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ATPS applied to extraction of small molecules – polycetides – and simultaneous clarification of culture media with filamentous microorganisms

Elis Esmanhoto, Beatriz Vahan Kilikian*

Departamento de Engenharia Química, Escola Politécnica, Universidade de São Paulo, C.P. 61548, CEP: 05424-970 São Paulo, SP, Brazil

Abstract

Aqueous two-phase systems (ATPS) were applied for extraction of small molecules (polycetides) – retamycin, an anthracyclin, and two red pigments, rubropunctamin and monascorubramin – from the whole culture media of *Streptomyces olindensis* and *Monascus purpureus*. ATPS allows, in one step, the separation of the small hydrophobic molecules in the PEG rich phase, from the filamentous microorganisms, which remains in the salt phase. Through experimental designs, the main variables and their levels were defined, as follows: for retamycin extraction, PEG 6000 (10%, w/w), phosphate at 20% (w/w) and pH 6.0 led to the higher partition coefficient, $K_r = 8.2$, and yield = 91.3%; for red pigments, the statistical analysis indicate PEG 6000 (20%, w/w) and phosphate at 15% (w/w), for a high partition coefficient, ($K_{pig} = 113$ and 150).
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Keywords: Aqueous two-phase system; PEG/salt; Polycetides; Clarification

1. Introduction

Aqueous two-phase systems (ATPS), mainly poly(ethylene glycol) (PEG)/salt systems, have been widely used for the bioseparation of enzymes and proteins because of their low cost and wide range of hydrophobicity differences between the two-phase systems [1–4]. Besides the type of polymer and salt, the molecular weight of the polymer, polymer and salt concentrations, ionic strength and pH of the medium, together with the target molecule characteristics (size, charge and hydrophobicity), are the main factors influencing the partition of the molecules [1,3,5].

Although ATPS are widely used for recovery of macromolecules, few studies about small molecules were carried out [6]. This work presents an option to purify small hydrophobic molecules – polycetides – from filamentous microorganisms, difficult to separate by means of centrifugation or filtration.

Some advantages of applying ATPS for whole culture medium: increase of recovery yields; feasibility for continuous operation; decrease of steps and process costs by joining clarification and partial purification. In the extraction of vancomycin, a glycopeptide antibiotic, partition coefficient above 100 was obtained [7], a high value regarding that small molecules are extracted almost equally to both phases [1]. Yang et al. [8] showed that ATPS were able to extract cephalosporin C from whole culture medium, separating early in the process, cells and desacetyl cephalosporin C, a by-product with a molecular structure close to the target molecule. Paquet et al. [9] studied partitioning of prestinamycins from whole culture medium and observed that cells were confined into the bottom phase and prestinamycins partitioned to the top phase.

The small molecules applied in this work are retamycin and red pigments, both polycetides. Retamycin, an anthracyclinic complex produced by *Streptomyces olindensis*, was isolated by the Department of Antibiotics of the University of Pernambuco, Brazil, in the 1960s. In antineoplastic experiments with Walker and Yoshida sarcomas, considerable tumor inhibition occurred [10] and positive results in the treatment of human leukemias demonstrated its potential as an anti-leukemia drug [11]. The red pigments produced by *Monascus purpureus* sp. (rubropunctamine and

* Corresponding author.
E-mail address: kilikian@usp.br (B.V. Kilikian).

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monascorubramine), are of great advantage in substitution of the highly toxic nitrite that is used nowadays to the color and biological preservation of meat and sausage products [12].

The aim of this study is to apply the ATPS extraction for whole culture medium of *S. olindensis* So20 and *M. purpureus* sp. in order to offer an alternative to the traditional methods of purifying antracyclines through extraction in organic solvents and, in both cases, to clarify the medium, thus eliminating the centrifugation or filtration step. The results were analyzed on the basis of the partition coefficient for red pigments, and partition coefficient and yield of retamycin, from whole culture media of *M. purpureus* sp and *S. olindensis* So20, respectively.

2. Experimental

2.1. Chemicals

Sodium citrate, citric acid, PEG 300, 1500, 4000 and 6000, were obtained from Merck–Schuchardt (Hohenbrum, Germany). Mono- and dibasic potassium phosphates were obtained from Carlo Erba–AnalytiCals (Rodano, Italy). All other chemicals were of analytical grade.

2.2. Cultures

2.2.1. Retamycin production

The mutant strain, *S. olindensis* So20, was cultivated in a semi synthetic medium (g/L): glucose, 10; yeast extract, 5; hydrolysate of casein, 0.1; tris(hidroxymethyl)-aminometan, 3.09; K_2SO_4, 0.25; and $MgCl_2{\cdot}6H_2O$, 10.12. A batch run was performed in 5 L bioreactor (Bioflo II, New Bunswick, USA) under the following conditions: working volume, 4 L; agitation rate, 500 rpm; air flow rate, 4 L/min; pH 7.0; temperature, 30 °C and culture time, 48 h. The extractions were did just at the end of the cultivation time to prevent problems like antibiotic degradation.

2.2.2. Pigments production

The microorganism applied to produce the pigments was *M. purpureus* CCT3802 (Tropical Foundation of Research André Tosello, Campinas, Brazil). Cultivation was done in a semi synthetic medium (g/L): glucose, 10.0; $MgSO_4{\cdot}7H_2O$, 4.8; KH_2PO_4, 1.5; K_2HPO_4, 1.5; $ZnSO_4{\cdot}7H_2O$, 0.01; monosodium glutamate, 7.6; NaCl, 0.4; $FeSO_4$, 0.01; yeast extract, 1.0. The cultivation conditions applied in shaker were as follows: shaker flasks working volume, 100 mL; agitation rate, 300 rpm; temperature, 30 °C and culture time, 72 h.

2.3. Extraction in aqueous two-phase systems

Phase systems were prepared in 15 mL graduated centrifuge tubes by weighting a PEG solution (50% (w/w) for PEG 1500, 4000 and 6000, and 100% (w/w) for PEG 300) and a stock solution of phosphate (40%) consisting of a mixture of appropriate amounts of KH_2PO_4 and K_2HPO_4 in order to obtain the desired pH. A sample of 2 g of whole culture medium containing retamycin or pigments was added to the system, which was made up to 10 g by the addition of water. All the systems were vortex-mixed for 45 s and in some runs of the experimental designs, mixed later in a rotary homogenizer at 16 rpm (agitator model AP-22, Phoenix, Araraquara, Brazil) for an extra time (30 or 60 min) in order to verify the required time to establish the equilibrium between the phases. Additional contact time relative to the vortex mixing was observed to be necessary for the equilibrium between the phases, when the extraction was done in whole culture medium [7]. Finally, the media were centrifuged under $1000 \times g$ for 5 min to speed up separation of the phases and samples of top and bottom phases were assayed for retamycin concentration or pigments absorbance.

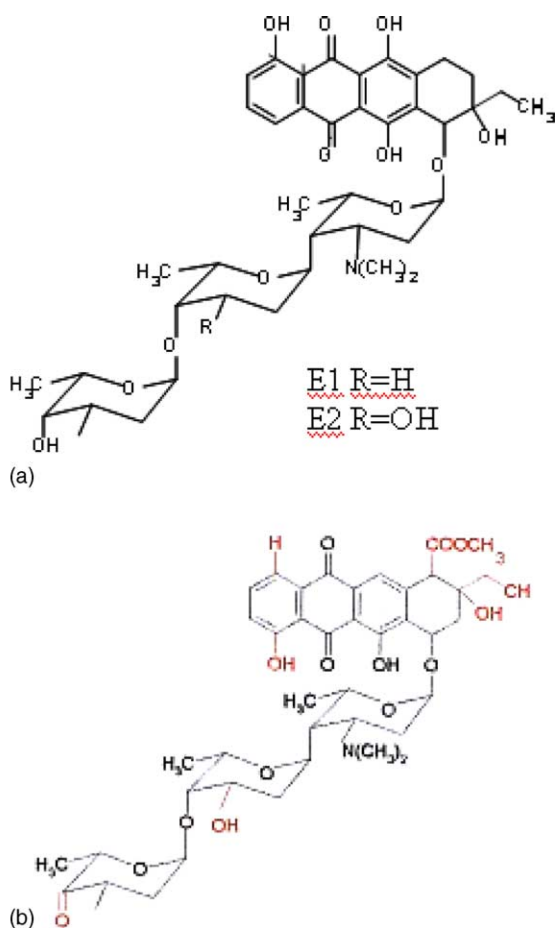
2.4. Assays

2.4.1. Retamycin quantification

Retamycin concentration was estimated by high-performance liquid chromatography (HPLC) (Waters, Milford, USA). Since retamycin is not commercialized, it was applied aclarubicin (product A5989, Sigma–Aldrich, USA), an antracyclinic antibiotic with a very close molecular structure (Fig. 1), as a standard for the calibration curve, made as follows: injection of 20 μL of standard solutions (10–1000 mg/L) to a reversed-phase column (C18 μBondapak-Waters, USA), operated in water/methanol gradient (10–90% of methanol in 25 min, from 90 to 100% until 30 min and maintaining 100% of methanol until 45 min). The absorbance measures ($\lambda = 492$ nm) showed 5 picks. Among them, the one at 31.6 min represents more than 90% of the total area, being this the aclarubicin, a product commercialized with at least 90% of this molecule, accordingly to SIGMA ALDRICH. The calibration curve is presented in Eq. (1):

$$\text{Aclarubicin (mg/L)} = 289.65\,\text{area} - 39.149, \quad r = 0.9966 \tag{1}$$

Finally, to establish the retention time of the retamycin molecules, samples of different culture times of *S. olindensis* were applied to the HPLC, including the medium before inoculation. The chromatograms depicted in Fig. 2 show a set of early picks, 4–9 min, of molecules of low hydrophobicity, which, in part, are already in the medium before the inoculation (Fig. 2a); a pick at 39.9 min, which is the methanol in the mobile phase, and a set of picks between 25 and 30 min, which appears after 56 h of culture (Fig. 2b) simultaneously to the violet color enhancement in the medium, the typical retamycin color. The area under this set of picks are considered to the Eq. (1), in order to estimate retamycin concentration.



(a)

(b)

Fig. 1. Molecular structure of retamycin E1 and E2 of *Streptomyces olindensis* (a) and aclarubicin (b) [13].

2.4.2. Red pigments quantification

Red pigments produced by *M. purpureus* CCT3802 were indirectly quantified with a spectrophotometer (BECKMAN COULTER, model DU530, USA), under scanning wavelengths that covers the red color range, 495–505 nm, and taking the maximum absorbance, which relies on 504 nm, for the samples analyzed.

2.5. Experimental designs

Experimental designs [14] were applied to the extractions of both, retamycin and red pigments (rubropunctamin and monascorubramin) under the next factors (input variables): PEG molar mass (X_1); pH (X_2); centrifugation force (X_3); additional contact time in the rotary homogenizer (X_4); PEG (X_5) and phosphate concentration (%, w/w) in the systems (X_6). The chosen response (output variable) was retamycin partition coefficient (K_r) or red pigments partition coefficient (K_{pig}) and retamycin yield (η), defined as follows:

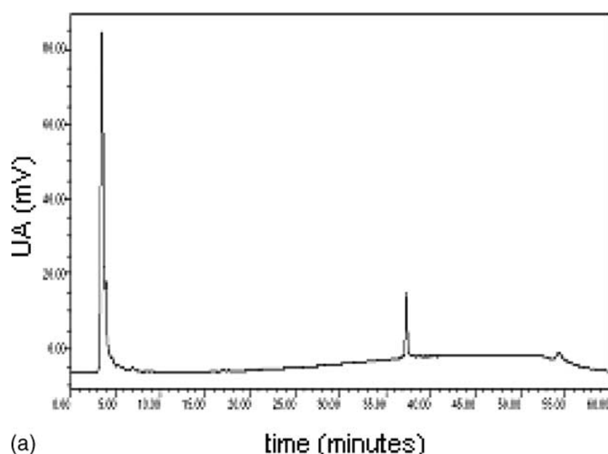
$$K_r = \frac{\text{retamycin concentration in top phase}}{\text{retamycin concentration in bottom phase}}$$

$$K_{pig} = \frac{\text{red pigments absorbance in top phase}}{\text{red pigments absorbance in bottom phase}}$$

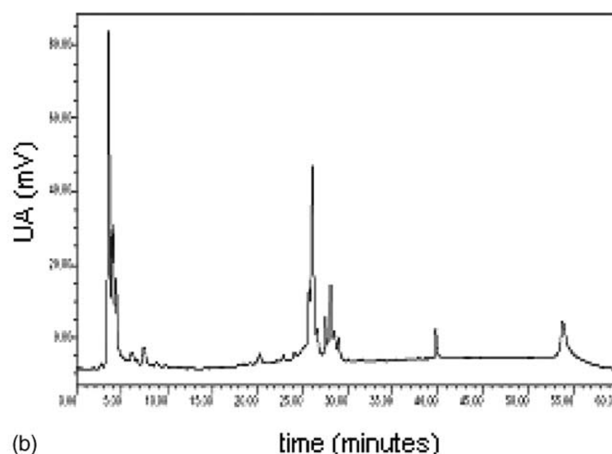
$$\eta = \frac{V_T C_{RT}}{V_0 C_{R0}} \times 100$$

A full factorial (2^4) experimental design with four repetitions in the central point were employed to evaluate the effect of the factors on the partition coefficient (K_r) and yield (η) of retamycin. The three levels (−1, 0 and +1) of the factors are 300, 1500 and 6000 for PEG molar mass (X_1); 6.0, 7.0 and 8.0 for pH (X_2); 900, 1350 and 1800 for g (X_3) and 0, 30 and 60 min for contact time (X_4).

For red pigments extraction, a full factorial (2^4) experimental design with two repetitions in the central point were employed to evaluate the effect of the factors on the partition coefficient (K_{pig}). The factors were defined after the experiments with retamycins, and their levels (−1, 0 and +1) are as follows: 1500, 4000 and 6000 for PEG molar mass (X_1); 7.0, 8.0 and 9.0 for pH (X_2); 10, 15 and 20% (w/w) for PEG concentration (X_5); 10.0, 12.5 and 15.0% (w/w) for phosphate concentration (X_6).



(a)



(b)

Fig. 2. Chromatograms of *Streptomyces olindensis* culture media before inoculation (a) and at 56 h of cultivation (b).

3. Results

3.1. Definition of an appropriate system for extraction from whole medium

Initial experiments of retamycin extraction from whole culture medium of *S. olindensis* So20 were done in PEG/citrate, PEG/sulfate and PEG/phosphate, all at pH 8.0, under variable concentrations of PEG and salt, in order to observe the partition of cells and antibiotic. The results (data not shown) show that despite the wide range of the molecular mass of PEG (400–6000) and salt concentration (20 and 40%) employed, cells partitioned to the bottom phase while the target molecule remains in the PEG rich phase.

The best system for retamycin extraction in PEG phase, from whole culture medium, is that with phosphate, because cells partitioned to the bottom phase while in PEG/citrate systems, cells partitioned close to the interface and PEG/sulfate systems showed unstably phase separation when the culture medium was added in.

Cells partitioning to the bottom phase, makes easy top phase separation, richer in PEG and where retamycin is accumulated. Otherwise, when cells remain in the interface, it is difficult to avoid some lost of the target molecule because interface medium must be discarded.

3.2. Extraction of retamycin PEG/phosphate systems from whole culture medium

For retamycin extraction from whole culture medium an experimental design full factorial (2^4) with four repetitions in central point was applied, accordingly to factors and levels presented in 2.5.

Table 1 presents the experimental design matrix, the conditions of the extractions and the values of K_r and yields, relative to the extraction of retamycin from whole culture medium. In all of the extractions, cells partitioned to the bottom phase. Cell partition coefficient was not calculated because the high viscosity imposed by PEG, makes filtration impossible; besides, traces of PEG in the filtration membranes presented interference in the dry mass membranes. Nevertheless, top phase was completely clear in all the extractions.

The highest values of K_r and yield were obtained in extractions 11 and 15, which varied by the centrifugation force. Table 2 presents the effects, standard errors and Student's

Table 2
Effects, standard errors and Student's *t*-test to K_r of Table 1

Factors**	Effects	*t*
Average	4.30 ± 0.93	46.28*
X_1	2.48 ± 0.21	−11.95*
X_2	−1.68 ± 0.21	−8.10*
X_3	0.17 ± 0.21	0.81
X_4	−1.32 ± 0.21	−6.35*
X_1X_2	−1.41 ± 0.21	−6.77*
X_1X_3	0.19 ± 0.21	0.93
X_1X_4	−0.32 ± 0.21	−1.54
X_2X_3	−0.14 ± 0.21	−0.69
X_2X_4	−0.36 ± 0.21	−1.72
X_3X_4	0.09 ± 0.21	0.45

X_1: PEG molar mass; X_2: pH; X_3: centrifugation force and X_4: contact time.
* Significant at 5% level ($t = 1.860$).

Table 1
Extraction conditions and results of a full factorial 2^4, with four center points for retamycin extraction in PEG/phosphate, from whole medium

Extraction	Factors and levels				Results[a]	
	X_1	X_2	X_3	X_4	K_r	η
1	6000	8.0	1800	60	3.7	45.9
2	300	8.0	1800	60	2.0	19.3
3	6000	6.0	1800	60	6.7	65.4
4	300	6.0	1800	60	3.4	52.8
5	6000	8.0	900	60	3.2	34.5
6	300	8.0	900	60	2.3	24.0
7	6000	6.0	900	60	6.0	49.5
8	300	6.0	900	60	3.3	48.4
9	6000	8.0	1800	0	4.9	60.1
10	300	8.0	1800	0	4.1	55.6
11	**6000**	**6.0**	**1800**	**0**	**8.3**	**90.5**
12	300	6.0	1800	0	3.5	52.6
13	6000	8.0	900	0	4.9	60.7
14	300	8.0	900	0	4.1	55.8
15	**6000**	**6.0**	**900**	**0**	**8.1**	**92.1**
16	300	6.0	900	0	3.4	50.3
1[b]	1500	7.0	1350	30	7.0	46.8
2[b]	1500	7.0	1350	30	3.3	43.2
3[b]	1500	7.0	1350	30	3.8	52.5
4[b]	1500	7.0	1350	30	4.0	55.6

X_1: PEG molar mass; X_2: pH; X_3: centrifugation force (g); X_4: contact time (min).
[a] Average K_r and η, relative to two extractions.
[b] Central point extractions.

Table 3
Analysis of variance (ANOVA) for the significant variables of retamycin extraction from whole culture medium, in linear model

Factors	Sum of squares (SS)	Degrees of freedom	Mean square	F value	P value
X_1	49.25	1	49.25	136.91	0.0000*
X_2	22.61	1	22.61	62.86	0.0000*
X_4	13.91	1	13.91	38.67	0.0000*
X_1X_2	15.82	1	15.82	43.98	0.0000*
Total error	12.59	35	0.36		
Total SS	114.19	39			

$R^2 = 0.890$; X_1: PEG molar mass, X_2: pH, X_4: contact time (min).
* Significant at 5% level.

t-test to average K_r. The significant effects were observed to PEG molar mass (X_1), pH (X_2), contact time (X_4) and the interaction between PEG molar mass and pH (X_1X_2). The sign of the factors indicates that the highest values of K_r (8.2) and yield (90.5) were obtained for PEG molar mass at 6000 (+), pH 6.0 (−) and under no extra contact time.

An analysis of variance (ANOVA) was made considering all the significant effects of Table 2 and is shown in Table 3. The low value of the determination coefficient ($R^2 = 0.89$) indicates that linear model is not adequate for this study, but the characteristics of the partitioning phenomenon were described, indicating therefore the main important process variables.

Although these data were obtained with intact cells, its interesting to exploit the application of ATPS to disrupted cells, regarding that most of antracycline purification processes apply cell disruption. The possibility of separating the product from cell debris in a process no sensitive to culture medium viscosity, like ATPS, is attractive, because processes like cross flow filtration and centrifugation, are strongly impaired by medium viscosity. Further purification, can be reached with reversed-phase adsorption, applied in Expanded Bed Adsorption (EBA), less sensitive to the PEG viscosity relative to compact adsorption beds.

3.3. Extraction of red pigments from whole culture medium

PEG/phosphate systems were applied also for red pigments extraction from whole culture medium of *M. purpureus* CCT3802. Excluding the centrifugation force (X_3) and the additional contact time (X_4) – they were not significant in the retamycin extraction – it was possible to include X_5 and X_6 (PEG and phosphate concentration respectively), accordingly to the levels shown in 2.5, which includes X_1 and X_2 also. Table 4 presents the experimental design matrix employed with the average values of K_{pig}, relative to two runs for each condition. The highest val-

Table 4
Extraction conditions and results of a full factorial design (2^4), with two central points for red pigments extraction in PEG/phosphate, from whole culture medium

Extraction	Factors and levels				K_{pig} [a]
	X_1	X_5	X_6	X_2	
1	**6000**	**20**	**15**	**9**	**113.3**
2	1500	20	15	9	79.7
3	6000	10	15	9	78.1
4	1500	10	15	9	8.8
5	6000	20	10	9	39.6
6	1500	20	10	9	7.9
7	6000	10	10	9	14.3
8	1500	10	10	9	N.a.
9	**6000**	**20**	**15**	**7**	**150.0**
10	1500	20	15	7	24.8
11	6000	10	15	7	44.9
12	1500	10	15	7	4.6
13	6000	20	10	7	38.8
14	1500	20	10	7	3.5
15	6000	10	10	7	3.4
16	1500	10	10	7	N.a.
1[b]	4000	15	12.5	8	21.0
2[b]	4000	15	12.5	8	6.9

N.a.: no phase separation, X_1: PEG molar mass; X_2: pH; X_5: PEG (%, w/w); X_6: phosphate (%, w/w).
[a] Average K_{pig}, relative to two extractions.
[b] Central point extractions.

Table 5
Effects, standard errors and Student's t-test to K_{pig} of Table 4

Factors**	PEG/phosphate system	
	Effects	t
Average	34.6 ± 3.7	–
X_1	38.7 ± 7.9	4.9*
X_5	32.5 ± 7.9	4.1*
X_6	44.2 ± 7.9	5.6*
X_2	18.4 ± 7.9	2.3
X_1X_5	7.4 ± 7.9	0.9
X_1X_6	18.0 ± 7.9	2.3
X_1X_2	2.5 ± 7.9	0.3
X_5X_6	15.0 ± 7.9	1.9
X_5X_2	6.3 ± 7.9	0.8
X_6X_2	14.4 ± 7.9	1.8

X_1: PEG molar mass; X_2: pH; X_5: PEG (%, w/w); X_6: phosphate (%, w/w).
* Significant at the 5% level.

Table 6
Analysis of variance (ANOVA) for the significant variables of red pigments extraction from whole culture medium, in linear model

Factors	Sum of squares (SS)	Degrees of freedom	Mean square	*F* value	*P* value
X_1	6648.3	1	6648.3	15.0730	0.00189*
X_5	4794.0	1	4794.0	10.8690	0.00578*
X_6	8528.0	1	8528.0	19.2249	0.00072*
X_2	1860.0	1	1860.0	4.2170	0.06071
Total error	5733.9	13	441.1		
Total SS	30716.52	17			

$R^2 = 0.81$. X_1: PEG molar mass; X_2: pH; X_5: PEG (%, w/w); X_6: phosphate (%, w/w).
* Significant at 5% level.

ues of K_{pig} (113 and 150) were obtained in extractions 1 and 9.

The analysis of effects (Table 5) show that all the factors had a positive influence on K_{pig}, and any one of the interactions considered (until 2) was significant. The ANOVA to main effects (molar mass of PEG, % (w/w) of PEG, % (w/w) of phosphate and pH) (Table 6), with a low determination coefficient (0.81), showed that a linear model is not appropriate to represent the partitioning coefficient. However, on the basis of the statistical analysis and the range applied to the factors, the best conditions to extract the pigments in top phase are: PEG 6000 (20%, w/w) and phosphate concentration (15%, w/w), which led to K_{pig} value of 113 and 150.

4. Conclusions

PEG/phosphate systems are able to extract retamycin and red pigments to the top PEG phase, while the filamentous microorganisms – *S. olindensis* and *M. purpureus* – remains in the salt bottom phase, allowing some degree of purification and simultaneous clarification.

Through experimental designs, the main variables and their levels were defined as follows: for retamycin extraction, PEG 6000 (10%, w/w), phosphate at 20% (w/w) and pH 6.0 led to the higher partition coefficient, $K_r = 8.2$ and yield = 91.3%; for red pigments, the statistical analysis led to PEG 6000 (20%, w/w) and phosphate (15%, w/w), for K_{pig} values of 113 and 150.

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