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Expression and response surface optimization of the recovery and purification of recombinant D-galactose dehydrogenase from *Pseudomonas fluorescens*

Shadi Rokhsartalab Azar[1+], Raika Naiebi[2+], Ameneh Homami[3], Zahra Akbari[4], Anvarsadat Kianmehr[4]*,
Rahman Mahdizadehdehosta[5]* and Faezeh Najafzadeh[1]*

[1]Department of Microbiology, Zanjan Branch, Islamic Azad University, Zanjan, Iran
[2]Department of Hematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[3]Medical Genetic and Molecular Biology Group, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[4]Department of Medical Biotechnology, School of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[5]Department of Biology, Islamic Azad University, Bandar Jask Branch, Bandar Jask, Iran

The enzyme D-galactose dehydrogenase (GalDH) has been used in diagnostic kits to screen blood serum of neonates for galactosemia. It is also a significant tool for the measurement of β-D-galactose, α-D-galactose and lactose as well. In this study, response surface methodology (RSM) was used to identify the suitable conditions for recovery of recombinant GalDH from *Pseudomonas fluorescens* in aqueous two-phase systems (ATPS). The identified GalDH gene was amplified by PCR and confirmed by further cloning and sequencing. *E. coli* BL-21 (DE3) containing the GalDH gene on a plasmid (pET28aGDH) was used to express and purify the recombinant enzyme. The polyethylene glycol (PEG) and ammonium sulfate concentrations and pH value were selected as variables to analyze purification of GalDH. To build mathematical models, RSM with a central composite design was applied based on the conditions for the highest separation. The recombinant GalDH enzyme was expressed after induction with IPTG. It showed NAD^+-dependent dehydrogenase activity towards D-Galactose. According to the RSM modeling, an optimal ATPS was composed of PEG-2000 14.0% (w/w) and ammonium sulfate 12.0% (w/w) at pH 7.5. Under these conditions, GalDH preferentially concentrated in the top PEG-rich phase. The enzyme activity, purification factor (PF) and recovery (R) were 1400 U/ml, 60.0% and 270.0%, respectively. The PEG and salt concentrations were found to have significant effect on the recovery of enzyme. Briefly, our data showed that RSM could be an appropriate tool to define the best ATPS for recombinant *P. fluorescens* GalDH recovery.

Keywords: Aqueous two-phase systems, D-Galactose dehydrogenase, Expression, Response surface methodology, Purification

D-Galactose dehydrogenase (GalDH; D-galactose: NAD^+ oxidoreductase; EC 1.1.1.48) belongs to the family of oxidoreductases that catalyzes the dehydrogenation reaction of β-D-galactopyranose to D-galacto-1,5-lactone and NADH[1]. GalDH has been identified in plants (e.g. green peas and *Arabidopsis thaliana*), algae (e.g. *Iridophycus flaccidum*), bacteria and mammals[2,3]. It is a significant tool for the measurement of β-D-galactose, α-D-galactose and lactose as well. The enzyme has been used in

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*Corresponding authors:
Tel: +98-766-5220701-4; Fax: +98-766-5220110
E-mail: najafzade_f@yahoo.com & R.Mahdizadeh@iau.ac.ir & kiabiotpro@yahoo.com
Abbreviations: ATPS, aqueous two-phase systems; CCFD, central composite face-centered design; *F*-test, Fisher's satirical test; GalDH, D-galactose dehydrogenase; LOF, lack of fit; MR_R, mean square of regression; MR_e, mean square of regression error; PEG, polyethylene glycol; PF, purification factor; *R*, recovery; RSM, response surface methodology; SOR, significance of the regression.
+These authors contributed equally to this work.

diagnostic kits to screen blood serum of neonates for galactosemia[4]. Galactosemia is an inborn metabolic disorder that without strict dietary control results in mental retardation, microcephaly and seizures. Newborn screening using GalDH is a simple method which has proved sensitive, reliable, rapid and cheap compared to other methodologies[5].

GalDH has been purified by conventional methods, including ammonium sulfate precipitation, followed by chromatography which are usually time-consuming and expensive[2,4]. Owing to the commercial importance of GalDH, developing the efficient and scalable alternative purification methods is of great interest. Liquid-liquid extraction using aqueous two-phase systems (ATPS) has been applied for recovery and purification of many industrial enzymes[6]. Compared with the traditional techniques, ATPS has the advantages, such as preserving the targeted biomolecules, low energy consumption and ease of scale-up[7,8]. However, despite the apparent simplicity,

partition of compounds in these systems is very complex due to the several factors involved. In fact, the classical optimization approach varying the level of one parameter at a time, while keeping the rest of the variables constant, is generally time-consuming[9]. For these reasons, mathematical modeling has been utilized to identify parameters mainly those that affect the partition of proteins in ATPS[10,11]. An effective statistical technique is the response surface methodology (RSM) which is a useful statistical tool for studying of systems[12,13].

In this communication, we have used ATPS technology for purification of recombinant *Pseudomonas fluorescens* GalDH. The RSM has been applied to identify the suitable operating conditions and also to simplify the optimization of process.

Materials and Methods

Polyethylene glycols (PEGs) with different molecular weights were purchased from Merck (Darmstadt, Germany). D-Galactose and NAD^+ were obtained from Sigma-Aldrich (St. Louis, MO, USA). The salts and all other chemicals were of analytical grade.

Isolation and screening of GalDH producing microorganism

Screening of GalDH producing bacteria was carried out by culture in selective medium containing galactose as a sole source of carbon and nitrogen. Each soil sample (1 g) was suspended in selective liquid medium that contained: 1% D-galactose, 1 g NaCl, 2 g K_2HPO_4, 0.5 g $MgSO_4$.$7H_2O$, 5 g yeast extract, 5 g polypeptone in 1 liter of deionized water, pH 7.0, then incubated with shaking at 140 rpm for 48 h. Serial dilutions up to 10^{-4} were prepared. From each dilution, 0.05 ml was taken and spread on agar plates and incubated at 37° C for 24 h until the isolates formed colonies. In order to identify GalDH producing strains, isolated bacteria were grown at 37°C for 24 h. The cells were harvested, washed with 0.9% NaCl solution and resuspended in 10 mM potassium phospahte buffer (pH 7.0) containing 1 mM EDTA and then disrupted by 9-KHz ultrasonic oscillator for 15 min. The cell debris was removed and supernatant solution was used for enzyme assay. GalDH activity was determined by monitoring the reduction of NAD^+ at 340 nm. Mixture assay contained 0.3% (w/v/) D-galactose, 100 mM Tris-HCl buffer (pH 8.6), 2.0 mM NAD^+ and the enzyme solution in a total volume of 1 ml[3,4]. Strain which exhibited the enzyme activity was identified and selected for our study.

Production of recombinant GalDH enzyme

Isolation of genomic DNA and plasmid purification was performed as described previously[14]. Primers were designed using DNASIS MAX software (DNASIS version 3.0, Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The *gdh* gene was amplified from the genomic DNA with specific primers GDHFw (5'-T*GGATCC*ATGCAACCGATTCGTCTCG-'3) and GDHRev (5'-GCG*AAGCTT* TTAATCGTAGAACGG C-'3), which contained the restriction sites for *Bam*HI and *Hind*III, respectively. PCR amplification was performed under following conditions; preincubation at 95°C for 1 min and then 30 cycles of 95°C for 1 min, 61°C for 1 min and 72°C for 2 min.

The PCR reaction product was cut with *Bam*HI and *Hind*III, and then ligated into the pET-28a (+) expression vector. The construct bearing the *gdh* gene was named pET28aGDH and transformed into the *E. coli* BL-21 (DE3). For expression of recombinant enzyme, *E. coli* BL21 (DE3) strain was grown overnight in Luria-Bertani (LB) medium containing 40 µg/ml of kanamycin at 37°C and 180 rpm. When cell density reached an OD_{600} of 0.6-0.8, GalDH was expressed by the addition of 0.6 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG). After 5 h induction at 30°C, cells were harvested and stored at –20°C for further use. Pelleted *E. coli* cells were suspended in lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0), mechanically broken by sonication using a pulse sequence of 15 s on and 10 s off for 20 min and clarified by centrifugation at 4000 rpm for 1 h[2].

Preparation of ATPS

The purification systems were prepared in 15 ml graduated tubes by mixing the appropriate amounts of PEG-2000, $(NH_4)_2SO_4$ and the enzyme solution. Systems were agitated for 1 h at room temperature and then centrifuged at 3000 rpm at 25°C for 40 min to speed up the phase separation. The volumes of the phases were determined and the samples from the two phases were carefully tested for enzyme assay and total protein concentration[6,7]. To evaluate the recovery of GalDH, different factors were considered[6]. The partition coefficient (K_E or K_P), which was defined as the ratio of the enzyme activity or protein concentration in the top phase divided by the correspondent value in the bottom phase. The recovery (R%) was the ratio of the enzyme activity in the top phase (A_t) to the initial activity added to the

system (A_{ori}). Purification factor (PF) was calculated as the specific activity in the top phase (SA_t) divided by the initial specific activity in the original sample (SA_{ori}).

Analytical methods

The enzyme activity was determined by monitoring the reduction of NAD^+ at 340 nm. Mixture assay contained 0.3% (w/v/) D-galactose, 100 mM Tris-HCl buffer (pH 8.6), 2.0 mM NAD^+ and the enzyme solution in a total volume of 1 ml. The change of absorbance at 340 nm was measured and corrected for blank values not including D-galactose. One unit of GalDH activity (U) was defined as the amount of enzyme catalyzing the formation of 1 μmol NADH per min under the assay conditions[4]. The total protein concentration was determined by a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard[15]. The purity of recombinant enzyme was tested by a 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). After separation, the gel was stained with Coomassie brilliant blue R-250 and then destained by diffusion in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid[14].

Design of experiments and statistical analysis

A three-factor central composite face-centered design (CCFD) was used to optimize the recovery of recombinant GalDH using Design-Expert software (version 8.0.4, State-Ease, Inc., USA). The selected variables were: concentration of PEG-2000 (X_1), $(NH_4)_2SO_4$ concentration (X_2) and pH (X_3). For each of the three variables, high (coded value + 1) and low (coded value −1) points were chosen on the basis of preliminary test about their effects on GalDH. The level and ranges chosen for the variables are shown in Table 1. The experimental data were analyzed by the response surface regression procedure using the following second-order polynomial equation:

$$Y_i = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j$$

where Y_i is the predicted response, b_0, b_i, b_{ii} and b_{ij} are regression coefficients for the intercept, first-order model coefficients, the linear mode coefficient for the interaction between variable i and j, respectively and $x_i^'$s are the coded independent variables. The analysis of variance (ANOVA) was used to estimate the statistical significant of the full quadratic models. The suitability of proposed model was evaluated by

Table 1—Coded and uncoded values of the variables in different experiments of central composite design

Assay	Coded and uncoded values of variables		
	PEG (%)(x_1)	Salt (%)(x_2)	pH (x_3)
1	15 (1)	11 (-1)	7 (-1)
2	15 (1)	11 (-1)	8 (+1)
3	14 (0)	12 (0)	7.5 (0)
4	15 (1)	13 (1)	7 (-1)
5	14 (0)	10.32 (-1.68)	7.5 (0)
6	14 (0)	12 (0)	6.66 (-1.68)
7	13 (-1)	13 (1)	8 (+1)
8	14 (0)	12 (0)	7.5 (0)
9	13 (-1)	13 (1)	7 (-1)
10	15.68 (1.68)	12 (0)	7.5 (0)
11	12.32 (-1.68)	12 (0)	7.5 (0)
12	15 (1)	13 (1)	8 (+1)
13	14 (0)	12 (0)	7.5 (0)
14	13 (-1)	11 (-1)	8 (+1)
15	14 (0)	13.68 (1.68)	7.5 (0)
16	14 (0)	12 (0)	7.5 (0)
17	14 (0)	12 (0)	8.34 (1.68)
18	14 (0)	12 (0)	7.5 (0)
19	13 (-1)	11 (-1)	7 (-1)
20	14 (0)	12 (0)	7.5 (0)

Fisher's satirical test (F-test) by testing for significance between sources, if variation in experimental results, i.e. the significance of the regression (SOR), the lack of fit (LOF), p-value and the coefficient of determination (R^2). The F-value was defined as the ratio of the mean square of regression (MR_R) to the error (MR_e), representing the significance of each controlled variable on the tested model. The regression equations were also summated to the F-test to determine the coefficient R^2.

Results and Discussion

PCR amplification and construction of expression plasmid

The gene encoding GalDH was PCR-amplified from the purified genome of *P. fluorescens*. As observed in Fig. 1, lane 1, 1.0 kb DNA fragment was obtained, which was gel purified and cloned into pET-28a. The recombinant plasmid was named pET28aGDH and transformed into *E. coli* BL21 (DE3). Among 50 transformants of *E. coli* strain, 15 colonies were selected for plasmid isolation. The restriction of pET28aGDH with *Nde*I and *Bam*HI confirmed the cloning of *gdh* gene (Fig. 1, lanes 2 and 3). The nucleotide sequence of the insert DNA of pET28aGDH was also determined by DNA sequencing.
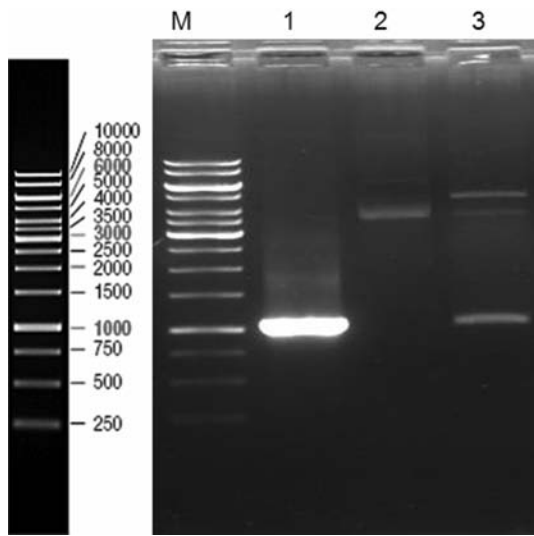
Fig. 1—PCR amplification and restriction analysis of the recombinant plasmid pET28aGDH [Lane M, 1-kb ladder; lane 1, PCR product of GalDH gene using genomic DNA from *P. fluoresense*; lane 2, purified recombinant plasmid pET28aGDH; lane 3, digestion products of pET28aGDH with *Nde*I and *Bam*HI]

Determination of significant parameters for mathematical modeling

The influence of PEG MW was studied using four different molecular weights, including 2000, 4000, 6000 and 8000 Da. The best partition coefficient (55.7%) was achieved with PEG-2000. GalDH showed high affinity in the top PEG-rich phase due to the lower MW of PEG. This behavior can be attributed to the exclusion effect that leads to the enzyme's movement from the top phase to the bottom[16,17]. In other words, increase of PEG MW from 2000 to 8000 resulted in less available space for GalDH in the upper phase, leading to the decrease of partition coefficient (36.5%). Similar results have also been reported for other proteins, such as BSA in PEG-potassium citrate partitioning system[18]. Therefore, PEG-2000 was selected as the best MW.

To study the effect of neutral salt on the partition features of GalDH, addition of NaCl 0-10% (w/w) in PEG-2000 14% (w/w) and $(NH_4)_2SO_4$ 12% (w/w) system was examined. Based on the obtained data, the highest K (55. 7%) was achieved without the addition of NaCl, suggesting that NaCl negatively affected the partitioning of desired enzyme. Addition of salt at high concentrations leads to aggregation followed by protein precipitation, because a large amount of water molecules are strongly bound to the salts. As a consequence, the interactions among proteins become more powerful than between protein and water[6].

Table 2—Results of enzyme activity, PF and R for each system based on the central composite design

Assay	Enzyme activity (U/ml) PF R (%)		
1	1356.50	47	220
2	1391.30	48.3	230
3	1426.0	61	256.25
4	1078.26	43	195
5	1043.47	42.61	200
6	973.91	53	195
7	1113.0	30	163
8	1495.65	58.9	268.75
9	1008.70	35	181.25
10	1078.26	39.81	259
11	1043.47	38	188
12	1182.60	35.02	209
13	1391.30	55	270
14	1113.0	36	205
15	973.91	26.25	175
16	1460.87	56.64	262.5
17	1078.26	42.73	198
18	1530.43	61.76	270
19	1321.74	28.98	220
20	1565.21	63	268

Similar observations on the influence of PEG MW and NaCl addition have been reported for other enzymes such as phenylalanine dehydrogenase[6] and proline dehydrogenase[19].

The range of pH applied in this work was chosen according to the pI of target enzyme (pI = 4.86). The optimal pH range for this pI in two-phase extraction is usually between 7.0 and 8.0[6,18]. This is a typical behavior for the enzymes which have negative charges[19]. The pH is an important parameter in optimization, so it was included in the experimental conditions. Briefly, pH, polymer and salt concentration, which are the most important variables affecting enzyme recovery and separation were chosen for modeling and optimization.

Optimization of GalDH recovery conditions using RSM

The design variables and their ranges were determined as follows: PEG concentration (13-15%, w/w), pH (7.0-8.0) and salt concentration (11-13%, w/w). The details of design matrix are presented in Table 1. Experiments according to the design matrix of variables were carried out and the results of enzyme activity, PF and R are shown in Table 2. As can be seen, the best results for responses were achieved with the system composed of PEG-2000 14.0% (w/w) and ammonium sulfate 12.0% (w/w) at pH 7.5.

Table 3 lists the significant parameters and statistical test results of the models. The model determination coefficient, R^2 was calculated to be 0.93, 0.93 and 0.96, for the enzyme activity, PF and R, respectively. The R^2 value is always between 0 and 1 and a value >0.75 indicates the suitability of the model[20,21]. The model F-value for the enzyme activity, PF and R of recombinant GalDH were 15.08, 14.91 and 31.89, respectively. In this study, all the P-values (probability of error) were less than 0.05. The larger F-values and smaller p-values showed that the variables would be significant. The data confirmed that pH, PEG and salt concentration significantly affected the enzyme purification.

Table 3—Analysis of variance (ANOVA) performed for enzyme activity, PF and R of GalDH

Source	Parameter	Responses		
		Enzyme activity	PF	R
	Sum of squares	6.15E + 05	2394.27	23814.61
	Mean of squares	68288.89	266.03	2646.07
Model	F-value	3. 9	14.91	31.89
	R^2	0.93	0.93	0.96
	Degree of freedom	9	9	9
	Sum of squares	21172.02	48.32	150.83
Pure error	Mean of squares	4234.4	9.66	30.17
	Degree of freedom	5	5	5
	Sum of squares	1.54E+05	130.07	679.04
	Mean of squares	30800.98	26.01	135.81
Lack of fit	p-value	0.0241	0.004	0.0035
	Degree of freedom	5	5	5

The response surface plots provide a method to visualize the relationship between responses and experimental levels of each variable[22]. Through the response surface plots, interactions of variables and optimum level of each variable for maximum response can be well understood. Since the influence of PEG and salt concentration was more appropriate than pH in the recovery of recombinant GalDH, therefore, these two factors were selected for displaying plots. The contour plots of the enzyme activity, PF and R against salt and PEG concentrations are depicted in Fig. 2. As observed, the contour plots of these responses (enzyme activity, PF and R) exhibited similar curving shapes, where the maximum values were obtained near the centers of the graph. The highest estimated enzyme activity, PF and R at this condition (PEG-2000 14.0% (w/w), $(NH_4)_2SO_4$ 12.0% (w/w) and pH 7.5) were 1400 U/ml, 60.0 and 270.0%, respectively. According to these plots, salt and PEG concentration had a prominent influence on the all responses (enzyme activity, PF and R).

Moreover, since the PF and R are the two most important factors for purification process[23], the overlay plots of these two parameters were also measured (Fig. 3). The gray region in Fig. 3 is a preferred experimental region with high PF (60.0) and R (270.0%). This data showed again the adequacy of proposed model and also the potential of RSM in the design of process. The purification of recombinant enzyme was evaluated by SDS-PAGE analysis (Fig. 4). Purified GalDH was found in the PEG-rich phase and appeared as a single protein band on Coomassie brilliant blue stained SDS-PAGE gel. The subunit MW of recombinant GalDH was found to be
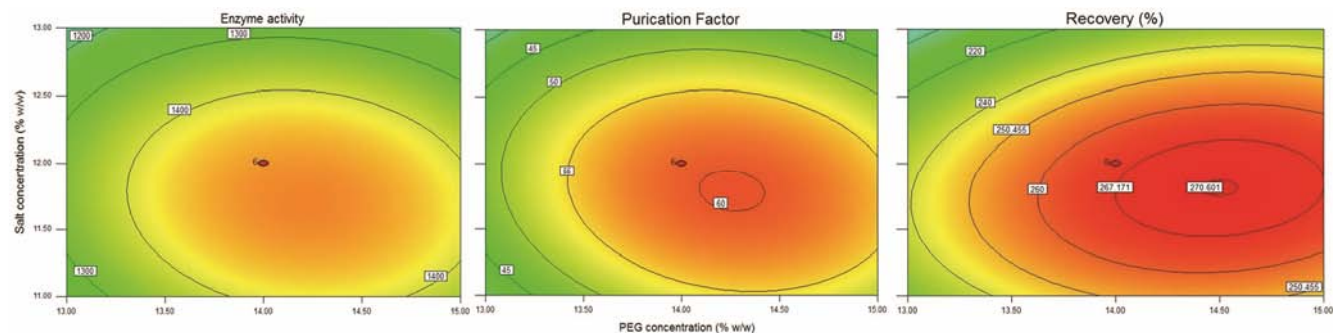

Fig. 2—Contour plot of enzyme activity (A), PF (B) and R (C) at different concentrations of PEG-2000 and $(NH_4)_2SO_4$. [The contour plots of these responses (enzyme activity, PF and R) exhibited similar curving shapes where the maximum values were obtained near the centers of the graph [The highest estimated enzyme activity, PF and R at this condition (PEG-2000 14.0% (w/w), $(NH_4)_2SO_4$ 12.0% (w/w) and pH 7.5) were 1400 U/ml, 60.0 and 270.0%, respectively. According to these plots, salt and PEG concentration had a prominent influence on the all responses (enzyme activity, PF and R). The numbers inside the figures show the constant value of response. The color change from green to red corresponds to increase in response]
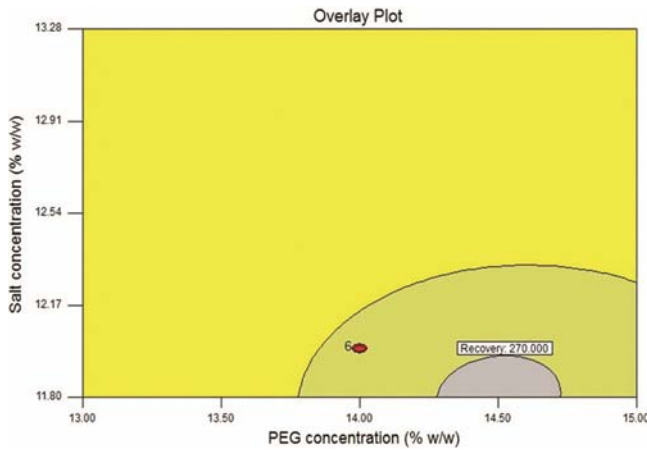
Fig. 3—Overlay plot of PF and *R* at different concentrations of PEG-2000 and $(NH_4)_2SO_4$ [The gray region is a preferred experimental region with high PF (60.0) and *R* (270.0%). This data again showed the adequacy of proposed model and also the potential of RSM in the design of process]
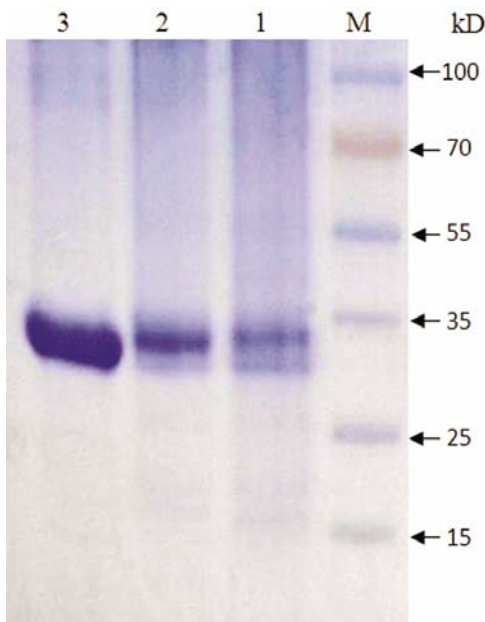

Fig. 4—SDS-PAGE profile of the purified recombinant GalDH showing purified GalDH in the PEG-rich phase and appearing as a single protein band [Lane M: protein molecular marker; lane 1: whole cell lysate before purification, lane 2: bottom phase of the optimized system, lane 3: purified enzyme from the top phase of optimized system. The subunit MW of recombinant GalDH was found to be about 34 kDa, which was similar to the reported value from *P. fluoresense*[1,2]]

about 34 kDa, which was similar to the reported value from *P. fluoresense*[1,2]. Generally, *P. fluoresense* GalDH has a molecular mass of about of 34 kDa.

Conclusion

The study demonstrated the potential of ATPS for recovery and purification of recombinant GalDH in a single step. In the present work, the gene encoding GalDH gene was isolated from *P. fluoresense*, cloned and expressed in *E. coli* BL21. The RSM combined to a proper factorial experimental design proved to be a powerful tool in designing and modeling the best two-phase condition for purification of the enzyme. The ATPS consisting of PEG-2000 14.0% (w/w), $(NH_4)_2SO_4$ 12.0% (w/w) and pH 7.5 was the most optimal system to perform GalDH partition. Under these experimental conditions, the values for the enzyme activity, PF and *R* were 1400 U/ml, 60.0 and 270.0%, respectively. The purified enzyme was also confirmed via SDS-PAGE.

Acknowledgements

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