	1.18	0.2913	
D-Alanine	7489.24	1	7489.24	0.018	0.8957	
E-Casein hydrolysate	9.853E+005	1	9.853E+005	2.32	0.1440	
F-Vitamin solution	24114.78	1	24114.78	0.057	0.8141	
AB	1.851E+006	1	1.851E+006	4.36	0.0504	
AC	1.126E+006	1	1.126E+006	2.65	0.1198	
AD	1.131E+006	1	1.131E+006	2.67	0.1189	
AE	4.275E+005	1	4.275E+005	1.01	0.3281	
AF	45512.76	1	45512.76	0.11	0.7468	
BC	48786.51	1	48786.51	0.11	0.7383	
BD	1235.61	1	1235.61	2.912E-003	0.9575	
BE	1.323E+006	1	1.323E+006	3.12	0.0935	
BF	1.723E+006	1	1.723E+006	4.06	0.0583	
CD	2.464E+006	1	2.464E+006	5.81	0.0263	
CE	1.023E+006	1	1.023E+006	2.41	0.1370	
CF	8.875E+005	1	8.875E+005	2.09	0.1644	
DE	2.264E+005	1	2.264E+005	0.53	0.4740	
DF	3.167E+006	1	3.167E+006	7.46	0.0132	
EF	27698.15	1	27698.15	0.065	0.8011	
A^2	3.573E+005	1	3.573E+005	0.84	0.3703	
B^2	1.970E+006	1	1.970E+006	4.64	0.0442	
C^2	2.406E+005	1	2.406E+005	0.57	0.4606	
D^2	3.412E+005	1	3.412E+005	0.80	0.3811	
E^2	4.611E+005	1	4.611E+005	1.09	0.3103	
F^2	9136.11	1	9136.11	0.022	0.8849	
Residual	8.061E+006	19	4.243E+005			
Lack of Fit	51211.00	1	51211.00	0.12	0.7384	not significant
Pure Error	8.010E+006	18	4.450E+005			
Cor Total	3.588E+007	46				
R-Squared	0.78					

Source: Author

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Semi-artificial method of induced breeding of the African catfish (*Clarias gariepinus,* Burchell, 1822) under varying broodstock ratios using Ovaprim®

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Modern methods of producing African catfish (*Clarias gariepinus*) fingerlings require that the male brooder is sacrificed to obtain milt for artificial fertilization of the eggs stripped from the female under hormonal induction. This study assessed the semi-artificial technique of producing catfish larvae with different broodstock ratios using Ovaprim®, a synthetic spawning inducing hormone. The treatments with 3 replicate each were: T1 (artificial spawning with 1:1 female: male ratio), T2 (semi-artificial spawning with 1:1 female: male ratio), and T3 (semi-artificial spawning with 2:1 female: male ratio). The relative fecundity of brooders in T1, T2, and T3 was 68 ± 6.31, 78 ± 12.29, and 65 ± 8.18, respectively with no significant difference ($P \geq 0.05$). Percent fertilization for T1 (81±1.52%), T2 (75 ± 2.51%) and T3 (62 ± 2.50%) was significantly different ($P \leq 0.05$). The observed percent hatchability (85 ± 2.51, 83 ± 3.21, and 82 ± 2.50%) in respect of T1, T2, and T3 was not statistically different ($P \geq 0.05$). Differences in total egg weight (96 ± 3.30, 72 ± 10.53, and 59 ± 0.50 g; p=0.099), and total larval production (57,700 ± 3672; 42,423 ± 6972 and 34,078 ± 762; p=0.002) for T1, T2 and T3, respectively, were statistically significant between artificial spawning and semi-artificial spawning. Larval survival was significant ($P \leq 0.05$) between T1 (84 ± 2.31) and T3 (92 ± 2.50) but both did not differ significantly ($P \geq 0.05$) from T2 (87 ± 2.51). In conclusion, the semi-artificial spawning of *C. gariepinus* with Ovaprim® could be beneficial to fish farmers if done at a broodstock sex pairing ratio of 1:1.

Key words: *Clarias gariepinus*, induced breeding, Ovaprim®, semi-artificial, aquaculture.

INTRODUCTION

In the past, fish farmers have collected their fish fingerlings from the wild, but due to challenges such as erratic supply and poor quality, it is not reliable to source fingerlings from natural waters to sustain commercial aquaculture (Olumuji and Mustapha, 2012; Ali et al., 2016, 2020). Increase in demand for fingerlings, necessitated by the remarkable growth in the fish culture industry, has exacerbated the requirement for artificial

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propagation of catfish fingerlings (Nwokoye et al., 2007). The major challenge to captive breeding of *Clarias gariepinus,* which is their inability to breed under captive conditions (Adebayo and Fagbenro, 2004) due to stress induced ovarian atresia (Lubzens et al., 2010), must however, be resolved to help meet this demand.

Thus, to instigate the release of eggs, hormones are used to overcome the limitations of producing the eggs in captivity. This is necessary for the catfish to proceed through spawning without the limitation of ovarian atresia. Hormonal induction is carried out either by injecting natural hormone extract from the pituitary gland of same fish species or other fish species (tilapia and carp) (Fagbenro et al., 1993) or the injection with synthetic hormones (Salami et al., 2006). Nwokoye et al. (2007) and Ngueku (2015) revealed that hormones such as Human Chorionic Gonadotrophin (HCG), Decorticosterone Acetate (DOCA), Pituitary extracts, Ovaprim®, Ovatide and Ovaryprim have been used to induce reproduction in the African catfish with success. However, in all the techniques developed to induce artificial reproduction in the African catfish, the female is stripped and the male is killed in order to obtain their eggs and milt, respectively. Some attempts at sparing the male catfish is by abdominal incision to extract milt from the gonads (Yisa et al., 2013), hand stripping of milt from live male (Viveiros et al., 2002) and removal of one testis and suturing (Egwui and Nwanko, 2015), but these techniques even though successful to some extent have limitations. For instance, abdominal incision requires special care to ensure survival and post-surgery recuperation, whereas hand stripping can fail because of anatomical blockage in African catfish males, and sutured males, used for breeding only twice. This work seeks to demonstrate that the African catfish can be bred commercially without sacrificing the male catfish, hence, reducing the overall cost of fingerling production. The technique of inducing the male African catfish in captivity to spontaneously release milt for fertilization during spawning, will not only save it for subsequent breeding but also provide a simpler method for breeding the African catfish under hatchery conditions.

MATERIALS AND METHODS

This study was carried out at Flosell Farms Limited, a reputable farm that produces tilapia and catfish (table fish and fingerlings), located at Sogakope in the Volta Region of Ghana. Mature brood stocks (27 females and 18 males) were obtained and kept in separate rectangular concrete tanks (one for males and another for females) for one month during which period they were fed with 35% crude protein Skretting Fish Feed (Skretting, Egypt) at 5% body weight. Twelve (12) females and nine (9) males were selected for the breeding experiment based on their ripeness. Ripeness of females was determined morphologically by the swelled abdomen and readiness to spawn (eggs ooze out easily when the abdomen is gently pressed). The reddening of the genital papilla was used as an indicator of ripeness in the males (Natea et al., 2017; Ngueku, 2015; Olumuji and Mustapha, 2012).

The total length (cm) and corresponding weight (g) of the brooders were measured prior to hormone administration. The condition of the brooders was determined using the Fulton's condition Factor (K) from the relationship (Ricker, 1975; Barnham et al., 1998):

$$K = (W/L^3) \times 100.$$

where W is the mean weight and L^3 is the total length of the fish in cm cubed.

Experimental design

The male and female brooders were grouped into three (3) treatments with three (3) replicates each. The treatment groups with female to male ratios were as follows:

Treatment 1 (T1, Control): Artificial spawning with 1:1 ratio
Treatment 2 (T2): Semi-artificial spawning with 1:1 ratio
Treatment 3 (T3): Semi-artificial spawning with 2:1 ratio

T1 (artificial spawning) was made up of one matured female and one matured male brooder, where both male and female brooders were injected with Ovaprim®, the synthetic spawning hormone (Syndel, USA) and kept in separate plastic tanks for a latency period of 11 h. After the latency period, the female was stripped and the male was sacrificed, and the gonads were removed to obtain the milt. This treatment was designated as the Control group because it is the most common method used by hatcheries in the production of catfish fingerlings (Abdulraheem et al., 2012). T2 and T3 were the semi-artificial spawning, where both female and male brooders were injected with Ovaprim® and left for 11 h to allow for natural spawning, fertilization and incubation. T2 had a female: male ratio of 1:1 and T3 consisted of a female: male ratio of 2:1.

Hormone injection

Ovaprim® was used as the spawning inducing hormone. All the brooders were disinfected in a salt bath (5 g sodium chloride per litre of water), after starving for a period of 24 h and weighed before Ovaprim® was administered. The brooders were injected intramuscularly with the hormone using a syringe with a needle inserted 2.00 to 2.50 cm at an angle of 45° (Abdulraheem et al., 2012). A dosage of 0.50 ml/kg brood stock weight (BW) and 0.25 ml/kg BW of the hormone was administered to the females and males, respectively.

Stripping and fertilization for artificial spawning

Injected female brooders were placed in circular plastic tanks (1.5 m^3) fill with water to 30 cm depth with continuous aeration and flow-through. The female brooders were stripped into dry bowls and weighed after the latency period. The total number of eggs was determined by taking three representative samples (1 g each) and counting the eggs with the aid of an egg counter. Relative fecundity was estimated as follows:

Relative Fecundity = (Total no. of eggs / body weight of female).

The males were sacrificed by dissecting to remove the testes. In order to collect sperms, the testes were cut into pieces with a sterile surgical blade and the milt was extracted and used to fertilize the eggs. The eggs were fertilized by mixing the milt with little quantity (25 ml) of saline solution in a Petri dish and milt mixture was poured over the eggs and mixed with a clean feather after adding clean

Table 1. Body weight, length and condition factor (K) for female *Clarias gariepinus* broodstocks.

Parameter	T1	T2	T3
Female body weight (g)	1000 ± 132.28[a]	600 ± 50.00[b]	616 ± 60.55[b]
Female body length (cm)	51 ± 2.51[a]	43 ± 2.46[b]	43 ± 2.54[b]
K – Factor	0.75 ± 0.05[a]	0.76 ± 0.07[a]	0.75 ± 0.09[a]

Values are means ± standard deviations. Means in the same row with different superscripts differ significantly ($P \leq 0.05$).
Source: Authors

water to activate the sperms and prevent the coagulation of the eggs. The fertilized eggs were spread evenly on an incubation tray, which was a 50 cm × 60 cm rectangular frame of Polyvinyl Chloride (PVC) pipes, overlaid with a 2 mm nylon mesh. The tray was placed in a circular plastic tank with water at 40 cm deep with continuous aeration in a flow-through system. Hatching occurred approximately 22 h after incubation and the hatching trays were removed from the tanks when the hatched larvae had penetrated the nylon mesh and gathered at the edges and at the bottom of the tank.

Semi-artificial spawning

For this treatment, injected female and males were placed in hapas (Cloth hatchery), made with nylon netting material (2 mm mesh size) within the circular plastic tanks and allowed to spawn. Brooders were carefully removed from the hapas with a scoop net after the 11 h latency period and weighed to determine the weight of eggs spawned. The mass of eggs spawned was determined by calculating the difference in weight of each female catfish before and after spawning. The overall number of eggs reproduced was estimated by multiplying the weight of spawned eggs by quantity of eggs in 1 g (Tiamiyu et al., 2015). Eggs spawned were incubated in the same tank for a period of 24 h. The hapas and unhatched eggs were removed to prevent fungal infection from egg shells. The relative fecundity was then estimated.

Estimation of percent fertilization, hatchability and survival

Percent fertilization was determined using Ella method (Ella, 1987) as described by Ataguba et al. (2012). A glass tube (30 cm in length and 2.5 mm in diameter) was used to siphon eggs from the egg mass. Samples were taken from areas of the hapa or hatching tray with the fertilized egg mass. Good and bad eggs were counted by viewing the glass tube against a source of light to determine total numbers of good and bad eggs. The fertilized eggs were seen as bright and shiny while unfertilized eggs appeared opaque and white. Fertilization success was estimated as:

%Fertilization = [(N-b)/N] × 100

where N = the total number of eggs spawned, b = number of bad eggs, b was estimated by the ratio:

b = [(*y/x*) × N],

where *y* = number of bad eggs counted and *x* = total number of eggs in the samples.

Hatchability and survival rates were calculated at 30 h and 5 days after hatching (Adebayo and Popoola, 2008). These rates were calculated as:

%Hatchability = [(No. of hatched eggs in a sample / Total no. of eggs in a sample) × 100]

%Survival = [(No. of larvae alive / Total no. of hatchlings) × 100]

Statistical analysis

Data collected were subjected to one-way Analysis of Variance (ANOVA) test. The test of significance (at α level of 0.05) for egg weight, egg number, relative fecundity, hatchability rate and survival rate were compared for statistical differences using Tukey's HSD with PRIMER 6.0 software.

RESULTS

Broodstock characteristics

The weight of female broodstock used for the experiment was in the range of 600 to 1000 g (Table 1). Artificial spawning with 1:1 female to male ratio (T1) had the highest mean female weight (1000 ± 132.28 g) followed by semi-artificial spawning with 2:1 ratio (T3). Semi-artificial spawning with 1:1 ratio (T2) showed the lowest mean female weight (600 ± 50.00 g). The statistical differences in mean female brood stock weight among T1, T2 and T3 was significant ($P \leq 0.05$), but mean weight variation in T2 and T3 was not significant (($P \geq 0.05$).

Induced spawning of *C. gariepinus* broodstock

Spawning was observed in all treatment groups as indicated in Table 2. Mean number of eggs counted was highest (67,573 ± 2,315.65) in artificial spawning (T1) and lowest (41,300 ± 350.00) in semi-artificial spawning with 2:1 ratio (T3) ($P \leq 0.05$), but the difference in means of semi-artificial spawning (T2) (50,633 ± 7,377.72) and T3 (41,300 ± 35.00) were not significant (($P \geq 0.05$). The mean total egg weight estimated was 96 ± 3.30, 72 ± 10.53 and 59 ± 0.50 g for T1, T2, and T3, respectively. The highest mean total egg weight (96 ± 3.30 g) was recorded in T1 and the lowest (59 ± 0.50 g) was in T3. Differences in means were significant ($P \leq 0.05$) between T1 and T3 but not between T2 and T3 ($P \geq 0.05$).

Mean relative fecundity was 68 ± 6.31, 78 ± 12.29 and 65 ± 8.18 for T1, T2 and T3, respectively (Table 2). T2 showed the highest value (78 ± 12.29) and T3 recorded the lowest value (65 ± 8.18) with no significant difference

Table 2. Spawning characteristics of *Clarias gariepinus* in the different treatments.

Parameter	T1	T2	T3
Egg number	67,573 ±2,315.65[a]	50,633 ±7,377.72[b]	41,300 ±350.00[b]
Total egg weight (g)	96 ±3.30[a]	72 ±10.53[b]	59 ±0.50[b]
Relative fecundity	68 ±6.31[a]	78 ±12.29[a]	65 ±8.18[a]

Values are means ± standard deviations. There is a significant difference between means in the same row with different superscripts ($P \leq 0.05$).
Source: Authors

Table 3. Mean percentage fertilization, hatchability, larval survival and mean larval production.

Parameter	T1 (Control)	T2	T3
Fertilization (%)	81±1.52[a]	75±2.51[b]	62±2.50[c]
Hatchability (%)	85±2.51[a]	83±3.21[a]	82±2.50[a]
Larval production	57,700±3,672[a]	42,423±6,973[b]	34,078±1,321[b]
Larval survival (%)	84±3.21[a]	87±2.51[a]	92±2.50[b]

Values are means ± standard deviations. There is a significant difference between means in the same row with different superscripts (P≤0.05).
Source: Authors

($P \geq 0.05$).

Fertilization, hatchability, larval production and larval survival

Table 3 shows the mean percent fertilization obtained during the experiment as 81 ± 1.52, 75 ± 2.51 and 62 ± 2.50% for artificial spawning (T1), semi-artificial spawning with 1:1 ratio (T2) and semi-artificial spawning with 2:1 ratio (T3), respectively. T1 recorded the highest value (81 ± 1.52%) with T3 having the lowest value (62 ± 2.50%) and differences among all treatment means were significant ($P \leq 0.05$). Mean percent hatchability were 85 ± 2.51% (T1), 83 ± 3.21% (T2) and 82 ± 2.50% (T3), with no significant difference ($P \geq 0.05$) as indicated in Table 3. Mean larval production was highest in T1 (57,700 ± 3,672) followed by T2 (42,423 ± 6,973) and lowest in T3 (34,078 ± 762). The difference in means were significantly different ($P \leq 0.05$) between artificial and semi-artificial but not significant between T2 and T3 ($P \geq 0.05$).

Mean percent larval survival recorded during the experiment was 84 ± 2.31, 87 ± 2.51, and 92 ± 2.50% with T3 recording the highest (92 ± 2.50%) followed by T2 (87 ± 2.51%) and T1 (84 ± 2.31%) (Table 3). The difference in means of the treatments was significant ($P \leq 0.05$) but there was no significant difference between T2 and T1 ($P \geq 0.05$).

DISCUSSION

The individual weights of the brood stocks, as well as the sex pairing weights are relevant in the determination of the fecundity, fertilization and hatchability, and the overall production of larvae by the African catfish. Brood stock weight between 300 and 800 g was ideal for spawning, since larger fish were difficult to handle and often resulted in significant loss of eggs (Graaf and Janssen, 1996). Other studies have reported the successful spawning of the African catfish weighing between 500 and 3000 g for both female and male brooders (Adebayo and Popoola, 2008; El-Hawarry et al., 2016; Okomoda et al., 2016). In a study conducted (Ataguba et al., 2012), brood stock combination of 824 g (female) and 619 g (male) resulted in the best fecundity, fertilization and hatchability. The variations observed in the weights of the brood stock used in this study (600 - 1000 g) could have accounted for the differences observed in total egg weight, egg number, percent fertilization and the eventual larval production. According to Atuguba et al. (2012), increase in brood stock size leads to significant increase in fertilization and hatchability of *C. gariepinus.*

The Condition Factor (K) recorded for both male and female brood stock were <1, indicating poorer state of well-being (Keyombe et al., 2015). The body condition of fish under natural conditions changes as a result of development of gonads, food abundance and other environmental factors (Pope and Willis, 1996), hence fecundity could have been affected by the condition of brooders used in this study. Well-fed female brood fish will give excellent result with respect to fecundity, latency period, and egg mass, size and yolk (Ngueku, 2015). Condition factor greater than 1 gives an indication of well-being of fish (Datta et al., 2013).

Results from this study indicated that spawning was

successful for all treatment groups (T1, T2 and T3). Relative fecundity across treatments was not significantly different and this gives a good indication of good response to Ovaprim®. Fecundity is used as an index to determine the reproductive capability of fish and the extent of the efficiency of the inducing agent (Okere et al., 2015). Ovaprim® significantly increases ovulation and spawning in matured female African catfish (Watson et al., 2009; Sharaf, 2012). The relative fecundity values (68 ± 6.31, 78 ± 12.29 and 65 ± 8.18) obtained in this research were lower than the 129.06 value obtained by Ngueku (2015) who concluded that well-fed female will give excellent result with respect to fecundity, latency period, and egg mass, size and yolk.

The mean weight of egg observed in this study was significantly higher ($p<0.05$) in artificial spawning treatment group than the semi artificial spawning treatment groups. This is accounted for by the significant variation in the mean weight of female brooders used in this experiment. Females used for artificial spawning were significantly heavier than those used for the semi artificial spawning, hence the difference in mean egg weight. A ripe female African catfish can release a mass of eggs that is about 15 to 20% of its total body weight with stripping, but usually spawns only 5 to 15% of the total body weight under semi artificial spawning techniques (Graaf and Janssen, 1996). The weight of eggs spawned as observed for the semi artificial spawning group in this experiment was found to be within the range of 9 to 12% of the body weight of brood stock used, which is similar to that reported (De Graaf et al., 1995).

Fertilization rate was best at 81 ± 1.52% in artificial spawning treatment group, and this is comparable with 83.7% (Ataguba et al., 2012), but lower than the 88.3% (Ngueku, 2015) and 92.7% reported by Kasi et al. (2015), due to difference in species and cultural systems. The difference observed in fertilization rates for the artificial and semi artificial breeding techniques from this study were statistically significant ($P \leq 0.05$).

Percent fertilization was lower for semi artificial treatment groups (T2 and T3) than the artificial treatment group (T1). Fish sperm motility after activation is usually short-lived, hence losing their capacity for fertilization of eggs (Cejko et al., 2016). The African catfish sperm has a relatively short (about 90 s) period of motility after contact with water, even though the eggs can remain active for a relatively longer period, fertilization is affected if the sperms do not reach the eggs (Biegniewska et al., 2010; Kucharczyk et al., 2019). Sperm activation tests was not carried out in this study but could have caused the difference in fertilization rates observed between the artificial and semi artificial methods of propagation, since fertilization occurred under artificial (T1) and natural (T2 and T3) conditions.

Percent hatchability was high for all treatment groups (85 ± 2.51, 83 ± 3.21 and 82 ± 2.50%) and the differences observed were not significant ($P \geq 0.05$). These results are similar to what was reported by Ataguba et al. (2012) and Kasi et al., (2015). Other authors (Delince et al., 1987; De Graaf et al., 1995) have however, reported lower values (4 - 59%) with different substrates and at different seasons. Adebayo and Popoola (2008) used different synthetic hormones and reported hatchability within the range of 51 to 73%. The use of Ovaprim® in this study resulted in a better hatchability, due to its influence on egg size compared with natural hormones. Eggs size positively correlates with hatchability and hatching rate is also influenced by breeding history, type and age of fish, as well as water quality. Particularly, water quality parameters recorded in this study were within the recommended range for hatching catfish eggs and could also account for the good hatchability.

Larval survival rate was observed to be highest (92 ± 2.5%) in semi artificial spawning (2:1 female to male ratio) and lowest (84 ± 2.31%) in artificial spawning (1:1 female to male ratio). These rates are higher than the 40 to 42% (Olumuji and Mustapha, 2012), 66 to 73% (Abdulraheem et al., 2012) in other studies. Survival rate of larvae is influenced by the size of the receptacle and aeration (Adebayo and Popoola, 2008), in addition to effect of high dissolved oxygen concentration (Nwadukwe and Ayinla, 1993; Sahoo et al., 2008). The higher survival rates observed in this study could be attributed to the effect of Ovaprim® on ovulated eggs. Ovaprim® induced the release of larger eggs compared with natural hormones and eggs size correlate positively with larval length and survival (Rideout et al., 2005). Larger eggs provide more energy for larvae development; the larger the yolk sac, the more energy is available for survival (Olaniyi and Akinbola, 2013; Kucharczyk et al., 2019) .

In this study, significantly ($P \leq 0.05$) high percent of larvae survived in the treatment T3 than in T1 and T2, due to larval density that was traced to the low larval production in T3, thus, giving larvae more dissolved oxygen and larger space to move than in T1 and T2 experiments, that had relatively higher numbers of larvae produced.

In conclusion, semi artificial spawning of *C. gariepinus* with Ovaprim® could be beneficial to fish farmers if done at a broodstock sex pairing ratio of 1:1.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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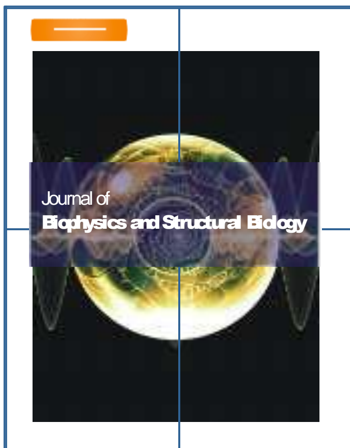

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