OPTIMIZING R-PHYCOERYTHRIN PURIFICATION FROM THE RED ALGAE *GRACILARIA VERRUCOSA* IN POLYMER-SALT AQUEOUS TWO-PHASE SYSTEM USING RESPONSE SURFACE METHODOLOGY

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ملخّص

الاستغلال الأمثل للتنقية صبغ R-phycoérythrine المستخرج من الطحلب الأحمر *Gracilaria verrucosa* وذلك بإستعمال نظام المحاليل المائيّة الثنائيّة و منهجية الإستجابة السطحية : إن صبغ R-phycoérythrinهو بروتين يعمل بمثابة إكسسوار ضوئي عندالطحالب الحمراء (Rhodophyceae) والبكتيريا الزرقاء، لديه العديد من التطبيقات التكنولوجية الحيوية .في هذه الدراسة، تم إستخراج هذا الصبغ من طحلب *Gracilaria verrucosa* بتقنية تقوم على التحلّل بلأنزيمات والطرد المركزي. تم إستعمال نظام المحاليل المائيّة الثنائيّة لفصله باستخدام البولي أثلين جلايكول (PEG) وسلفاط المغنيسيوم (MgSO4).سنة عوامل تؤثرفي نظام المحلول المائي تمت دراستها وهي وزن PEG الجزيئي وتركيزه وتركيزMgSO4 وكمية محلول الصبغ وتركيز كلوريد الصوديوم ودرجة الحرارة .وتم إجراء دراسة مفصلة باستخدام واستعمال منظومة Les plans d'expériences .كما تم إستعمال نسبة الامتصاص A565 /A280 /معيارا للقاء. بالنّسبة لعمليّة الفصل فإنّ نظام المحلول المائي الأمثل فهو يتكوّن من البولي أثلين جلايكول له وزن جزئي يساوي 6000 ونسبة تركيز بالنّظام تساوي 19% كما يحتوي النظام على سلفاط البوتسيوم وذلك بنسبة تركيز تساوي 16% . وكمية من المحلول الخام للطحلب تساوي 4, 4غ/غ ونسبة تركيز كلورور الصوديوم 0,3 ودرجة حرارة بعمل فيها هذا النظام تساوي 25 درجة مائوية وقد مكنتنا هذه الطريقة من تحسين نقاوة الصبغ لتصل إلى ثلاثة مرات مقارنة مع المادة الخام المستخرجة من الطحلب بطريقة التحلل الأنزيمي. يعتبر هذا العمل هو الأوّل من نوعه لإستعماله نظام المحاليل المائيّة الثنائيّة لتنقية الصبغ. كما تبيّن من خلال هذا العمل أنّ النمذجة الرياضية لعملية التنقية جيّدة جدّا(R^2=0,82)

الكلمات المفاتيح : طحلب الغراسلاريا - صبغ R– Phycoérythrine – نظام المحاليل المائيّة الثنائيّة – تنقية

RÉSUMÉ

Optimisation de la purification de la R-phycoérythrine de l'algue rouge *Gracilaria verrucosa* à l'aide d'un Système Aqueux Biphasique en utilisant la méthodologie des surfaces de réponse : La R-phycoérythrine (RPE) est une protéine qui agit en tant que accessoire photosynthétique chez les algues rouges (Rhodophyceae) et les cyanobactéries. Ce pigment possède plusieurs applications biotechnologiques. Dans cette étude, la phycoérythrine est extraite par hydrolyse enzymatique suivi par centrifugation à partir de l'algue *Gracilaria verrucosa*. La RPE est purifié à partir l'extrait brut par un système aqueux biphasique. Le système a été optimisé en utilisant du polyethylène glycole (PEG) et du sulfate de magnésium (MgSO4). Six facteurs qui affectent la pureté de la RPE ont été étudiés; à savoir le poids moléculaire de PEG, la concentration de PEG, la concentration du MgSO4, la quantité d'extrait de RPE, la concentration de NaCl et de la température. Une étude détaillée a été réalisée en utilisant un plan central composite (PCC) et la méthodologie des surfaces de réponse (MSR). Le rapport d'absorbance A565 / A280, le critère de pureté considéré, est égale à 3,5. Les conditions optimales sont les suivantes : 19% (w / w) de PEG6000, 16% (w / w) phosphate, 4,8% (w/ w), la quantité d'extrait de RPE, 0,3% (w/ w) de NaCl et une température optimale de 22 ° C. L'analyse de variance présentait un fort coefficient de détermination (R^2) du modèle de régression qui est de 0,80. Ceci est le premier rapport sur la purification de la RPE par un système aqueux biphasique moyennant l'utilisation de la méthodologie des plans d'expériences et celle des surfaces de réponse.

Mots clés : *Gracilaria verrucosa*; systeme aqueux biphasique; R-phycoérythrine; purification; Surface de réponse.

Abstract

R-Phycoerythrin (RPE) is a protein which acts as a photosynthetic accessory pigment in red algae (Rhodophyta) and cyanobacteria, it has many biotechnological applications. In this study, phycoerythrin,

from *Gracilaria verrucosa* (Rhodophyta) was extracted by enzymatic hydrolysis and centrifugation. Phycoerythrin was purified from this crude extract via an aqueous two-phase system (ATPS). Here the ATPS process was optimized using polyethylene glycol (PEG)/Magnesium sulfate ($MgSO_4$). Six factors that affect RPE purity were studied; namely PEG's molecular weight, PEG's concentration, $MgSO_4$ concentration, R-phycoerythrin loading, NaCl concentration and temperature. A detailed study was carried out using central composite design (CCD) in response surface methodology (RSM). The absorbance ratio A_{565}/A_{280}, a criterion for purity, was 3.5, and was predicted for a system with 19% (w/w) PEG6000, 16%(w/w) phosphate, 4.8% (w/w) RPE loading, 0.3% (w/w) NaCl loading and an optimal temperature of 22°C. Analysis of variance exhibited a high coefficient of determination (R^2) value of 0.80 and ensured that the quadratic model in tandem with the experimental data was a satisfactory one. This is the first report on RPE purification by aqueous two phase system using statistical experimental design and RSM.

Keys worlds: *Gracilaria verrucosa*; Aqueous two-phase system; R-phycoerythrin; purification; Response

INTRODUCTION

Gracilaria is a genus of red algae that includes 150 species reported from different parts of the world, and represents a rich source of agar and protein. Dried *Gracilaria* contains up to 30% protein (Mabeau et Fleurence, 1993) and 48 % agar (Orduña-Rojas et al., 2008; Skriptsova et Nabivailo ,2009) by weight. *Gracilaria* is one of the world's most cultivated and valuable seaweeds (Buschmann et al., 2008; Oliveira et al., 2008; Yarish et Pereira, 2008). The gross production biomass of *G. verrucosa* in China, is estimated to be about 948 282 metric tons, and represents 93.7 % of the world's total production. Taiwan, South Africa, Namibia, the Philippines, and Vietnam are also involved in its production (FAO, 2004).

Phycobiliproteins are produced in the main from cyanobacterium and red algae (Bermejo et al., 2002). Constituents of phycobiliproteins are B-phycoerythrin (B-PE) from unicellular red alga, allophycocyanin, (AP) sourced mainly from cyanobacteria and R-phycoerythrin (R-PE) sourced from red macroalgae. *Gracilaria* have the ability to accumulate high levels of R-phycoerythrin (Wang, 2002). R-phycoerythrins are fluorescent, and have high quantum efficiency; a large stokes shift and excitation and emission bands at visible wavelengths. Their special absorption spectrum has three-peak spectrum with absorption maxima at 565, 539 and 498 nm, respectively. In immunodiagnostics 488 nm is often used as an excitation wavelength, where R-phycoerythrin is better than B-phycoerythrin because the 498 nm maximum is more available (Telford et al., 2001). Stable proteins, phycoerythrins, can be easily linked to antibodies and other proteins by conventional protein cross linking techniques, without altering their spectral characteristics. R-phycoerythrin has immense application potential particularly as fluorescent tags, food additives, cosmetics additives, amongst others (Sekar et Chandramohan, 2008).

Essentially, phycobiliproteins are required to have an adequate purity, and they, therefore, need to be prepared from cultivated algae or from natural macroalgal stocks. The criteria which are commonly used to assess purity in prepared phycobiliproteins are; a visible absorption spectrum and the ratio of the visible maximum absorbance to the absorbance at 280 nm. Polyacrylamide gel electrophoresis (PAGE) and SDS–PAGE are also employed to complement or enhance the purity evaluation. The purification of R-phycoerythrin from red algae is very difficult because the crude extract is always very sticky as polysaccharides are released from the algal cells during homogenizing. Difficulties associated with the purification of phycobiliproteins are often because the chromatographic column is blocked (Wang, 2002). Several methods have been developed for the separation and purification of R-phycoerythrin (Galland-Irmoulia et al., 2000 ; Rossano et al., 2003 ; Liu et al., 2005 ; Niu et al., 2005 ; Sun et al., 2009), but they are either more tedious or time consuming. The major drawback for almost all these methods is the large number of steps involved. The higher the number of steps, the higher the loss in product yield (Kula et al., 1982). Furthermore, scaling up these methods is very expensive and difficult. The aqueous two

phase system (ATPS) is one potential purification technique for downstream processing. The efficiency of ATPS was first realized for the isolation of cells, cell particles, and proteins. Currently, ATPS is used for the separation of various biomaterials and living organisms such as; plant and animal cells, microorganisms, fungi, fungal spores, viruses, chloroplasts, mitochondria, membrane vesicles, proteins, and nucleic acids (Hatti-Kaul, 2008). ATPS has also been recommended as a useful method for the primary preparation of phycobiliproteins from microalga (Benavides, 2004). It is a better alternative to existing methods, particularly, for the early processing stages, as it is suited to the scale of operation, the low processing time, and the enrichment of the product; it is a continuous operation for the separation and purification of desired enzymes/proteins from a complex mixture (Albertsson, 1986 ; Raghavarao et al., 1995 ; Rito-Palomares et al., 2001).

The aim of this work is to investigate the feasibility of using ATPS to recover R-phycoerythrin from a *G. verrucosa*, by optimizing separation and identifying the key variables that rule partitioning. A 2^k full factorial design and response surface methodology will be used to identify the significant factors and develop a model. The model will predict the response of the ATPS and obtain the optimal conditions for R-phycoerythrin partitioning.

EXPERIMENTAL

CHEMICALS

Polyethylene glycols (PEG) with molecular weights of 2000, 4000, 6000, 8000, 10000, NaCl and Mg_2SO_4 were obtained from Sigma–Aldrich (France). All polymers were used without further purification. Potassium phosphate dibasic anhydrous (K_2HPO_4), Bovine serum albumin (BSA), sodium phosphate monobasic anhydrous (NaH_2PO_4), and sodium chloride (NaCl) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

R-PHYCOERYTHRIN EXTRACTION

Two commercial polysacharidases, xylanase (E.C. 3.2.1.32) and cellulases (E.C. 3.2.1.4) were provided by Fulka and used without further purification. The xylanase was from sourced from *Trichoderma viride* and the cellulases were from *Aspergillus niger*. The xylanase was assayed as follows: remazol brilliant blue R-xylan, Fulka N° 66960, as substrate, 3.51UImg^{-1} at PH 6.0 and 40°C. Cellulase was assayed as follows: 1.44UImg^{-1} at PH 5.0 and 37°C with carboxymethylcellulose as substrate. All tests were run twice. Enzymatic hydrolysis of *G. verrucosa* was carried out in 0.5 l Erlenmeyer flasks equipped with a magnetic stirrer as a reactor. The volume of the reaction solution was 0.3 l. Substrate suspended in buffer solution was stirred to form a well dispersed slurry before starting the hydrolysis, by adding a given amount of concentrated cellulase and xylanase solution. Temperature was kept stable in a thermostated water bath. Two phases were obtained by centrifugation at 6000g for 20 min at 20°C. The supernatant was removed and stored at 4°C and the pellet was frozen at – 20°C.

The spectral analysis of the supernatants was performed with a spectrophotometer at wavelengths between 400 and 600. The amount of phycoerythrin in the supernatant was estimated using Beer Lmbert's law (Denis et al., 2009), where the phycoerythrin extinction coefficient was $2.10^6 M^{-1}$ cm^{-1}at 565 nm and the phycoerythrin molecular weight was 24.000 Da. Total sugars, from the hydrolyzed polysaccharides, were determined using the phenol-sulphuric acid reagent described by (Dubois et al., 1956) D-glucose was the calibration standard.

PREPARATION OF AQUEOUS TWO-PHASE SYSTEM PARTITIONNING

PEG–phosphate two-phase systems were prepared by adding defined amounts of PEG solution 40% (w/w), magnesium sulfate solution 30 % (w/w), NaCl solution 100 %, followed by the R-phycoerythrin solution in 15 ml graduated tubes. The final weight of the system was adjusted to 10 g by the addition of buffer solution at the desired PH. The pH of the phosphate solution was adjusted by mixing appropriate amounts of di-potassium hydrogen phosphate and sodium di-hydrogen phosphate solutions. Fresh R-phycoerythrin solution was prepared before all sets of experiments. The two-phase systems were mixed by vortexing for 1 min, and then allowed to stand for 1 h at room temperature (22 °C), and sebsequently centrifuged for 10 minutes at 1800

rpm, in a bench centrifuge, to facilitate phase separation. The volumes of the phases were determined, and the samples from the two phases were isolated and examined.

EXPERIMENTAL DESIGN

A two-level, six-factor, fractional factorial design was applied to determine the optimal ATPS for R-phycoerythrin purification, according to Myers and Montgomery, (2002) with the assistance of commercial software, STATISTICA 8.0. A central composite design (CCD) was used to evaluate the effect of PEG molecular weight (X1), PEG concentration (%, w/w) (X2), MgSO4 concentration MgSO4 (% w/w) (X3), R-phycoerythrin load (% w/w) (X4), NaCl concentration (% w/w) (X5) and incubation temperature (°C) (X6) on RPE yield in the top phase. The effects of these factors on responses A statistical model for optimising yield of R-phycoerythrin, as described earlier by (Rahimpour et al., 2006), employing the significant variables was determined by response surface regression procedures. The data obtained for each parameter, with the above experimental design, were fitted to full second-order models. Analysis of variance (ANOVA) for the models was performed and the model significance was examined by Fisher's statistical test (F-test). Specifically we tested for significant differences between sources of variation in the experimental results, the significance of the regression, the lack of fit, and the coefficient of multiple determination (R^2). The full second-order models (models containing all two parameter interactions) were not accepted using the latter tests. They were improved by eliminating the model terms until the determined conditions were fulfilled (Myers and Montgomery, 2002 ; Rahimpour et al., 2006 ; Davis, 1993). Where the F-significance was lower than 95%; the reduced model was accepted. Otherwise, the discarded parameters with the highest significance were added until the condition was fulfilled. Where one of these conditions was not satisfied, the model was accepted when $R^2 > 0.95$ which meant that more than 95% of the data is explained by the model. After substitution of normalized factors with real values, the following equation was obtained:

were investigated at five levels (-2,-1, 0, 1, 2) based on primary investigations using single model systems.

This design was composed of 2^{6-1} fractional factorial design (run1-32), 14 star points (run 33-44) and 2replicates (run 45 – 46). Thus 46 experiments were needed in total. Each of the six variables studied, were selected according to the results obtained with preliminary tests, taking into consideration the required experimental conditions and relevant literature. Table I presents the coded and actual values. The coded level of each factor was calculated according to (Myers and Montgomery, 2002) the equation that follows:
Coded value = actual value − [(high level + lowlevel)/2]/ (high level − lowlevel)/2 (1)

MODEL BUILDING FOR OPTIMIZING THE PARTITIONNING CONDITIONS

$$\mathbf{Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\Sigma\beta_{ij} x_i x_j;\ i = 1; 2; \ldots; k;\ j = 1; 2; \ldots; k;\ i \neq j} \quad \mathbf{(2)}$$

Where Y is the R-phycoerythrin purity, β_0 is the intercept, bi is the first-order model coefficients, bij is the linear mode coefficient for the interaction between variable i and j, and Xi's are variables under examination.
Response functions were analyzed by canonical analysis (Myers and Montgomery, 2002). The stationary point, if it exists, is the solution to Eq (3) and may represent a point of maximum response, a point of minimum response, or a saddle point.

$$\frac{\partial Y}{\partial x_1} = \frac{\partial Y}{\partial x_2} = \ldots = 0 \quad \mathbf{(3)}$$

The algebraic signs of the eigenvalues explain the nature of its stationary point. If the values are all negative, it is a maximum; if all positive, it is a minimum, and if the signs are mixed it is a saddle point.

ANALYTICAL PROCEDURES

R-phycoerythrin purity was determined in relation to 565–280 nm absorbance (purity of R-phycoerythrin = Abs565 nm/Abs280 nm). Concurrent with data reported from previous work (Rossano et al., 2003), the absorption spectrum of this protein exhibits a peak at 565 nm. However, a ratio greater than five corresponds to a highly purify R-phycoerythrin (defined as pure commercial R-phycoerythrin; Sigma).

For all of the systems studied the partition experiments of R-phycoerythrin in ATPS revealed that this protein exhibited a strong top-phase preference (data not shown), which implies that the majority of the target protein was concentrated in the top phase. The top-phase preference of the that was concentrated in this phase. As a consequence, it was very difficult to evaluate the impact of the system parameters upon the partition behaviour of R- phycoerythrin, by monitoring the protein partition coefficient (K). R-phycoerythrin resulted in partition coefficients of greater than 100.0. This result may be explained by problems associated with the detection of the presence of R-phycoerythrin in the bottom phase, which was caused by the very low amount of the protein

Consequently, the purity of R-phycoerythrin from the top PEG-rich phase as the response variable was used to evaluate the effect of system parameters on the behavior of the protein in ATPS.

Table I. Process variables in coded and actual units

Variable	PEG (Da)	PEG (%,w/w)	MgSO4 (%,w/w)	RPE (%,w/w)	Nacl (%,w/w)	Temperature (T°C)
Levels	X_1	X_2	X_3	X_4	X_5	X_6
-2	**2000**	**16**	**11**	**4**	**0**	**15**
-1	**4000**	**18**	**13**	**4.5**	**0.2**	**20**
0	**6000**	**20**	**15**	**5**	**0.3**	**25**
1	**8000**	**22**	**17**	**5.5**	**0.4**	**30**
2	**10000**	**24**	**19**	**6**	**0.5**	**35**

RESULTS

The optimum conditions for R-phycoerythrin recovery were obtained from preliminary recovery tests using experimental design methodology, by determining the experimental space around the conditions. Design independent variables and their range were classified as follows: PEG molecular weight (2000 – 10000), PEG concentration (16 – 24%, w/w), salt concentration (11–19%, w/w), RPE load (4 - 6 %, w/w), NaCl (0 – 0.5 %, w/w) and temperature (15 – 35 °C). We ignored systems where phase separation was not achieved because of low concentrations of the phase-forming components. Statistical optimization through experimental design offered the opportunity to find the optimal levels of process variables under any given condition; by establishing the relationship between factors and the predicted responses. Response surface methodology, based on central composite design, was used for optimizing R-phycoerythrin recovery. 46 experimental runs with different combinations of six factors were carried out. The experimental response for each run is shown in Table II. The data was analyzed by multiple regression analysis. The regression coefficient of the proposed accuracy model and their P-values (probability of error) that were used to determine the significant parameters are given in Table III.

A second-order polynomial equation (Eq.4) was derived to represent R-phycoerythrin as a function of the independent variables tested.

$$\mathbf{Y = 1.95 - 0.04x_1 + 0.04x_2 + 0.01x_3 - 0.17x_4 + 0.04x_5 - 0.09x_6 - 0.09x_1^2 + 0.07x_2^2 + 0.01x_3^2 - 0.25x_4^2 - 0.10x_5^2 - 0.18x_6^2 - 0.24x_1 x_2 - 0.05x_1 x_3 + 0.08x_1 x_4 + 0.03x_1 x_5 + 0.03x_2 x_3 - 0.11x_2 x_4 - 0.04x_2 x_5 - 0.112x_2 x_6 - 0.02x_3 x_4 - 0.08x_3 x_5 + 0.07x_3 x_6 + 0.03x_4 x_5 + 0.09x_4 x_6 + 0.12x_5 x_6 \quad (3)}$$

Where Y is the response variable (RPE purity), $\mathbf{x_1}$, $\mathbf{x_2}$, $\mathbf{x_3}$, $\mathbf{x_4}$, $\mathbf{x_5}$ and $\mathbf{x_6}$ are the coded values of PEG molecular weight, PEG concentration (%, w/w), MgSO4 concentration MgSO4 (% w/w), R-phycoerythrin load (% w/w), NaCl concentration (% w/w) and incubation temperature (°C), respectively.

The coefficient of determination (R^2) and adjusted R^2 value were 0.80 and 0.60, respectively. This indicates that the model could explain 80 % of the

variability in the response. R^2 increases as the model terms (variables) are added to the model. By using the regressor variables in the model, adjusted R^2 could be used to check any decrease in the variability of responses. In general, the adjusted R^2 will not increase with addition of variables; in fact, if unnecessary terms are added the value of R^2 adj will often decrease. In this case, R^2 and R^2 adj are not similar, which indicates that not all the terms used in model are necessary for building the correct model (Myers and Montgomery, 2002).

each source of variance (e.g. regression, lack of fit, etc.) is compared relative to residual (the prediction error) variance. The probability that any error was significant was determined (P-value) by comparing the derived F-value relative

Table II. Central composite design (CCD). Factors are x_1- PEG Molecular weight (Da); x_2- PEG Concentration (%,w/w); x_3 - MgSO4 (%,w/w); x_4- RPE (%,w/w); x_5- Nacl (%,w/w); x_6- Temperature (T°C). The factors levels are 2000 and 10000 Da for x_1,16 and 24 (%,w/w) for x_2, 11 and 19 (%,w/w) for x_3, 4 and 6 (%,w/w) for x_4, 0 and 0.5 (%,w/w) for x_5 and 15 and 35 (T°C) for x_6.

	X_1		X_2		X_3		X_4		X_5		X_6		**Purity (A_{564}/A_{280})**
1	-1	4000	-1	18	-1	13	-1	4.5	-1	0.2	-1	20	1.36
2	-1	4000	-1	18	-1	13	-1	4.5	+1	0.4	-1	20	1.47
3	-1	4000	-1	18	-1	13	+1	5.5	-1	0.2	-1	20	0.92
4	-1	4000	-1	18	-1	13	+1	5.5	+1	0.4	1	30	1.08
5	-1	4000	-1	18	+1	17	-1	4.5	-1	0.2	1	30	1.59
6	-1	4000	-1	18	+1	17	-1	4.5	+1	0.4	1	30	1.55
7	-1	4000	-1	18	+1	17	+1	5.5	-1	0.2	-1	20	1.33
8	-1	4000	-1	18	+1	17	+1	5.5	+1	0.4	-1	20	0.64
9	-1	4000	+1	22	-1	13	-1	4.5	-1	0.2	-1	20	2.16
10	-1	4000	+1	22	-1	13	-1	4.5	+1	0.4	-1	30	1.51
11	-1	4000	+1	22	-1	13	+1	5.5	-1	0.2	-1	30	1.00
12	-1	4000	+1	22	-1	13	+1	5.5	+1	0.4	-1	30	1.47
13	-1	4000	+1	22	+1	17	-1	4.5	-1	0.2	1	20	3.00
14	-1	4000	+1	22	+1	17	-1	4.5	+1	0.4	1	20	2.26
15	-1	4000	+1	22	+1	17	+1	5.5	-1	0.2	1	20	1.49
16	-1	4000	+1	22	+1	17	+1	5.5	+1	0.4	-1	30	1.48
17	+1	8000	-1	18	-1	13	-1	4.5	-1	0.2	-1	30	1.20
18	+1	8000	-1	18	-1	13	-1	4.5	+1	0.4	-1	30	1.66
19	+1	8000	-1	18	-1	13	+1	5.5	-1	0.2	-1	20	1.50
20	+1	8000	-1	18	-1	13	+1	5.5	+1	0.4	-1	20	1.73
21	+1	8000	-1	18	+1	17	-1	4.5	-1	0.2	-1	20	1.70
22	+1	8000	-1	18	+1	17	-1	4.5	+1	0.4	1	30	1.47
23	+1	8000	-1	18	+1	17	+1	5.5	-1	0.2	1	30	1.37
24	+1	8000	-1	18	+1	17	+1	5.5	+1	0.4	1	30	2.32
25	+1	8000	+1	22	-1	13	-1	4.5	-1	0.2	-1	20	1.84
26	+1	8000	+1	22	-1	13	-1	4.5	+1	0.4	-1	20	1.55
27	+1	8000	+1	22	-1	13	+1	5.5	-1	0.2	-1	20	1.01
28	+1	8000	+1	22	-1	13	+1	5.5	+1	0.4	-1	30	0.69
29	+1	8000	+1	22	+1	17	-1	4.5	-1	0.2	-1	30	1.076
30	+1	8000	+1	22	+1	17	-1	4.5	+1	0.4	-1	30	1.33
31	+1	8000	+1	22	+1	17	+1	5.5	-1	0.2	1	20	1.44
32	+1	8000	+1	22	+1	17	+1	5.5	+1	0.4	1	20	0.70
33	-2	2000	0	20	0	15	0	5	0	0.3	0	25	1.50
34	2	10000	0	20	0	15	0	5	0	0.3	0	25	1.59
35	0	6000	-2	16	0	15	0	5	0	0.3	0	25	1.86
36	0	6000	2	24	0	15	0	5	0	0.3	0	25	2.62
37	0	6000	0	20	-2	11	0	5	0	0.3	0	25	2.69
38	0	6000	0	20	2	19	0	5	0	0.3	0	25	1.33

39	0	6000	0	20	0	15	-2	4	0	0.3	0	25	1.26
40	0	6000	0	20	0	15	2	6	0	0.3	0	25	0.61
41	0	6000	0	20	0	15	0	5	-2	0	0	25	1.20
42	0	6000	0	20	0	15	0	5	2	0.5	0	25	1.87
43	0	6000	0	20	0	15	0	5	0	0.3	-2	15	1.44
44	0	6000	0	20	0	15	0	5	0	0.3	2	35	1
45	0	6000	0	20	0	15	0	5	0	0.3	0	25	2
45	0	6000	0	20	0	15	0	5	0	0.3	0	25	2.3

Table III. Regression coefficient table for R-phycoerythrin purity in coded level of variables. Variables: x_1- PEG (Da); x_2- PEG (%,w/w); x_3- MgSO4 (%,w/w); x_4- RPE (v/v); x_5- Nacl (M); x_6- Temperature (T°C).

Source	Coefficient	t-value	P-value
Constant	**1.95**	**7.36**	**0.00**
x_1	-0.04	-0.65	0.51
x_2	0.04	0.68	0.49
x_3	0.01	0.14	0.89
x_4	**-0.17**	**-2.64**	**0.01**
x_5	0.04	0.63	0.53
x_6	-0.09	-1.32	0.20
x_1^2	-0.09	-1.23	0.23
x_2^2	0.07	0.94	0.35
x_3^2	0.01	0.21	0.83
x_4^2	**-0.25**	**-3.16**	**0.00**
x_5^2	-0.10	-1.27	0.21
x_6^2	**-0.18**	**-2.27**	**0.03**
$x_1 x_2$	**-0.24**	**-3.24**	**0.004**
$x_1 x_3$	-0.05	-0.76	0.45
$x_1 x_4$	0.08	1.14	0.26
$x_1 x_5$	0.03	0.46	0.64
$x_1 x_6$	0.00	-0.07	0.93
$x_2 x_3$	0.03	0.46	0.64
$x_2 x_4$	-0.11	-1.52	0.14
$x_2 x_5$	-0.04	-0.57	0.57
$x_2 x_6$	-0.11	-1.32	0.20
$x_3 x_4$	-0.02	-0.35	0.73
$x_3 x_5$	-0.08	-1.09	0.29
$x_3 x_6$	0.07	0.88	0.38
$x_4 x_5$	0.03	0.44	0.66
$x_4 x_6$	0.09	1.04	0.311
$x_5 x_6$	0.12	1.69	0.10

Table IV. Analysis of variance (ANOVA) of second order polynomial model for optimization of R-phycoerythrin purification by aqueous two phase systems

	SS	d.f	MS	F	p
(1)PEG (Da)(L)	0.0646	1	0.0646	5.7455	0.2516
PEG (Da)(Q)	0.2263	1	0.2263	20.1157	0.1397
(2)PEG(%,w/w)(L)	0.0705	1	0.0705	6.2701	0.2419
PEG(%,w/w)(Q)	0.1772	1	0.1772	15.7479	0.1572
(3)MgSO4(%,w/w)(L)	0.0030	1	0.0030	0.2671	0.6963
MgSO4(%,w/w)(Q)	0.0144	1	0.0144	1.2820	0.4606
(4)RPE(%,w/w)(L)	1.0468	1	1.0468	93.0519	0.0658
RPE(%,w/w)(Q)	1.6123	1	1.6123	143.3149	0.0531
(5)Nacl (%,w/w)(L)	0.0601	1	0.0601	5.3442	0.2599
Nacl (%,w/w)(Q)	0.2442	1	0.2442	21.7110	0.1346
(6)Temperature (°C)(L)	0.2590	1	0.2590	23.0233	0.1308
Temperature (°C)(Q)	0.8171	1	0.8171	72.6323	0.0744
1L by 2L	1.5716	1	1.5716	139.7017	0.0537
1L by 3L	0.0866	1	0.0866	7.6958	0.2203
1L by 4L	0.1945	1	0.1945	17.2893	0.1503
1L by 5L	0.0329	1	0.0329	2.9238	0.3369
1L by 6L	0.0010	1	0.0010	0.0889	0.8156
2L by 3L	0.0327	1	0.0327	2.9084	0.3376
2L by 4L	0.3459	1	0.3459	30.7501	0.1136
2L by 5L	0.0493	1	0.0493	4.3843	0.2836
2L by 6L	0.2584	1	0.2584	22.9710	0.1309
3L by 4L	0.0181	1	0.0181	1.6133	0.4246
3L by 5L	0.1763	1	0.1763	15.6690	0.1575
3L by 6L	0.1165	1	0.1165	10.3559	0.1918
4L by 5L	0.0292	1	0.0292	2.5980	0.3535
4L by 6L	0.1642	1	0.1642	14.5942	0.1630
5L by 6L	0.4238	1	0.4238	37.6711	0.1028
Lack of Fit	2.5323	17	0.1490	13.2408	0.2132
Erreur Pure	0.0113	1	0.0113		
Erreur résiduel	2.5360	17	0.1492		
Total SC	12.0923	45			

SS: Sum of squares; d.f : Degree of freedom; MS: Mean square; F: F value; P: probability
L: Linear effect; Q: Quadratic effect R^2=0.80

to the F-value in the standard F-table, corresponding to the degrees of freedom of the two sources of variance. The F-test revealed that the regression was statistically significant at a confidence level of 99.9%. An analysis of variance found that the F-value of the model (regression) was greater than the tabular F-value with the same number of degrees of freedom of two sources of variance, indicating that the treatment differences between treatments were highly significant. The P-value, for lack of fit, was 0.365, which also indicated that the probability of error for lack of fit was very high. The model therefore represents the

actual relationships of parameters well within the range selected.

Response surface curves were plotted to understand the interaction effects of the variables and to identify the optimal levels of each parameter to attain R-phycoerythrin purity. Canonical analysis of Eq. (3) gave general idea of the surface. The stationary point of the fitted response surface was $\mathbf{x_s}$ (0.12, -0.45, 0.52, -0.26, 0.2,-0.07), and was precisely within the experimental region where the predicted value was 3.54. The optimal values of PEG (MW), PEG (%,w/w), MgSO4 (%,w/w), RPE(%,w/w), NaCl (%,w/w) and temperature (°C), were estimated in actual units, and were 6000, 19 %, 16 %,4.8,0.3,and 25 respectively. The canonical analysis, based on the stationary point, resulted in the following equation:

$$\mathbf{Y = 3.54 - 0.37w_1^2 - 0.28w_2^2 - 0.19w_3^2 - 0.03w_4^2 + 0.02w_5^2 + 0.33w_6^2} \quad \mathbf{(6)}$$

Where, $\mathbf{w_1}$, $\mathbf{w_2}$, $\mathbf{w_3}$, $\mathbf{w_4}$, $\mathbf{w_5}$ and $\mathbf{w_6}$ are eigenvalues based on coded data and Y is the R-phycoerythrin purity. The mixed signs of eigenvalues indicated that the predicted response surface of the stationary point is saddle shaped.

Figure 1 – 3 represents the response surfaces obtained for the interaction effects of the tested variables. The data presented in the response plots indicated that R-phycoerythrin purity increased with an increase in the PEG molecular weight, RPE, NaCl and temperature up to 6000 Da, 4.8 (%,w/w), 1.3 (%,w/w) and 25 °C respectively, after which the purity decreases. The shape of contour plots indicates the nature and extent of the interaction. It is clearly shown in the response surface and contour plots that there is a significant interaction between PEG molecular weight and PEG concentration only.

The effects of PEG concentration and RPE loading, NaCl concentration and Temperature were shown in Fig.2. The response surface plot shows that an increase in PEG concentration, there is a minor shift in optimal RPE, NaCl and Temperature toward the lower values, though this is not a very significant effect.

Similarly, the interaction effects plotted for NaCl, Temperature and RPE showed that there are no significant interactions between these variables which affect R-phycoerythrin purity (Fig.3). However, we could confirm that optimal RPE range lay between 4.5 and 5.5 (%,w/w), that optimal NaCl was between 0.2 and 0.4 (%,w/w) with optimal temperature close to 25 °C.

The adequacy of the predicted model was examined by additional independent experiments at the suggested optimal conditions. An optimum R-phycoerythrin purity of 2.9 was obtained in the top phase. The agreement between the predicted and experimental results verifies the validity of the response model and the existence of an optimal point. All the above experiments were performed at the optimal values of PEG (MW), PEG (%,w/w), MgSO4 (%,w/w), RPE(%,w/w), NaCl (%,w/w) and temperature (°C), as estimated in actual units (6000, 19 w/w, 16 w/w, 4.8 w/w, 0.3 (w/w) and 25°C respectively.

DISCUSSION

The red pigments from *G. verrucosa* that were isolated by ATPS from a crude extract, exhibited three distinct absorption maxima at 498, 538 and 565 nm. Such features led to the classification of this protein as an R-phycoerythrin. To design an aqueous two-phase primary recovery process, the influence of systems parameters on R-phycoerythrin purity was examined using single model systems.

The model, based on response surface methodology, predicted with good accuracy, the optimal conditions for recovery of R-phycoerythrin by extraction in a PEG-salt system. Besides showing the optimum conditions for RPE purity, the model describes, an interaction between x_1 and x_2. These interactions caused the optimal level of one variable to change in response to change in the other variables. This interaction is substantial and it might have complicated the optimization if it had been conducted using the "one variable at a time" approach.

The model predicted that the purity and recovery of RPE from ATPS decreased with increasing PEG molecular weight (6000 - 10000). This was attributed to the increasing hydrophobicity of the polymer, which causes more aggregation during refolding (Franco et al., 1996 ; Rito-Palomares et al., 2000). Larger PEG molecules have larger

available surfaces and may adsorb RPE molecules which would result in decreased RPE purity. In lower molecular weight PEGs (<4000), the polymer does not spread across the protein surface to cover all the hydrophobic parts on the proteins; which is the likely cause of aggregation. In the case of RPE, a decrease in RPE purity may be explained by a migration of contaminant proteins from the bottom phase or interface into the top phase. The optimum region of R-phycoerythrin recovery shifted towards the higher salt concentrations. The latter may be due to an increase in interaction that kept the protein in solution combined with the stabilizing effect of salt on the refolded protein (Rito-Palomares et ai., 2000). According to (Cerasoli et al., 2003), the stabilizing effect of salts depends on the nature of anions and cations; where strongly hydrated anions such as phosphate and sulfate and the weakly hydrated cations such as potassium are considered the most stabilizing.

A decrease in RPE purity with the increased NaCl content in the system was indicated. The purity value of RPE decreases from 3.5 to 2.4 when NaCl concentration increased from 0.3M to 0.5M. The occurrence of this effect may be due to increased protein solubility as a result of the addition of neutral electrolytes like NaCl. The increased net charge of the protein, due to the binding of the ion, should increase the electrostatic free energy of the protein and the resulting repulsive forces probably led to the decrease in the stability of the protein (Arakawa, Timasheff, 1982 ; Vojdani, 1996). This electrostatic repulsive force should also prevent protein association or aggregation; in other words, it should increase the solubility of the RPE in the system. NaCl has a known protein salting-in property. Thus, we suggest a dissociation of the RPE form with the increase of the NaCl content in the system.

R-phycoerythrin recovery was optimal at 25 °C, increased temperature promoted protein aggregation due to a decrease in the required activation energy (Fisher et al., 1993). R-phycoerythrin stability to heat variation was monitored spectromectrically by (Galland-Irmoulia, et al., 2000). Absorbance was slightly decreased when temperature increase from 20 °C to 30 °C. Beyond this range, however, spectral modifications occurred, probably, as a result of peak denaturation and absorbance at 565 nm progressively disappeared. The 499 nm peak showed the greatest stability to high temperature. Process conditions were selected for ATPS based on the influence of the system parameters upon the purity and top phase recovery of RPE from ATPS, which resulted in a product with a purity of 3.5. The ATPS system is characterized by three unit operations. In contrast, the processes reported by (Wang, 2002 ; Galland-Irmoulia et al., 2000 ; Niu et al., 2006), involve many units of operation for the primary recovery stage, in order to obtain a product with similar characteristics. The recovery process proposed here (ATPS extraction), increased the RPE purity up to 3.5, which raises the potential commercial application of this process as an alternative to the practical purification of RPE produced by *G. verrucosa*.

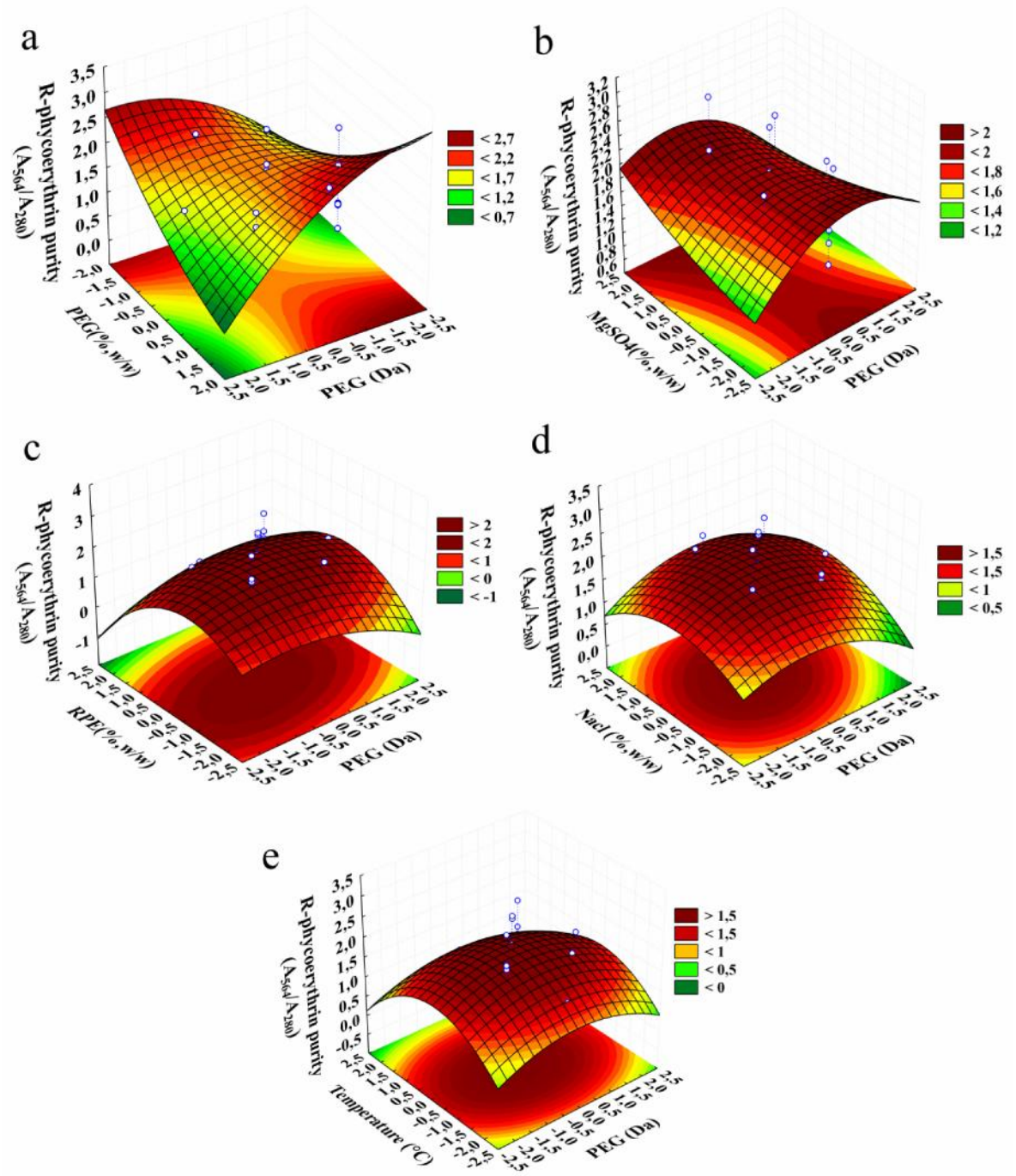

Fig.1. Three dimensional contour plots for R-phycoerythrin (RPE) purity. RSM plots were generated using the data shown in Table III. Inputs were the 46 experimental runs carried out under the conditions established by the CCD design. (a) RPE purity (A_{564}/A_{280}) as a function of PEG molecular weight (Da) and PEG concentration (%,w/w). (b) RPE purity (A_{564}/A_{280}) as a function of PEG molecular weight (Da) and MgSO4 (%,w/w). (c) RPE purity (A_{564}/A_{280}) as a function of PEG molecular weight (Da) and RPE concentration (%, w/w). (d) RPE purity (A_{564}/A_{280}) as a function of PEG molecular weight (Da) and Nacl (%,w/w) concentración. (e) RPE purity (A_{564}/A_{280}) as a function of PEG molecular weight (Da) and temperature (°C). The value of the missing independent variable in each plot was kept at the center point.

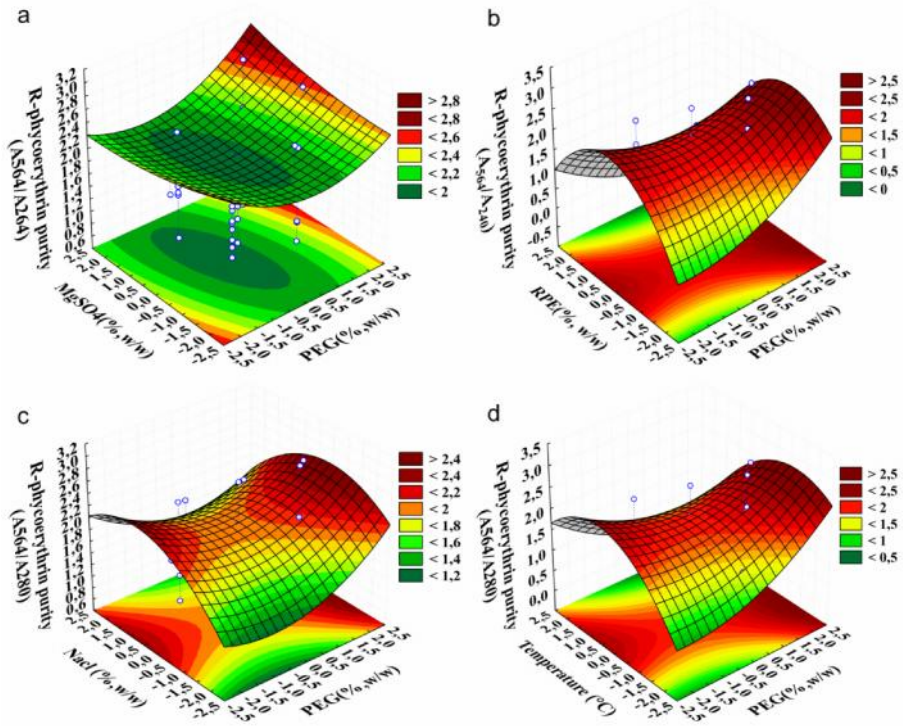

Fig.2. Three dimensional contour plots for R-phycoerythrin (RPE) purity. RSM plots were generated using the data shown in Table III. Inputs were the 46 experimental runs carried out under the conditions established by the CCD design. (a) RPE purity (A_{564}/A_{280}) as a function of PEG concentration (%,w/w) and MgSO4 concentration (%,w/w). (b) RPE purity (A_{564}/A_{280}) as a function of PEG concentration (%,w/w) and RPE loading (%,w/w). (c) RPE purity (A_{564}/A_{280}) as a function of PEG concentration (%,w/w) and Nacl (%,w/w) concentration. The value of the missing independent variable in each plot was kept at the center point. (d) RPE purity (A_{564}/A_{280}) as a function of PEG concentration (%,w/w) and temperature (°C). The value of the missing independent variable in each plot was kept at the center point

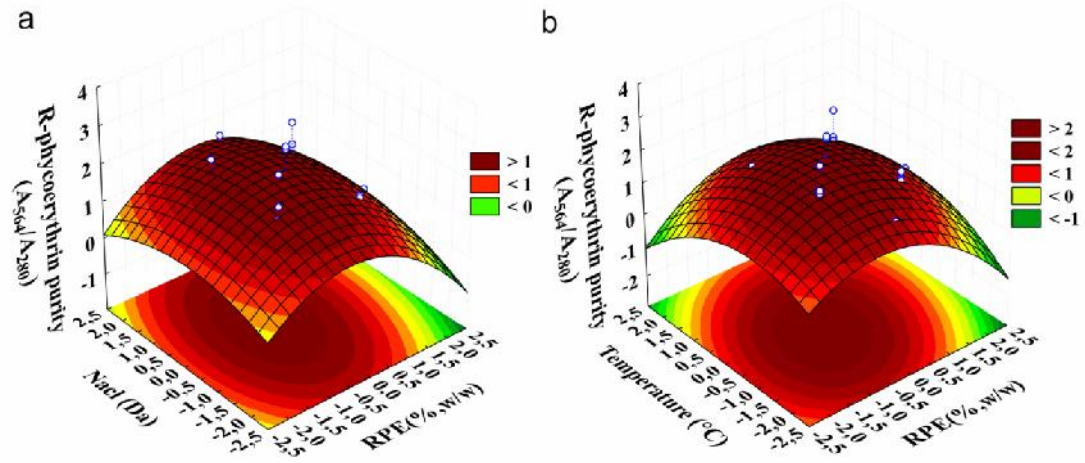

Fig.3. Three dimensional contour plots for R-phycoerythrin (RPE) purity. RSM plots were generated using the data shown in Table III. Inputs were the 46 experimental runs carried out under the conditions established by the CCD design. (a) RPE purity (A_{564}/A_{280}) as a function of RPE concentration (%,w/w) and Nacl concentration (%,w/w). (b) RPE purity (A_{564}/A_{280}) as a function of RPE concentration (%,w/w) and T°C. The value of the missing independent variable in each plot was kept at the center point.

CONCLUSIONS

The work presented here shows the feasibility of using ATPS as an effective and rapid means for the recovery of R-phycoerythrin using phases composed of PEG/Magnesium sulfate systems. The CCD used in this work allowed the definition of an appropriate model for the factors studied, which in turn, led to the definition of an optimum condition. The model equations predicted that all the parameters that were investigated had a significant influence on active recovery, and led to a choosing an optimal system that provided maximal purity (>3). Interactions between the six factors were absent, indicating that the driving force affecting RPE purity was purely affected by the magnitude of the factors employed. A good agreement was obtained between the experimental and predicted results, thus verifying the validity of the model.

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