ACTA ABP BIOCHIMICA POLONICA

Vol. 62, No 2/2015
185–190
http://dx.doi.org/10.18388/abp.2014_870

Regular paper

Optimization of culture conditions for flexirubin production by *Chryseobacterium artocarpi* CECT 8497 using response surface methodology

Chidambaram Kulandaisamy Venil[1]✉, Zainul Akmar Zakaria[2] and Wan Azlina Ahmad[1]✉

[1]Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia; [2]Institute of Bioproduct Development, UniversitiTeknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

Flexirubins are the unique type of bacterial pigments produced by the bacteria from the genus *Chryseobacterium*, which are used in the treatment of chronic skin disease, eczema etc. and may serve as a chemotaxonomic marker. *Chryseobacterium artocarpi* CECT 8497, an yellowish-orange pigment producing strain was investigated for maximum production of pigment by optimizing medium composition employing response surface methodology (RSM). Culture conditions affecting pigment production were optimized statistically in shake flask experiments. Lactose, L-tryptophan and KH_2PO_4 were the most significant variables affecting pigment production. Box Behnken design (BBD) and RSM analysis were adopted to investigate the interactions between variables and determine the optimal values for maximum pigment production. Evaluation of the experimental results signified that the optimum conditions for maximum production of pigment (521.64 mg/L) in 50 L bioreactor were lactose 11.25 g/L, L-tryptophan 6 g/L and KH_2PO_4 650 ppm. Production under optimized conditions increased to 7.23 fold comparing to its production prior to optimization. Results of this study showed that statistical optimization of medium composition and their interaction effects enable short listing of the significant factors influencing maximum pigment production from *Chryseobacterium artocarpi* CECT 8497. In addition, this is the first report optimizing the process parameters for flexirubin type pigment production from *Chryseobacterium artocarpi* CECT 8497.

Key words: *Chryseobacterium artocarpi* CECT 8497; yellowish-orange pigment; medium optimization; Box-Behnken Design; bioreactor

Received: 12 August, 2014; revised: 10 January, 2015; accepted: 30 March, 2015; available on-line: 15 May, 2015

INTRODUCTION

Bacterial pigments are receiving increased scientific attention owing to their biodegradability and natural colours for applications in industries such as textile, food, cosmetics etc. (Venil *et al.,* 2013). Moreover, the present concerns over the toxicity of synthetic pigments have also resulted in an increased interest in natural pigments (Wang *et al.,* 2011). The output of natural colorants is increasing at a rate of 10% every year in the market (Wang *et al.,* 2006). As a result, gradually natural pigments are replacing synthetic pigments. Among the natural pigments, bacterial pigments are of traditional use in oriental countries and have been a subject of intense research in the present decades because of their potential for industrial applications. Most researchers have focused on the production of yellow, violet and red pigment from different bacteria (Chen *et al.,* 2006; 2013; Wang *et al.,* 2012). Nevertheless, study of pigment (flexirubin) from *Chryseobacterium* sp. has not been documented well.

Flexirubins are the unique type of bacterial pigments with terminal alkyl substitution consisting of ω-phenyl octaenic acid chromophore esterified with resorcinol and are used in the treatment for chronic skin disease, eczema, gastric ulcers, etc. (Kim, 2013). Recent studies of the genus *Chryseobacterium* have documented the significance of flexirubin as a biocontrol agent, antioxidant, sulfobacin A, protease producer (Scheuplein *et al.,* 2007; Wang *et al.,* 2007; Chaudhari 2009; Kim *et al.,* 2012). These studies demonstrate that the genus *Chryseobacterium* is a novel source of bioactive compounds deserving depth study and having a great potential for biotechnological applications. Therefore, it is of great importance to discover new microorganisms producing flexirubin effectively with better bioactivity.

The optimal design of the culture medium is very important for the successful use of microbes in industries, as medium composition can significantly affect product yield. It has been reported that pigment production by bacteria is affected by culture conditions (temperature, agitation and pH) and by nutrient components (carbon, nitrogen and inorganic salts) and is regulated by quorum sensing molecule (Wang *et al.,* 2009). In this study, response surface methodology (RSM) was employed to examine the cultivation conditions on pigment production by *Chryseobacterium artocarpi* CECT 8497. RSM is a powerful statistical tool that has been successfully applied to optimize the fermentation parameters and in investigating the interactive effect of process variables and in building a mathematical model that accurately describes the overall process (He *et al.,* 2009). The most common design, i.e., Box Behnken design (BBD) has been widely employed for the optimization of bioprocesses variables such as fermentation media, cultivation and process conditions.

BBD is a spherical, revolving response surface methodology design that consists of three interlocking 2^2 factorial design having points, all lying on the surface of a sphere surrounding the centre of the design (Kumar

✉e-mail: Chidambaram Kulandaisamy Venil: ckvenil@gmail.com; Wan Azlina Ahmad: azlina@Kimia.fs.utm.my

Abbreviations: RSM, response surface methodology; BBD, Box-Behnken design; FTP, flexirubin type pigment

et al., 2007). This offers essential information for executing optimization process while simultaneously incorporating RSM. The objective of this study was to evaluate the optimum level of medium constituents for the enhanced production of pigment from *C. artocarpi* CECT 8497 by employing experimental design (BBD) as a statistical problem solving strategy and also to scale up batch fermentation in 50 L bioreactor based on the optimized conditions. To the best of our knowledge, there are no reports about the optimization of yellowish-orange pigment production from *Chryseobacterium* sp.

MATERIALS AND METHODS

Bacteria. *C. artocarpi* CECT 8497^T (=KCTC 32509^T) isolated from an orchard at Universiti Teknologi Malaysia (UTM), Skudai, Malaysia was used in this study (Venil *et al.,* 2014a). The yellowish-orange pigment produced by this strain was characterized and identified as a flexirubin (Venil *et al*., 2014b). The culture was maintained in Nutrient agar medium at 4°C and subcultured every month. Nutrient broth (NB) medium was used for the revival and pre-culture of the strain from a frozen stock. Chemicals used were all of analytical grade.

Culture conditions. The *C. artocarpi* CECT 8497 was cultivated in 20 mL NB in 100 mL Erlenmeyer flask at 30°C and 200 rpm agitation speed until it reached 1.0 optical density at 600 nm (OD_{600}). Culture (2 mL) was then transferred as inoculum into 100 mL of the fresh medium in 500 mL Erlenmeyer flask and incubated at 30°C at a shaking speed of 200 rpm for 24 h. The sample (5 ml) was withdrawn from the flask to measure the optical density (OD_{600}) using UV-VIS spectrophotometer (Hach DR 5000) and the remaining sample was extracted and quantified for pigment yield as described below.

Extraction and quantification of pigment. The culture broth (100 ml) was centrifuged at 8000 rpm for 10 min at 4°C and the supernatant was discarded. The cell pellet was then rinsed with deionized water, followed by centrifugation at 8000 rpm for 5 min at 4°C to recover the cells by decanting the supernatant. The recovered cells were extracted using 5% acetone according to the method of Williams *et al.* (1956). The mixture of cells and acetone was treated by ultrasonication until the cells were completely bleached. The pigment was then separated from the cells by centrifugation at 10000 rpm for 5 min at 4°C and it was also confirmed that there was no residual pigment in the cell pellets after the extraction. The optical density of the resulting solution was measured using UV-Vis spectrophotometer at 450 nm and the dry weight of the pigment was calculated.

Optimization of process parameters. Experimental design and optimization by RSM. BBD was used to optimize the medium constituents for pigment production. The effect of physical parameters (pH, temperature, agitation) and medium components (various carbon source, nitrogen source and inorganic salts) were screened for enhancing yellowish-orange pigment production in our preliminary study.

Process parameters affecting the pigment production were investigated and validated using (BBD). Three factors were selected, namely, lactose, L-tryptophan and KH_2PO_4 and successfully employed using BBD for the optimization of pigment production (mg/L). Design factor levels were coded as –1 (low), 0 (central point) and +1 (high). This RSM was applied to the experimental data obtained using the statistical software, Design-Expert 9.0.0.7 (Stat-Ease, trial version). Statistical terms and their definitions used in the Design-expert software were well defined elsewhere (Montgomery, 2004). Linear and second order polynomial were fitted to the experimental data to obtain regression equations. The sequential F-test, lack-of-fit test and other adequacy measures were used in selecting the best model (Muthukumar *et al.,* 2003). To analyze a process or system including a response Y, where Y depends on the input factors X_1, X_2…..X_n, the relationship between the response and input process parameters were described as:

$$Y = f(x_1,x_2.....) + \varepsilon \quad (1)$$

where f is the real response function whose format is unknown, and ε is the residual error which describes the differentiation that can be incorporated by the function f. Since the correlation between the response and the input variables can be described as a response surface of the x_1, x_2, ..x_n coordinates in the graphical sense, the investigation of these relationships is named as the RSM. Considering all the linear terms, square terms and the interaction effects, the quadratic response model can be described as in Eqn. (2), where β_0 is a constant, β_i the slopes or linear effects of the design factors x_i and x_j and β_{ij} is the quadratic effects of the interactions.

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

In the present study, BBD was applied to investigate and validate the process parameters affecting pigment production by *C. artocarpi* CECT 8497.

Batch fermentation in 50 L bioreactor. For batch fermentation, 100 L stirred bioreactor (Biotron LiFlus SP 100 L, Korea) was used. Production medium (45 L) was inoculated with 5 L seed culture in its late exponential stage. When pH decreased to 6.0, it was maintained at 7.0 by automatic addition of 4M H_3PO_4 or 4 M KOH using a computer coupled peristaltic pump. The feeding substrate, lactose, L-tryptophan and KH_2PO_4 respectively 11.25 g/L, 6 g/L and 650 ppm were pumped into the bioreactor using a computer coupled peristaltic pump according to the results of the pre-experiments. After 24 h of incubation, 50 L of the culture was centrifuged in continuous centrifuge (Hanil J 1250, Korea) and the pellet was extracted with 5% acetone and quantified.

Software and data analysis. The results of the experimental design were analyzed and interpreted using Design Expert Version 9.0.0.7 statistical software (Stat-Ease Inc., Minneapolis, Minnesota, USA).

RESULTS AND DISCUSSION

Modification of NB based medium to enhance pigment production

NB enables better growth of *Chryseobacterium* sp. than any other media (Im *et al.,* 2011). Nevertheless, the optimal components in this medium affecting pigment production have not been determined systematically so far. To enhance pigment production, the NB medium was supplemented with various carbon, nitrogen and inorganic salt sources and examined individually for their influence in pigment production.

Our results and literature data indicate that carbon source plays a crucial role in enhancing pigment production (Giri *et al.,* 2004; Bharmal *et al.,* 2012). From Fig. 1 it was noted that pigment production increased with the addition of lactose and sucrose, while other

Table 1. Effect of different composition in the medium on pigment production by *C. artocarpi* CECT 8497 (cultivated at pH 7, 30°C and 200rpm)

	Nutrient Broth	Lactose (in NB)	L-tryptophan (in NB)	KH_2PO_4 (in NB)	RSM	50 L Bioreactor
Pigment production (mg/L)	72.14	123.86	125.64	97.67	504.36	521.64
Increasing fold	–	1.72	1.74	1.35	7	7.23

RSM, response surface methodology

carbon sources do not have much effect on enhancing pigment production. The pigment production increased to 1.72 fold when supplemented with lactose compared to the control (NB) and lactose was selected as the additional carbon source into the culture medium for optimizing pigment production. The medium comprised of 0.5% L-tryptophan supplementation exhibited 1.74 fold increases in pigment production compared to the control (Table 1). The result indicated that *C. artocarpi* CECT 8497 could utilize lactose and L-tryptophan to synthesize the pigment and increase it substantially after 8 h of incubation time. The effect of inorganic salt component is shown in Fig. 1 and the results indicated that KH_2PO_4 had a positive effect and exhibited 1.35 fold increases in the pigment production compared to the control. Inorganic salts may induce pigment production due to catabolite repression (Bharmal *et al.,* 2012). The results showed that the pigment production significantly increased when lactose, L-tryptophan and KH_2PO_4 were added in NB and the effect of different concentrations were studied extensively by using RSM.

Box-Behnken design

BBD was employed to evaluate the interaction among the significant factors *viz.*, lactose, L-tryptophan and KH_2PO_4 and also to determine their optimal levels. A set of 17 experiments with six replicates at the center point was conducted. In the present study, experiments were planned to obtain a quadratic model. The BBD experimental design along with predicted and observed response for each experiment is summarized in Table 2. It also shows the production of pigment (mg/L) corresponding to the combined effect of all the three components in the specific ranges. The optimum levels of selected variables were obtained by solving the regression equation and by analyzing the response surface contour and surface plots. The results of the second-order response surface model fitting in the form of ANOVA are given in Table 3.

The adequacy of the model was indicated by the determination coefficient (R^2=0.9854), which explained 98.54% of the response variability. The high value of adjusted R^2 (0.9666) further supported the accuracy of the model. A second order polynomial function was fit to the experimental pigment yield resulting in the following regression equation:

Z\=4\83\.5\2\+\4.\68A\–\2\8.\74B\–\2\4.\67C\–
\3\.5\7AB\–\7\.3\5AC\–\4\.0\9BC\–\2\07\.5\0.4 – 2

where Y is the response (pigment production) and A, B and C are the concentrations of lactose, L-tryptophan and KH_2PO_4, respectively.

The statistical significance of the second-order model equation was evaluated by F-test analysis of variance as shown in Table 3 which exposed that this regression is statistically highly significant for pigment production. As shown in Table 3, the model was statistically valid given an F-test with a low probability value (P_{model}<0.0001). The lack of fit value was not significant (P=0.0120), indicating that the equation was adequate for predicting the pigment production under all conditions. The low coefficient of variation (CV=7.75%) suggested that the model was precise and reliable.

The 3-D response surface plots obtained using the Design Expert software are presented in Fig. 2–4. The main goal of the response is to track efficiently the optimum values of the variables in such a way that the response is maximized. By analysing the plots, the best response range can be calculated. Fig. 2 showed the interaction between lactose and L-tryptophan, which revealed that both the components have significant effect on the pigment production (lactose 11.25 g/L; L-tryptophan 6

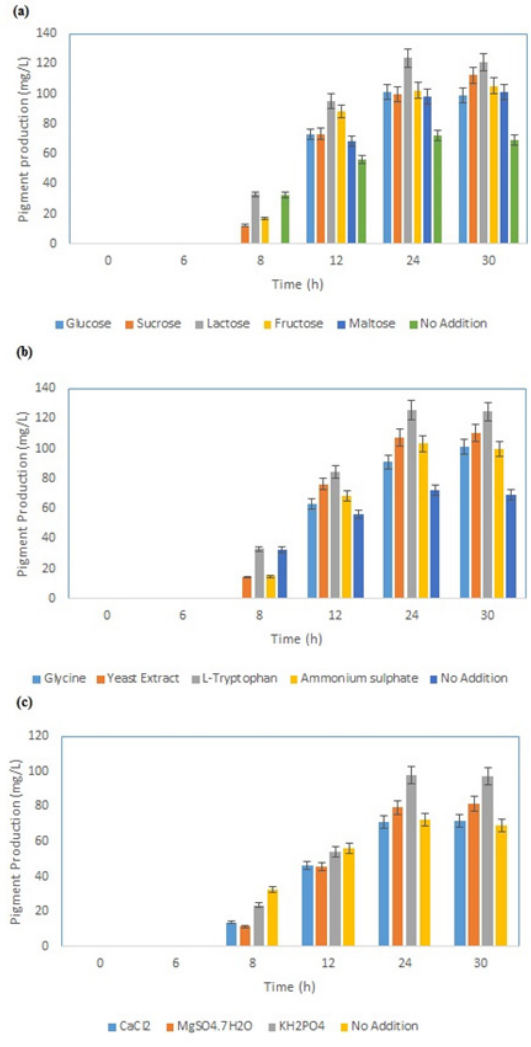

Figure 1. The effect of carbon (a), nitrogen (b) and inorganic salt (c) on pigment production by *C. artocarpi* CECT 8497

Table 2. Box-Behnken design matrix along with the experimental and predicted values of pigment production

Run	A: Lactose (g/L)	B: L-Tryptophan (g/L)	C:KH_2PO_4 (ppm)	Yellowish-Orange pigment (mg/L)	
				Experimental	Predicted
1	12.50	6.00	1000.00	172.22	169.24
2	10.00	4.00	650.00	212.98	224.10
3	11.25	4.00	1000.00	350.18	327.19
4	11.25	6.00	650.00	480.51	478.14
5	11.25	4.00	300.00	378.4	333.29
6	11.25	6.00	650.00	501.48	498.15
7	12.50	4.00	650.00	203.46	198.47
8	11.25	6.00	650.00	483.98	490.43
9	10.00	6.00	300.00	199.12	209.05
10	12.50	8.00	650.00	170.39	181.47
11	12.50	6.00	300.00	249.21	255.42
12	10.00	6.00	1000.00	151.53	162.63
13	11.25	6.00	650.00	472.51	467.42
14	11.25	8.00	300.00	297.54	280.51
15	11.25	6.00	650.00	483.11	491.30
16	10.00	8.00	650.00	194.2	197.02
17	11.25	8.00	1000.00	252.95	268.41

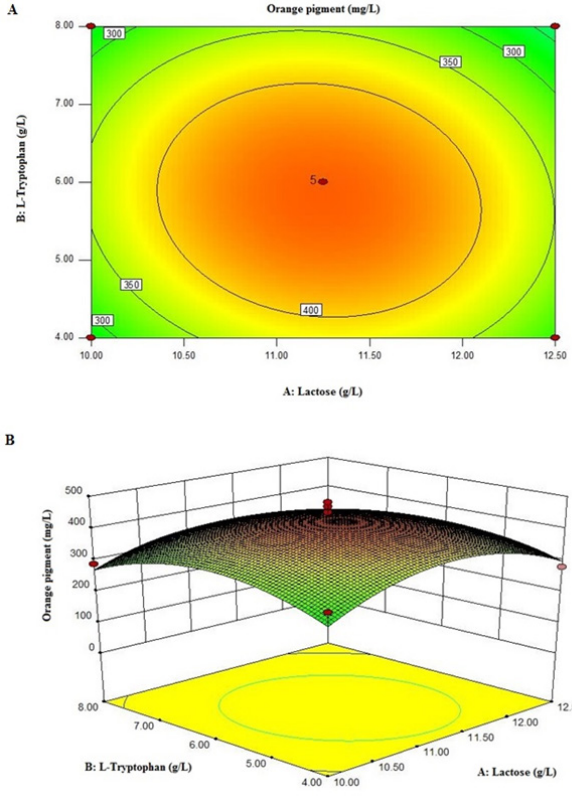

Figure 2. Response surface graph showing the interaction effect of lactose and L-tryptophan on pigment production by *C. artocarpi* CECT 8497.

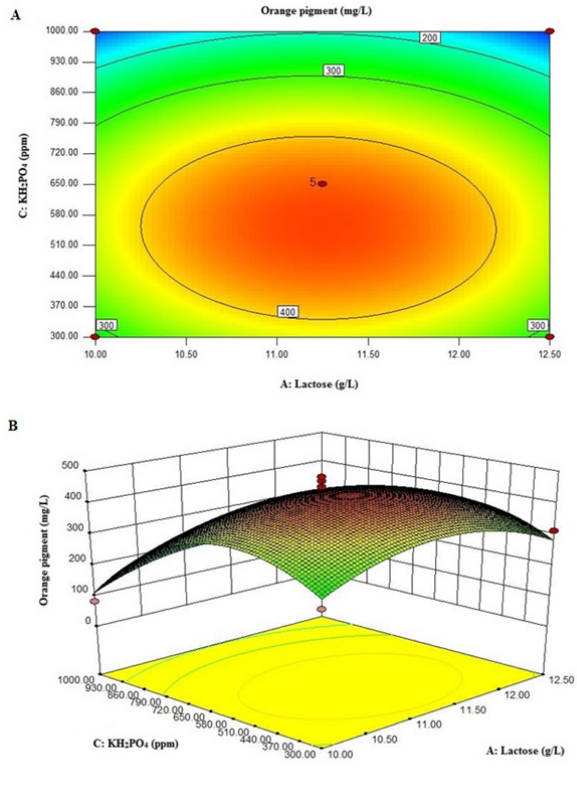

Figure 3. Response surface graph showing the interaction effect of lactose and KH_2PO_4 on pigment production by *C. artocarpi* CECT 8497.

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	Sum of Squares	Df	Mean square	F value	p-value Prob>F
Model	2.703E+005	9	30032.17	52.46	< 0.0001
Residual	4007.49	7	572.50		
Lack of Fit	3681.95	3	1227.32	15.08	0.0120
Pure Error	325.54	4	81.39		
Cor Total	2.743E+005	16			

R^2 0.9854; Adj R^2 0.9666; Pred R^2 0.7834; AdeqPrecisior 17.335.

g/L), while pigment production decreased with further increasing concentrations of lactose and L-tryptophan. The effect of interaction between lactose and KH_2PO_4 (Fig. 3) reveals that the pigment production increased with an increase in lactose concentration from 10 upto 11.5 g/L, and KH_2PO_4 concentration increased from 300 to 600 ppm and declined when the concentrations were increased. Figure 4 shows the interaction between L-tryptophan and KH_2PO_4. The pigment production decreased with increasing concentration of KH_2PO_4.

Validation of the quadratic model

In order to confirm the above mentioned optimized medium constitution and conditions, an experiment for pigment production was carried out in duplicate. Under the calculated optimal culture conditions, the maximum pigment production was 504.36 mg/L, that agreed well with the predicted value (501.16 mg/L), which was 7 folds of that obtained at the original conditions before optimization. Hence *C. artocarpi* CECT 8497 used in this study has higher pigment productivity potential and can be used in bio-pigment production.

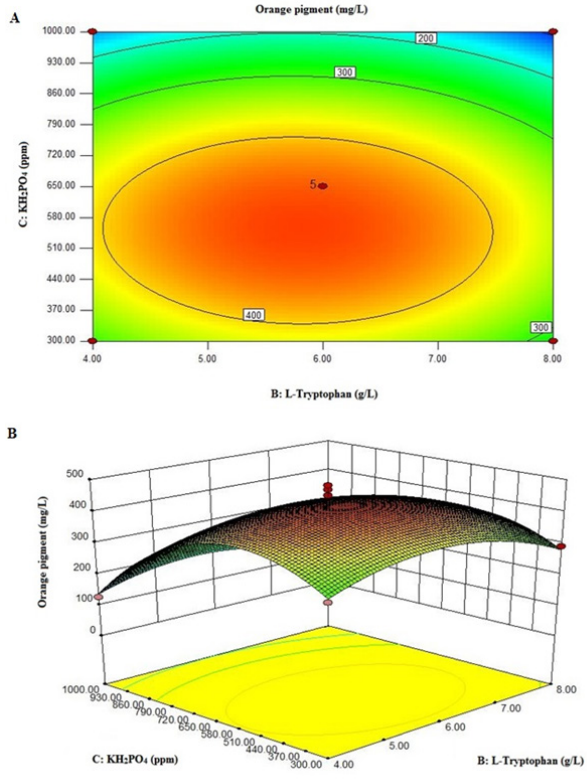

Figure 4. Response surface graph showing the interaction effect of L-tryptophan and KH_2PO_4 on pigment production by *C. artocarpi* CECT 8497.

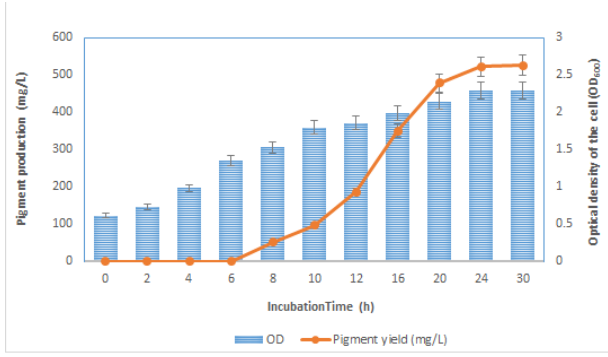

Figure 5. Time course profile of *C. artocarpi* CECT 8497.

Batch fermentation in 50 L bioreactor

Flexirubin production was scaled up from flasks to 50 L bioreactor. Several culture strategies have been evaluated to improve the pigment production and the most widely used system is cultivation of bacteria with different feeding strategies, i.e., monitoring dissolved oxygen, pH, aeration rate and agitation speed as feedback parameters. The experiments were conducted using the optimized conditions as this condition promotes both cell growth and pigment production. The optimal values of agitation speed, aeration rate and intial pH were 200 rpm, 2 L/min, and 7.04, respectively. Figure 5 shows time course of cell growth and pigment yield during cultivation of the strain in 50 L bioreactor. The OD_{600} reached a maximum svalue of 2.39 at 24 h. The pigment production was initiated after 6 h of cultivation and reached the maximum production of 521.64 mg/L after 24 h of incubation and exhibited 7.23 fold increase compared to the conditions before the optimization. The capital and operating costs will decrease if this bioreactor system is used for the bulk production of pigment.

CONCLUSION

In this study, statistical methods were employed to minimize time and cost-effective problems associated with conventional methods. The results of this study have shown that the pigment production by *C. artocarpi* CECT 8497 is largely influenced by chemical components: lactose, L-tryptophan and KH_2PO_4. The results of the BBD evaluation indicated that optimum conditions to achieve maximum pigment production were lactose,

L-tryptophan and KH_2PO_4 were 11.25 g/L, 6 g/L and 650 ppm, respectively. Under optimized conditions, the maximum pigment produced was 521.64 mg/L in 50 L bioreactor. This model can be used for economic production of flexirubin-type pigment for industrial scale. Further studies are required for a full characterization of the pigment particularly assessment of its potential in food and therapeutic uses.

Acknowledgement

Author C.K. Venil is grateful to the Universiti Teknologi Malaysia for the award of Post-Doctoral Fellowship. The authors would like to thank the Research University grants (Q.J 130000.2526.02H84, Q.J 130000.2526.03H83), Ministry of Agriculture (MoA), Malaysia for Techno fund grant (TF0310F080) for financial support of research activities.

REFERENCES

Bharmal MH, Jahagirdar N, Aruna K (2012) Study on optimization of prodigiosin production by *Serratiamarcescens* MSK1 isolated from air. *Int J Adv Biol Res* **2**: 671–680.

Chaudhari PN, Wani KS, Chaudhari BL, Chincholkar SB (2009) Characteristics of sulfobacin A from a soil isolate *Chryseobacterium gleum*. *Appl Biochem Biotechnol* **158**: 231–241.

Chen D, Han Y, Gu Z (2006) Application of statistical methodology to the optimization of fermentative medium for carotenoids production by *Rhodobacter sphaeroides*. *Process Biochem* **41**: 1773–1778.

Chen WC, Yu WJ, Chang CC, Chang JS, Huang SH, Chang CH, Chen SY, Chien CC, Yao CL, Chen WM, Wei YH (2013) Enhancing production of prodigiosin from *Serratia marcescens* C3 by statistical experimental design and porous carrier addition strategy. *Biochem Eng J* **78**: 93–100.

Giri AV, Anandkumar N, Muthukumaran G, Pennathur G (2004) A novel medium for the enhanced cell growth and production of prodigiosin from *Serratia marcescens* isolated from soil. *BMC Microbiol* **4**: 1–10.

He J, Zhen Q, Qiu N, Liu Z, Wang B, Shao Z, Yu Z (2009). Medium optimization for the production of novel bioflocculant from *Halomonas* sp. V3a' using response surface methodology. *BioresourceTechnol***100**: 5922-5927.

Im WT, Yang JE, Kim SY, Yi TH (2011) *Chryseobacterium ginsenosidi mutans* sp. nov., a bacterium with ginsenoside converting activity isolated from soil of a *Rhusvernicifera*-cultivated field. *Int J Syst Evol Microbiol* **61**: 1430–1435.

Kim HS, Sang MK, Jung HW, Jeun YC, Myung IS, Kim KD (2012) Identification and characterization of *Chryseobacterium wanjuense* strain KJ9C8 as a biocontrol agent of Phytophthora blight of pepper. *Crop Protect* **32**: 129–137.

Kim SK (2013) Marine Biomaterials: Characterization, isolation and applications. *CRC Press*, Taylor & Francis.

Kumar A, Prasad B, Mishra IM (2007) Process parametric study for ethane carboxylic acid removal onto powder activated carbon using Box-Behnken design. *ChemEngTechnol* **30**: 932–937.

Montgomery DC (2004) *Design and analysis of experiments*. 5th edn, John Wiley & Sons, Singapore.

Muthukumar M, Mohan D, Rajendran M (2003) Optimization of mix proportions of mineral aggregates using Box-Behnken design of experiments. *Cem Concr Compos* **25**: 751–758.

Scheuplein RJ, Mizutani A, Yamaguchi S (2007) Studies on the non-pathogenecity of *Chryseobacteriumproteolyticum*and on the safety of the enzyme: protein-glutaminase. *Regul Toxicol Pharmacol* **49**: 79–89.

Venil CK, Nordin N, Zakaria ZA, Ahmad WA (2014a) *Chryseobacterium artocarpi* sp. nov., isolated from the rhizosphere soil of *Artocarpus integer*. *Int J Syst Evol Microbiol* **64**: 3153–3159.

Venil CK, Zakaria ZA, Usha R, Ahmad WA (2014b) Isolation and characterization of flexirubin type pigment from *Chryseobacterium* sp. UTM-3T. *Biocatal Agric Biotechnol* **3**: 103–107.

Venil CK, Zakaria ZA, Ahmad WA (2013) Bacterial pigments and their applications. *Process Biochem* **48**: 1065–1079.

Wang H, Jiang P, Lu Y, Ruan Z, Jiang R, Xing XH (2009) Optimization of culture conditions for violacein production by a new strain of *Duganella* sp. B2. *Biochem Eng J* **44**: 119–124.

Wang H, Ren Z, Li P, Gu Y, Liu G, Yao J (2011) Improvement of the production of a red pigment in *Penicillium* sp. HSD07B synthesized during co-culture with *Candida tropicalis*. *BioresourceTechnol* **102**: 6082-6087.

Wang H, Wang F, Zhu X, Yan Y, Yu X, Jiang P, Xing XH (2012) Biosynthesis and characterization of violacein, deoxyviolacein and oxyviolacein in heterologous host, and their antimicrobial activities. *Biochem Eng J* **67**: 148–155.

Wang HS, Pan YM, Tang ZQ (2006) Isolation and characterization of melanin from *Osmanthus fragrans* seeds. *Food SciTechnol* **39**: 496–502.

Wang SL, Yanga CH, Lianga TW, Yen YH (2007) Optimization of conditions for protease production by *Chryseobacterium taeanense* TKU001**.** *Bioresour Technol* **99**: 3700–3707.

Williams RP, Green JA, Rappoport DA (1956) Studies on pigmentation of *Serratia marcescens*. I. Spectral and paper chromatographic properties of prodigiosin. *J Bacteriol* **71**: 115.