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Optimization of adenosine 5′-triphosphate extraction for the measurement of acidogenic biomass utilizing whey wastewater

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Abstract

A set of experiments was carried out to maximize adenosine 5′-triphosphate (ATP) extraction efficiency from acidogenic culture using whey wastewater. ATP concentrations at different microbial concentrations increased linearly as microbial concentration decreased. More than 50% of ATP was extracted from the sample of 39 mg volatile suspended solids (VSS)/l compared to the sample of 2.8 g VSS/l. The ATP concentrations of the corresponding samples were 0.74 ± 0.06 and 0.49 ± 0.05 mg/l, respectively. For low VSS concentrations ranging from 39 to 92 mg/l, the extracted ATP concentration did not vary significantly at 0.73 ± 0.01 mg ATP/l. Response surface methodology with a central composite in cube design for the experiments was used to locate the optimum for maximal ATP extraction with respect to boiling and bead beating treatments. The overall designed intervals were from 0 to 15 min and from 0 to 3 min for boiling and bead beating, respectively. The extracted ATP concentration ranged from 0.01 to 0.74 mg/l within the design boundary. The following is a partial cubic model where η is the concentration of ATP and x_k is the corresponding variable term (k = boiling time and bead beating time in order): $\eta = 0.629 + 0.035x_1 – 0.818x_2 – 0.002x_1x_2 – 0.003{x_1}^2 + 0.254{x_2}^2 + 0.002{x_1}^2x_2$. This model successfully approximates the response of ATP concentration with respect to the boiling- and bead beating-time. The condition for maximal ATP extraction was 5.6 min boiling without bead beating. The maximal ATP concentration using the model was 0.74 mg/l, which was identical to the experimental value at optimum condition for ATP extraction.

Introduction

Anaerobic process involves many different species of symbiotic microorganisms, which are broadly divided into two groups: acidogenic and methanogenic bacteria. It has recently been suggested that an overall process enhancement must be based on an understanding of the behavior of acidogens, especially optimization and mathematical modeling of acidogenesis (Hwang et al. 2001; Yang et al. 2003) since they play a primary role in producing major substrates, short-chain organic acids, for methanogens.

In almost all anaerobic process evaluations, accurate estimation of changes in the mass of the microbial population plays very basic and an important roles in determining the system performance. The volatile suspended solids (VSS) test is the most widely used method of quantifying microorganisms in environmental engineering (Yu et al. 2002). This method, however, can give inaccurate results when used to represent microbial concentration if the wastewater contains a fraction of suspended organic materials, because the VSS test cannot differentiate between microbial cells and suspended organic particles in the

wastewater. These environments include agricultural and food processing wastewaters, which are mostly treated by anaerobic processes (Hwang et al. 2001). Therefore, ways of measuring microbial concentration other than the VSS test are essential for better comprehension of operations and limitations of the anaerobic systems. The choice of method depends on the purpose of the analysis and the type of system being investigated. A method used to analyze wastewater, however, should be inexpensive, simple, and reliable. For this reason, attempts have been made to use microbial activity as an indirect parameter to indicate the presence of microorganisms. The most promising way of measuring the microbial activity in suspended organic wastewaters is to analyze specific biochemicals such as adenosine 5′-triphosphate (ATP), nucleic acids, or phospholipids, which universally exist in living cells (Ranalli et al. 2001; Richardson et al. 2002; Yu et al. 2002). Among these biochemicals, ATP is considered to be the most practical one to estimate microbial biomass because ATP is ubiquitously present in living cells and the amount of ATP per cell remains fairly constant (Ishida et al. 2002).

The firefly bioluminescence assay is widely used to measure ATP. This reaction involves the ATP-dependent oxidation of luciferin catalyzed by luciferase, resulting in the emission of light (Nelson 1991):

$$\text{luciferin} + \text{ATP} + \text{Mg}^{2+} + \text{O}_2 \xrightarrow{\text{Luciferase}} \text{oxyluciferine} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{light}.$$

Here ATP functions to link the enzyme luciferase to its substrate luciferin. Under conditions where ATP is only limiting, the light intensity emitted is proportional to the concentration of ATP (Lundin 2000). The bioluminescence method offers a highly sensitive and rapid result in ATP analysis. ATP can be extracted from a wastewater sample mixed with microorganisms, and the concentration in the extract can be determined using this method (Alfenore et al. 2003; Yu et al. 2002).

In an ATP assay, it is necessary to disintegrate the bacterial membrane structure to release cellular ATP, which in turn is measured using the bioluminescence reaction. Boiling and/or bead beating, using thermal shock and mechanical impact, respectively, are widely used methods in cell lysis and would be the most economical and practical methods for extracting ATP. These methods are used together, with boiling usually followed by bead beating or alone for environmental samples (Howeler et al. 2003; Yang et al. 2002; Yu et al. 2002). Enzymatic cell lysis, commonly used in molecular biology, would not be practical for use in wastewater engineering mainly due to the high cost. Contrary to using chemical extractants (Velazquez & Feirtag 1997), a recently developed ATP extraction method using boiling deionized water (DW) does not interfere with the bioluminescence, and is more sensitive than other methods tested (Yang et al. 2002). Although these methods become more popular to disintegrate cell membrane in environmental molecular technology, it must be noted that maintaining a consistent and maximal efficiency in the ATP extraction is extremely important for the reliable analysis. Nevertheless, optimization of ATP extraction from such highly suspended organic wastewater by a systematic approach is lacking in literature. Therefore, this study was conducted to optimize conditions where the efficiency of acidogenic ATP extraction from highly suspended organic wastewater, with respect to the simultaneous effects of boiling and bead beating, is maximized using a response surface methodology (RSM).

Materials and methods

Operation of a continuous acidogenesis system and microbial identification

A lab-scale continuously stirred tank reactor (CSTR) with a working volume of 1.5 l was used for culturing acidogens. Anaerobic seed sludge from a local municipal wastewater treatment plant was cultivated in the system to enrich a mixed population of acidogens by combining biokinetic and chemical controls (Hwang et al. 2001). The system was operated with dilute whey wastewater of 20.0 ± 0.5 g chemical oxygen demand (COD)/l at 0.5 ± 0.02 days hydraulic retention time (HRT). Yeast extract of 1 g/l was added to provide trace minerals. Temperature and pH were maintained at 35° C and at 6.0 with 3.0 N NaOH, respectively. The operating conditions remained constant throughout the experiment.

An effort was made to ensure that the enriched culture was a mixed population of acidogens by analyzing the bacterial community structure using a molecular technique. DNA was extracted from the acidogenesis system with an automated nucleic acid extractor (Magtration System 6GC, Precision System Science). Two universal primers for bacteria, Bac338F (5′-ACTCCTACGGGAGG-CAGC-3′) and Bac1099R (5′-GCAACGAGCG-CAACCC-3′), were used to amplify the bacterial small subunit (SSU) rDNA from the total acidogenic DNA (Jackson et al. 2001). Each polymerase chain reaction (PCR) mixture of 50 μl contained 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP), 10 pmol of each primer, 5U of *Taq* DNA polymerase (Takara *Taq*, Takara), and 0.5 μg of the extracted DNA. PCR amplification was carried out in a thermal cycler (PTC-100, MJ research Inc.) with the following program: an initial denaturation at 94 °C for 4 min; followed by 35 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 7 min.

The PCR fragments were purified using a purification kit (QIAquick PCR Purification, Qiagen) and cloned in *Escherichia coli* DH5α using a commercial cloning vector (pGEM-T Easy Vector, Promega) according to the manufacturer's manual. Cloned plasmids were isolated from randomly selected colonies of the library using a commercial kit (QIAprep Plasmid Miniprep, Qiagen) and used as templates for the DNA sequencing analysis. Sequencing was performed with T7 and SP6 primers using an automated sequencer (ABI PRISM 3100, Perkin-Elmer). The sequencing results were compared to the reference sequences in the GenBank and Ribosomal Database Project II databases. A sequence analysis software (DNAMAN, Lynnon Biosoft) was used to analyze similarity of sequences and estimate their phylogenetic relationship. The phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei 1987). The stability of the clustering of the tree was evaluated using bootstrap analysis of 1000 data sets.

Optimization using response surface methodology

In this research, we examined the ATP extraction efficiency associated with simultaneous changes of boiling and bead beating times. The initial starting points for boiling and bead beating times were selected based on the literature (Howeler et al. 2003; Yang et al. 2002; Yu et al. 2002). Response surface methodology (RSM), a collection of mathematical and statistical techniques for building empirical models (Box & Draper 1987; Hwang et al. 2001), was applied to locate the optimum treatment condition for maximizing the ATP extraction efficiency within the investigated region of the independent variables. A sequential procedure of collecting data, estimating polynomials, and checking the adequacy of the model was used. The method of least squares was used to estimate the parameters in the polynomials (Lee et al. 2003). Once the optimum region was found, a polynomial of higher degree was used to approximate the response because of the curvature in the surface. An analysis was then performed to locate the optimum treatment condition, which was the set of independent variables such that the partial derivatives of the model response with respect to the individual independent variable equaled zero. The central composite in cube (CCC) design (Montgomery 2001), which consisted of an orthogonal 2^2 factorial design augmented by a center and 2×2 axial points, was employed in this study.

Analytical methods

A gas chromatograph (Agilent 6890 Plus GC, Agilent) equipped with an Innowax capillary column (Agilent) and a flame ionization detector was used to quantify short-chain organic acids and ethanol. Helium was used as a carrier gas at a flow rate of 2.5 ml/min with a split ratio of 10:1. Solids were analyzed according to the procedures in Standard Methods (APHA-AWWA-WEF 1998). COD was measured by the closed reflux colorimetric method, and total carbohydrate (TC) was analyzed by the phenol-sulfuric acid method (APHA-AWWA-WEF 1998). The concentration of ammonia and organic nitrogen was measured according to the Kjeldahl method (APHA-AWWA-WEF 1998). A high performance liquid chromatograph (Agilent 1100 HPLC, Agilent) equipped with a refractive index detector was used to quantify lactose.

ATP was extracted as follows: A sample from the acidogenesis system was diluted to have a

desired VSS concentration. One milliliter of the diluted sample was centrifuged at 12,000 *g* for 10 min. The supernatant was decanted and boiling deionized water (DW) of 1 ml was added (Yang et al. 2002). The mixture was further heated in a boiling water bath for a desired time, followed by bead beating according to the experimental design. Bead beating was performed using 0.5 g of 0.1 mm diameter sterile zirconia/silica beads in a bead beater (Mini-BeadBeater, BioSpec Products). The extract was then filtered through a 0.45 μm membrane filter (Acrodisc Syringe Filter, Pall Corporation) to remove suspended particles. Twenty microliters of the filtrate was mixed with 100 μl of luciferin-luciferase reagent solution, and a microplate luminometer (Luminoskan Ascent, Labsystems) was used to measure bioluminescence. The assay solution was prepared using a commercial kit (ENLITEN ATP Assay System, Promega). All ATP extractions were performed in triplicate.

Results and discussion

Reactor performance and microbial identification

Table 1 summarizes the physical and biochemical characteristics of the whey wastewater used in this study. The total volatile solids (TVS) of 18.4 g/l indicated the amount of potentially biodegradable substances in the whey wastewater, which was a ratio of 92% compared to the total solids (TS). Approximately, 94% of the TVS were presented as a dissolved form. Lactose was the major soluble organic substance in the wastewater because the theoretical oxygen demand exerted by 16.4 g/l of lactose is 18.4 g/l (i.e., (16.4 g lactose/l)×(1.12 g O_2/g lactose)), which was nearly 100% of the soluble COD. The rest of the volatile solids, 6%, were likely to be protein and fat (Hwang & Hansen 1998).

Table 1. Physical and chemical characteristics of the whey wastewater

Parameters	Concentration (mg/l)
Total COD	19,730 (617)[a]
Soluble COD	17,490 (705)
Lactose	16,350 (713)
Protein	1,881 (75)
TKN	340 (15)
Organic N	301 (14)
NH_4^+	39 (1)
Total solids	20,130 (55)
Total volatile solids	18,360 (23)
Volatile dissolved solids	17,350 (92)
Total suspended solids	1120 (68)
Volatile suspended solids	1055 (65)

[a]Standard deviations are in parentheses.

Methane production ceased after approximately 6 turnovers of the acidogenic reactor. Steady state conditions with respect to lactose degradation and organic acid production were maintained after 10 turnovers. Nearly all lactose, 97.6%, was utilized while acetic acid, butyric acid, and ethanol were major products in the effluent, which were 4.8, 1.5, and 1.0 g/l, respectively.

Because microbial cells were also suspended in the system, efforts were made to approximate the contribution of microbial mass to the VSS concentration (i.e., 2.8 g/l) in the effluent from the system. If the VSS input of 1.1 g/l (Table 1) is totally refractory organics, a minimum of 61% of effluent VSS (i.e., (2.8–1.1)/2.8) must be composed of microbial mass. However, this is highly unlikely to be the case because suspended organics in whey wastewater are mostly composed of milk protein and fat, which are readily biodegradable organic substances (Hwang & Hansen 1998). The yield coefficient of acidogens with lactose in whey wastewater ranges from 0.16 to 0.29 g VSS/g lactose (Kisaalita et al. 1989; Yu et al. 2002), which translates 2.6–4.7 g microbial VSS/l in this system. Therefore, it could be concluded that microorganisms comprised a major portion of VSS in the reactor.

We constructed a clone library of the bacterial SSU rDNA fragments, amplified by PCR using the total acidogenic DNA extracts from the acidogenic reactor, to analyze the structure of the bacterial community. A total of 49 clones from the library were randomly selected and sequenced. Figure 1 and Table 2 show the phylogenetic affiliation of major phylotypes derived from this library with respect to other known bacteria and uncultured rDNA clones from various environments. Abbreviations indicate uncultivated SSU rDNA clones from various environments: A11, from human fecal samples (Zoetendal et al. 1998); p-2184–s959-3 and p-1565-b5, p-60-a5, and p-2746–24E5 from pig gastrointestinal tract (Leser et al. 2002). The sequences obtained in this study are described in bold characters. The numbers

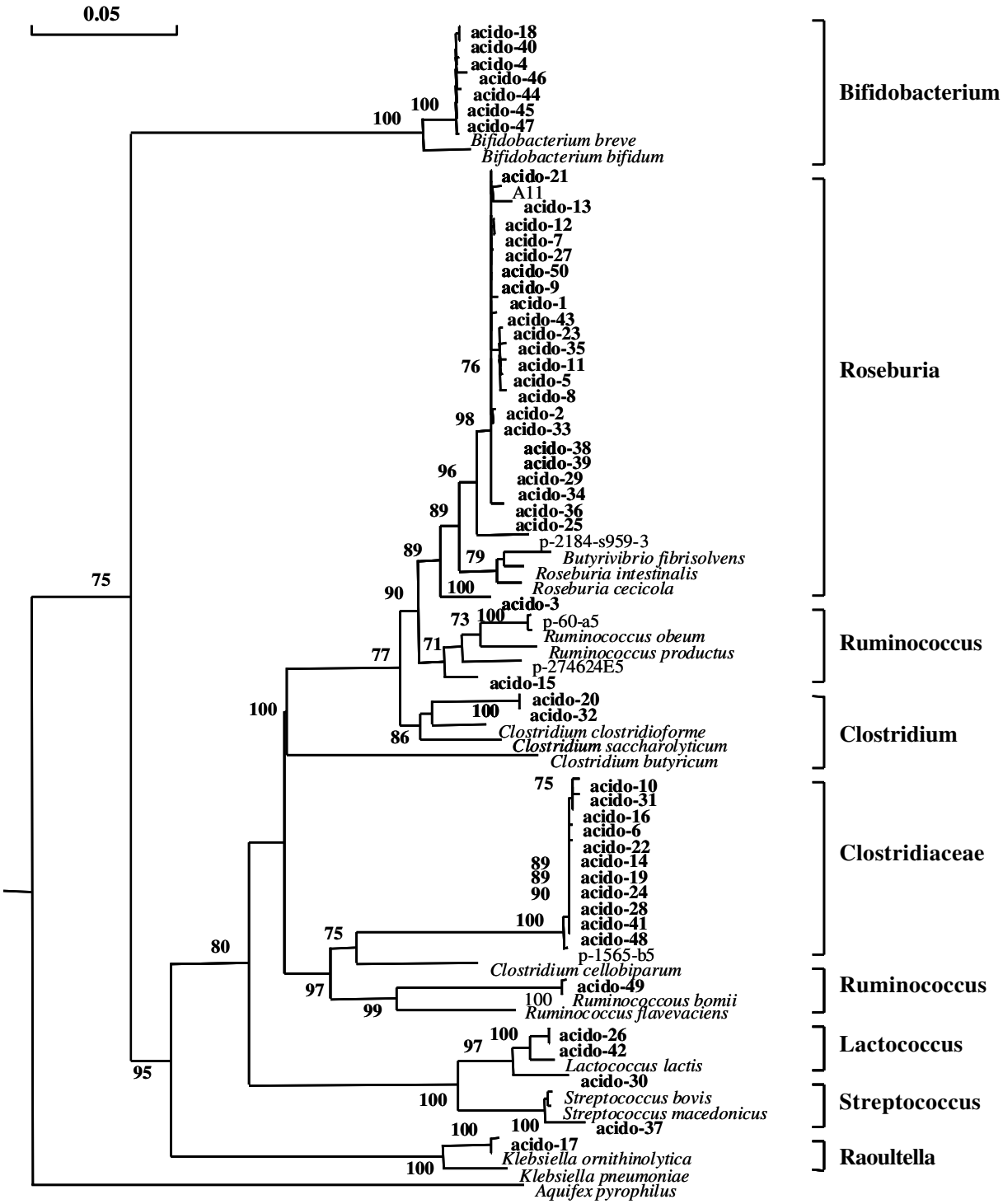

Figure 1. Phylogenetic placement of SSU rDNA sequences from the continuous acidogenesis system.

above internal segments are the percentages of 1000 bootstrap replicates and the scale bar represents 5% sequence difference.

All clones were assigned to the known acidogenic genera *Bifidobacterium, Clostridim, Lactococcous, Raoultella, Roseburia, Ruminococcus*,

Table 2. Similarity values of 16S rDNA sequences retrieved from the acidogenic inoculum system

Genus	Nearest relative species	Clones	Similarity
Roseburia	*R. intestinalis* (G-)[a]	acido-1, 2, 5, 7, 8, 9, 11, 12, 13, 21, 23, 25, 27, 29, 33, 34, 35, 36, 38, 39, 43, 50	96.3%
Clostridium	*C. cellobioparum* (G+)	acido- 6, 10, 14, 16, 19, 22, 24, 28, 31, 41, 48	88.9%
	C. clostridioforme (G+)	acido-20, 32	95.1%
Bifidobacterium	*B. breve* (G+)	acido- 4, 18, 40, 44, 45, 46, 47	99.7%
Lactococcus	*L. lactis* (G+)	acido-26, 42	99.7%
Raoultella	*R. ornithinolytica* (G-)	acido-17	99.5%
Streptococcus	*S. macedonicus* (G+)	acido-37	97.6%
	S. bovis (G+)	acido-30	94.7%
Ruminococcus	*R. obeum* (G+)	acido-3	96.7%
	R. bromii (G+)	acido-49	99.9%

[a] Gram types of the species are in parentheses.

Streptococcus, and an acidogenic family *Clostridiaceae*. Approximately 45% of the all clones from this study (i.e., 22 of 49 clones) belonged to *Roseburia*, which is a butyrate-producing acidogenic genus. About 22% of the clones (i.e., 11 of 49 clones) had a low similarity of less than 90% with a known genus in the reference databases. However, it was determined that these clones belonged to *Clostridiaceae* at the family level. The rest of the clones in the library were identified as *Bifidobacterium breve* (7 clones), *Lactococcus lactis* (2 clones), *Rauoltella ornithinolytica* (1 clone), *Ruminococcus bromii* (1 clone), and *Streptococcus macedonicus* (1 clone).

Optimization of ATP extraction

Prior to the optimization of ATP extraction, the effect of different microbial concentrations on ATP extraction was investigated. A total of six different VSS concentrations set up in ratios of 1:5:10:30:50:70 using the reactor effluent of 2.8 g VSS/l were tested. Because optimal time for additional boiling was unknown at this stage, ATP was extracted by adding boiling DW followed by 10 min additional boiling (Yang et al. 2002; Yu et al. 2002). ATP concentrations at different dilutions increased linearly as dilution rate increased (Figure 2). More than 50% of ATP could be extracted at 70-fold dilution (i.e., 39 mg VSS/l) compared to that in the undiluted sample (i.e., 2.8 g VSS/l), where the ATP concentrations were 0.74 ± 0.06 and 0.49 ± 0.05 mg ATP/l, respectively. For low VSS concentrations ranging from 39 to 92 mg/l, the extracted ATP concentration was

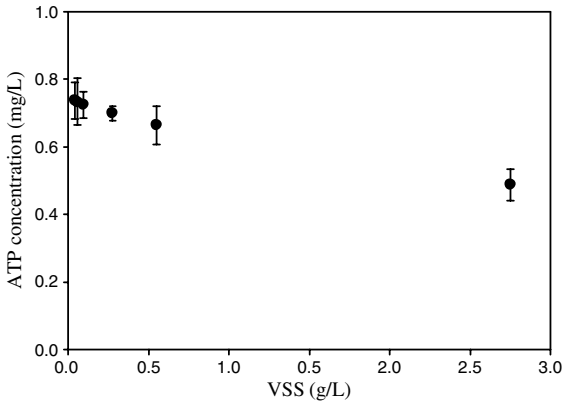

Figure 2. The effect of different microbial concentrations on ATP extraction. The data are expressed as means ± standard deviation with error bar, $n = 3$.

0.73 ± 0.01 mg ATP/l, which indicated that the ATP extraction efficiency did not vary significantly at a low VSS concentration. However, care should be taken to ensure that the sample is not diluted too much because inclusion of a small aggregated particle of microorganisms may produce a relatively large variation in ATP concentration at a high dilution.

The first region of exploration for the first-order model was decided to be 10 ± 5 min of boiling and 2 ± 1 min of bead beating (Howeler et al. 2003; Yang et al. 2002; Yu et al. 2002). Based on analysis of the initial nine trials as described previously, the amount of ATP in the extract increased with decreasing in both boiling and bead beating times in the investigated region. Therefore, a new region of exploration was used and was 5 ± 5

and 1 ± 1 min for boiling and bead beating, respectively (Figure 3). Because a new region of exploration contains no additional treatment of boiling and bead beating (Figure 3), a sequential approach of approximating the response using a linear followed by a quadratic design boundary (Hwang & Hansen 1997) would not be necessary. Therefore, models from first-order to partial cubic were sequentially tested with the trials (Table 3) to find a maximum in the response using a central composite design augmented with five center and four axial data points. Repeated observations at the new center point (i.e. 5 min of boiling and 1 min of bead beating) were used to estimate the experimental error. The *p*-values of regression, lack of fit, and corresponding coefficients were also tested. Residual plots as well as variances were simultaneously analyzed to ascertain if multiple models were statistically significant in describing the response. Based on statistical inspection of the coefficients of the models, a partial cubic model without an interaction term (Eq. 1) was used to approximate the response surface of the augmented trials, which was

$$\eta = 0.629 + 0.035x_1 - 0.818x_2 - 0.002x_1x_2 - 0.003x_1^2 + 0.254x_2^2 + 0.002x_1^2x_2 \quad (1)$$

where η is concentration of ATP extracted from sample, and x_1 and x_2 represent the boiling time and bead beating time, respectively.

Regression was significant and lack of fit was not significant for the model at 0.1% α level. All terms of the model were significant at 1% α level with a high regression coefficient of 0.999 and a small residual standard deviation of 0.012. The optimal treatment condition to maximize ATP extraction was calculated by setting the partial derivatives of the function to zero with respect to the corresponding independent variables. The estimated optimum was 5.6 min boiling without bead beating. The calculated model output at the optimal condition was 0.73 ± 0.04 mg ATP/l.

Two- and three-dimensional response surfaces of the modified partial cubic model for the ATP concentration, with estimated optimums, are shown in Figure 4. The response surface showed a flat sheet with constant contour lines. This meant that the two independent variables were not interdependent and bead beating exerted an adverse effect on ATP extraction.

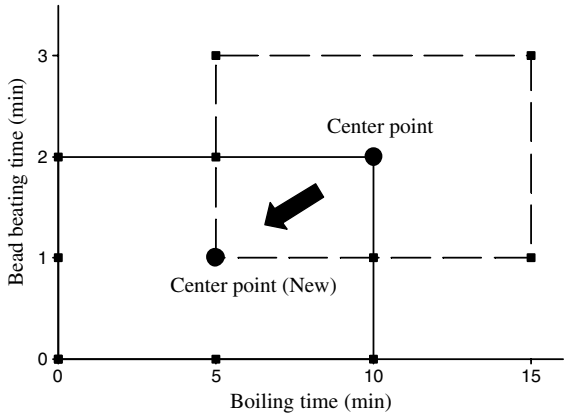

Figure 3. Experimental design boundary for initial and new region of exploration. Dashed line: design boundary for initial region; solid line: design boundary for new region. The arrow indicates the direction of movement of explored region.

Table 3. Experimental conditions and results of the central composite design

Trials	Conditions of variables		ATP concentration (mg/l)
	Boiling time (min)	Bead beating time (min)	
1	0	0	0.622
2	0	2	0.002
3	10	0	0.661
4	10	2	0.004
5[a]	5	1	0.102 (0.008)
6	0	1	0.078
7	5	0	0.736
8	5	2	0.005
9	10	1	0.090

[a] Experiment was replicated five times and the response presented average values (standard deviation).

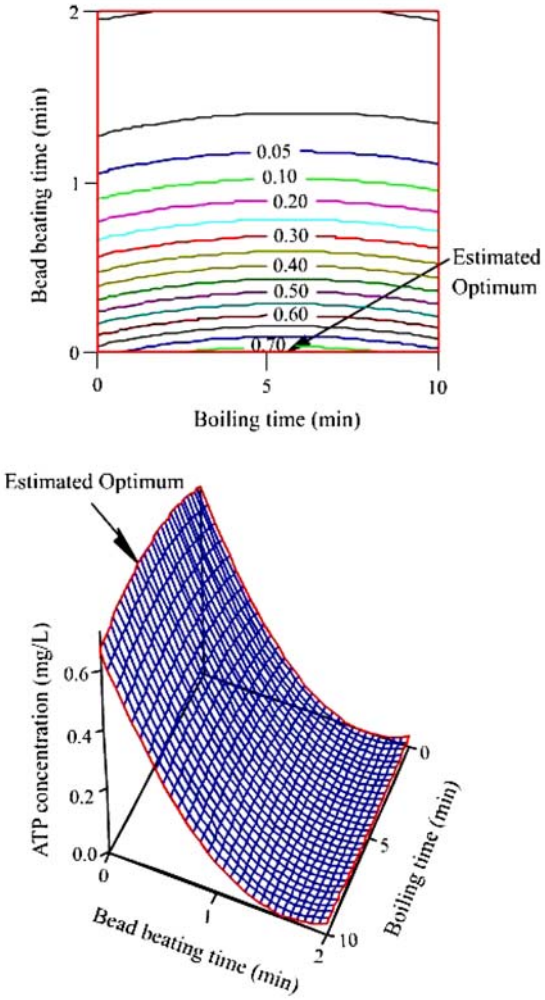

Figure 4. Two- and three-dimensional response surfaces of the modified partial cubic model for the ATP concentration with respect to treatment times of boiling and bead beating.

The adequacy of the fit of the model (i.e., Eq. 1) was verified by comparing the maximum model output of the ATP concentration with an experimental value, 0.74 ± 0.03 mg/l, at an optimum condition, then residual plots for all observed values were examined for any weakness in the models (Box & Draper 1987). The residual plots for the model and data set showed no patterns or trends with variance of less than 0.017 (data not shown). A check of the constant variance assumption also could be addressed because a random plot of residuals meant homogeneous error variances across the observed values. Excellent predictions of maximum responses along with constant variance

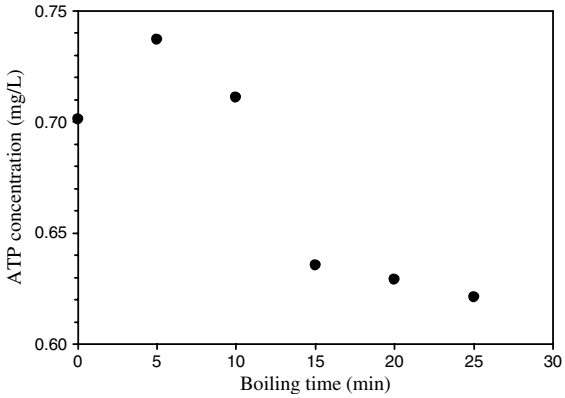

Figure 5. The concentration of ATP extracted at different boiling times without bead beating. The data are expressed as means ± standard deviation with error bar, $n = 3$.

in residual plots indicated the adequacy of the models, which meant the modified partial cubic model could fit the response surface of the ATP concentration with respect to boiling and bead beating.

Figure 5 represents the concentration of ATP extracted from samples of 55 ± 4.6 mg VSS/l at different boiling times without bead beating. The maximum extraction of 0.74 ± 0.03 mg ATP/l at 5 min additional boiling, which was close to the experimental value at the estimated optimum condition, was another indication of model adequacy. More than 10 min of additional boiling caused considerable reduction in the ATP extraction efficiency. Therefore, it could be concluded that the RSM with an orthogonal experimental design could be used to locate the condition that maximized the acidogenic ATP extraction using whey wastewater within the investigated experimental region.

Conclusions

A set of experiments was carried out to maximize ATP extraction efficiency from acidogenic culture using whey wastewater. ATP extraction efficiency varied more than 50% with different VSS concentrations ranging from 39 mg/l to 2.8 g/l. This indicated that microbial concentration in the sample could affect the ATP extraction efficiency. Therefore, it is desirable to extract ATP at a constant level of VSS concentration for reliable assay results. The acidogenic culture

showed high and fairly constant ATP extraction efficiency in a range of VSS concentration from 39 mg/l to 92 mg/l. RSM was successfully applied to determine the optimum treatment condition to maximize the ATP extraction efficiency, which was 5.6 min boiling without bead beating. The calculated value for concentration of extracted ATP was 0.73 ± 0.04 mg/l at the optimum condition. The ATP extraction efficiency decreased up to 16% at more than 10 min of boiling. The range of VSS concentrations along with the ATP extraction conditions investigated in this work would be a practical guideline to use an ATP assay in acidogenesis of whey wastewater or similar highly suspended organic wastewaters.

Acknowledgements

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