Pre-optimization of Medium for Biobutanol Production by a New Isolate of Solvent-producing *Clostridium*

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A Plackett-Burman design was used to pre-optimize the medium composition for biobutanol production using a unique isolate of solvent-producing *Clostridium* YM1. Various nutrient factors affecting biobutanol production were screened using the Plackett-Burman design. These factors included: glucose, tryptone, yeast extract, peptone, ammonium acetate, KH_2PO_4, K_2HPO_4, $MgSO_4$, $FeSO_4$, Na_2CO_3, and NaCl. The results were analyzed by an analysis of variance (ANOVA), which showed that glucose, tryptone, yeast extract, peptone, K_2HPO_4, Na_2CO_3, and $MgSO_4$ had significant effects on biobutanol production. However, ammonium acetate, KH_2PO_4, and $FeSO_4$ had insignificant effects. The established model from the ANOVA analysis had a significant value of $P_{model}>F$ = 0.0245 and an R^2 value of 0.999. The estimated maximum biobutanol production was 9.01 g/L, whereas the optimized medium produced 10.93 g/L of biobutanol.

Keywords: Biobutanol production; Novel butanol-producing strain; Plackett-Burman design; Medium optimization

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INTRODUCTION

Butanol is considered a promising renewable energy source and a future biofuel having potential to replace gasoline. Butanol has more advantageous fuel properties than ethanol, such as higher energy content, less sensitivity to temperature, less corrosivity, and the absence of any required modification in combustion engines (Jang *et al.* 2012; Lee *et al*. 2008). Butanol can be produced biologically through a well-known fermentation process called acetone-butanol-ethanol (ABE) fermentation using solvent-producing *Clostridium* strains. However, ABE fermentation has many shortcomings, including low production of butanol due to butanol toxicity, high cost of substrates (63%) (Jones and Woods 1986), and complications in recovery due to the presence of by-products such as ethanol, acetone, and acids. Hence, isolation and identification of new strains that produce larger amounts of biobutanol and optimization of culture conditions are vital. These can contribute to solving the problem of poor biobutanol production.

The Plackett-Burman design (PBD) is a two level factorial design that allows us to establish experiments with some number between these fractional factorial designs. It was used for the first time in 1946 (Plackett and Burman 1946).

PBD as statistical design is a linear model (Noguchi *et al.* 2012). It has been successfully used to pre-optimize alkaline protease production (Vaidya *et al.* 2009), bio-

ethanol production (Yingling *et al.* 2011), and phenolic compounds extraction (Anastácio and Carvalho 2013; Dopico-García *et al.* 2007).

PBD was used in this study because of the large number of nutritional factors (11 factors) to be investigated in terms of their effects on biobutanol production. This design can examine N factors in N+1 experiments.

The objective of this study was to optimize the production of biobutanol by screening the effect of nutrient factors using the Plackett-Burman design.

EXPERIMENTAL

Isolation of Solvent-producing *Clostridium*

Submerged soil samples were collected from a system of rice intensification (SRI) paddy fields located in Ban 9, Parit 3, Sekinchan, Selangor, Malaysia. The soil samples were transferred immediately into 100 mL serum bottles containing 50 mL sterilized RCM medium, which was pre-sparged with nitrogen to create anaerobic conditions.

The cultures were incubated thereafter at 30°C, and the gas production was observed for 5 days. The gas-producing cultures were then used to inoculate RCM agar plates at 30°C under anaerobic conditions using a generation kit for 2 days. Single colonies were transferred to new RCM agar plates and also incubated at 30°C under anaerobic conditions. Next, Gram staining was carried out to study the cell shape and the reaction with Gram stain. Only Gram positive, rod-shaped cells and gas-producing cultures were taken for further investigations.

The ability of the cultures to produce solvents (ABE) was checked using an acetone test. In this test, 5% sodium nitroperoside solution and ammonium solution (40%) were used. Positive acetone production was indicated by a change in the color of the culture suspension from yellow to purple.

Media Preparation and Butanol Fermentation

To evaluate the ability of isolated strains to produce ABE (able to produce acetone, Gram positive, rod cell shape, and able to form spores), RCM was used as a medium, 30°C as an incubation temperature, 10% inoculum size, and under anaerobic conditions.

Among ABE producer strains isolated, YM1isolate showed the highest ABE production and was selected for ABE production using different media, including reinforced clostridial media (RCM), anaerobic sugar (AnS) medium, P2 medium, and tryptone yeast extract acetate medium (TYA).

RCM medium contained 30 g/L glucose, 10 g/L peptone, 10 g/L beef extract, 3 g/L yeast extract, 5 g/L sodium chloride, 0.5 g/L cysteine HCl, 3 g/L sodium acetate, and 0.5 g/L agar. TYA medium was also used to prepare the inoculum and it was used as a fermentation medium and consisted of the following: 30 g/L glucose, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.4 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.4H_2O$, 0.01 g/L $FeSO_4.5H_2O$, 1.0 g/L yeast extract, and 0.5 g/L cysteine. A final concentration of 80 µg/L biotin and 1 mL of a solution containing 1 mg/L 4-aminobenzoic acid were added to 1 L of P2 medium. AnS medium consisted of the following components: 30 g/L glucose, 10 g/L peptone, 5 g/L yeast extract, 3 g/L K_2HPO_4, 1 g/L NaCl, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgCl_2.6H_2O$, 0.2 g/L $CaCl.2H_2O$, and 1 g/L Na_2CO_3.

ABE and Acids Analysis

The ABE and acids (acetic and butyric acids) concentration were measured using gas chromatography (7890A GC-System; Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and 30 m capillary column (Equity1; 30 m × 0.32 mm × 1.0 µm film thickness; Supelco Co, Bellefonate, PA, USA). The oven temperature was programmed to increase from 40 to 130°C at a rate of 8°C/min. The injector and detector temperatures were set at 250 and 280°C, respectively. Helium was the carrier gas and was set at a flow rate of 1.5 mL/min (Al-Shorgani *et al.* 2012).

Plackett-Burman Design (PBD)

PBD was used for screening the most significant fermentation parameters affecting biobutanol production by solvent-producing *Clostridium* isolated from system of rice intensification (SRI) soil. Each independent variable was investigated at two levels, high and low, which are indicated by +1 and -1, respectively. The details of the PBD experimental design are shown in Table 1. The variables with a *P* value less than 5% were considered to have a significant effect on biobutanol production. The PBD was created using Design-Expert version 6.0.8 software (State-Ease Inc., USA). The design involved 11 factors, namely: glucose, tryptone, yeast extract, peptone, ammonium acetate, KH_2PO_4, K_2HPO_4, $MgSO_4$, $FeSO_4$, Na_2CO_3, and NaCl. The aforementioned factors were coded as X1, X2, X3, X4, X5, X6, X7, X8, X9, X10, and X11, respectively.

Table 1. The Level of Variables Affecting Biobutanol Production by YM1IsolateUsed in the Plackett-Burman Design

Code	Factor	Low level (-1)	High level (+1)
X1	Glucose	20	50
X2	Tryptone	3	9
X3	Yeast extract	1	4
X4	Peptone	2	7
X5	Ammonium acetate	1	5
X6	KH_2PO_4	0.1	1
X7	K_2HPO_4	0.1	1
X8	$MgSO_4$	0.1	1
X9	$FeSO_4$	0.001	0.1
X10	Na_2CO_3	1	5
X11	NaCl	0.1	1

PBD with two-level design factors for testing n factors (n= number of runs) in k = n+1 (k= main effects) were used. The higher level value was coded as +1, and the lower level was coded -1, as shown in Table 1. Twelve runs of the PBD were done, as illustrated in Table 3.

Calculation of the effect of individual factors on biobutanol production was based on the first order equation as follows,

$$E = \beta_0 + \Sigma\beta_i X_i \quad (1)$$

where E is the effect of the factor under study (biobutanol production), β_0 and β_i are the constant coefficients, and X_i is the coded independent variables or parameters. The response was analyzed by an analysis of variance (ANOVA) to obtain the significance of the fitted model and the significance of the effect of the individual factors on the response (biobutanol production).

RESULTS AND DISCUSSION

Effect of Different Media on Butanol Production by Isolate YM1

In a preliminary study, experiments were done to produce butanol using different media (RCM, AnS, P2, and TYA). Out of these four media, growth was found to be faster and more extensive in RCM (data not shown), while TYA was the best medium for butanol production (Table 2). Therefore, a specific medium to optimize the biobutanol production by the new isolate of *Clostridium* (YM1 isolate) was designed.

Table 2. Biobutanol Production Using Different Media by Isolate YM1

Medium	Glucose (g/L)		Solvent production (g/L)				Acids production (g/L)		ABE Yield (g/g)
	Initial	Residual	Acetone	Butanol	Ethanol	ABE	Butyric	Acetic	
RCM	30	7.67	0.93	1.60	0.02	2.54	0.99	0.05	0.10
TYA	30	0.64	2.66	6.20	0.07	8.93	0.35	0.04	0.24
AnS	30	18.03	0.95	3.24	0.02	4.22	0.65	0.04	0.29
P2	30	0.94	1.56	5.69	0.06	7.31	0.65	0.05	0.25

Evaluation of Parameters Affecting Biobutanol Production

Screening is a very important step, especially when the researcher has many parameters and is unsure what levels are likely to produce optimal or nearly optimal responses. The selection of the levels of the parameters is a difficult part of the experimental design; experience and literature can help in choosing these factors (Strobel and Sullivan 1999).

The effects of the eleven medium nutrients, namely, glucose, tryptone, yeast extract, peptone, ammonium acetate, KH_2PO_4, K_2HPO_4, $MgSO_4$, $FeSO_4$, Na_2CO_3, and NaCl on biobutanol production in batch culture of newly isolated *Clostridium* (YM1) were tested by PBD. The effects of these nutrient components on the biobutanol production and significance levels are illustrated by Table 4.

Statistical analysis showed that the effects of glucose, yeast extract, peptone, tryptone, K_2HPO_4, $MgSO_4$, Na_2CO_3, and NaCl had significant effects on biobutanol production. However, ammonium acetate, KH_2PO_4, and $FeSO_4$ were insignificant factors and had no effect on the production of biobutanol (Table 4).

Table 3. Plackett-Burman Experimental Design for Evaluation of Parameters Affecting Biobutanol Production and the Response Values (Experimental and Predicted)

Run order	Parameters											Biobutanol (g/L)	
	X1	*X2*	*X3*	*X4*	*X5*	*X6*	*X7*	*X8*	*X9*	*X10*	*X11*	Observed	Predicted
1	-1	1	-1	-1	-1	1	1	1	-1	1	1	3.24	3.22
2	1	1	-1	-1	1	1	1	-1	1	-1	-1	7.75	7.77
3	-1	-1	1	-1	1	-1	1	1	1	-1	1	3.75	3.78
4	1	1	1	1	-1	-1	1	-1	-1	-1	1	4.66	4.64
5	1	-1	1	-1	1	1	-1	-1	-1	1	1	2.35	2.38
6	-1	1	1	1	1	1	-1	1	-1	-1	-1	3.38	3.40
7	1	-1	-1	1	1	-1	1	1	-1	1	-1	9.01	9.03
8	1	-1	-1	1	-1	1	-1	1	1	-1	1	8.68	8.66
9	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.39	4.36
10	-1	1	-1	1	1	-1	-1	-1	1	1	1	2.31	2.34
11	1	1	1	-1	-1	-1	-1	1	1	1	-1	4.12	4.09
12	-1	-1	1	1	-1	1	1	-1	1	1	1	2.51	2.48

Table 4. ANOVA Analysis for Selected Factorial Model

Source	Sum of squares	DF	Mean square	*F* -value	*t*-value	Prob> F	
Model	64.95	10	6.49	1004.96	202.1675	0.0245	significant
Glucose	19.95	1	19.95	3086.74	55.55844	0.0115	
Tryptone	3.34	1	3.34	517.06	-22.7389	0.0280	
Yeast extract	14.38	1	14.38	2225.19	-47.172	0.0135	
Peptone	3.06	1	3.06	473.16	21.75228	0.0292	
KH_2PO_4	0.06	1	0.06	9.90	3.146966	0.1959	
K_2HPO_4	3.84	1	3.84	593.40	24.35984	0.0261	
$MgSO_4$	3.92	1	3.92	606.15	24.62006	0.0258	
$FeSO_4$	0.88	1	0.88	136.29	11.6744	0.0544	
Na_2CO_3	4.94	1	4.94	764.55	-27.6505	0.0230	
NaCl	3.24	1	3.24	501.51	-22.3944	0.0284	
Residual	0.006463	1	0.006463				
Cor total	64.96	11					

R^2 = 0.999, R= 0.999, Std. Dev. = 0.08

The most significant nutrients affecting biobutanol production were glucose (P = 0.0115), yeast extract (P = 0.0135), Na_2CO_3 (P = 0.0230), $MgSO_4$ (P = 0.0258), K_2HPO_4 (P = 0.0261), tryptone (P = 0.0280), NaCl (P = 0.0284), and peptone (P = 0.0292). The model interaction had a low probability value (P_{model} > F = 0.0245) and F-value of 1004.96, which indicated that the model equation is reliable in its interpretation of the system interactions. The estimated correlation measures for the model regression equation are the multiple correlation coefficients R and R^2. The R^2 value was found to be 0.999, which indicated that the model could explain 99.9% of the variables content that contributed positively to the response, and only less than 0.1% of the total variations were not clarified by the model. Meanwhile, the R value was closer to 1 (0.9989), which represented good correlation between the experimental and predicted values.

The regression model is considered to have a very strong correlation when the R^2 value is greater than 0.9 (Chen *et al.* 2009). Hence, this model showed fit to the variation and the R^2 value represented a very good fit between the observed and predicted values of biobutanol production (Table 3). Meanwhile, the experimental results indicated the obtained values were very close to the predicted values.

The model equation for the individual parameters' interaction (as a first order equation) can be shown as follows:

$$\text{Butanol} = 3.66 + 0.09 \times \text{glucose} - 0.18 \times \text{tryptone} - 0.74 \times \text{yeast extract} + 0.21 \times \text{peptone} + 0.17 \times KH_2PO_4 + 1.28 \times K_2HPO_4 + 1.29 \times MgSO_4 + 5.58 \times FeSO_4 - 0.33 \times Na_2CO_3 - 1.36 \times \text{NaCl} \quad (2)$$

The effect of variables on biobutanol production was presented by a Pareto plot (Fig. 1), which is arranged from the maximal effect in the upper portion to the minimal effect in the lower portion. The Pareto plot shows that the three most important nutrients affecting biobutanol production were glucose, yeast extract, and Na_2CO_3. In Table 5, the effect estimates and coefficient estimates of the variable interactions are listed.

Table 5. Coefficient, Effect Estimate, and Confidence Level of Variables Affecting Biobutanol Production by YM1 Isolate

Factor	Coefficient estimate	Effect estimate	Confidence level (%)
Glucose	1.32	2.63	98.9
Tryptone	-0.53	-1.08	97.2
Yeast extract	-1.14	-2.23	98.7
Peptone	0.50	1.03	97.1
Ammonium acetate	a	-0.05	a
KH_2PO_4	0.06	0.15	80.4
K_2HPO_4	0.56	1.15	97.4
$MgSO_4$	0.60	1.16	97.4
$FeSO_4$	0.24	0.55	94.6
Na_2CO_3	-0.68	-1.31	97.7
NaCl	-0.58	-1.06	97.2

a: not included in the model

The estimate of the effect of variables on biobutanol production, as shown in Fig. 2, from the greatest to least positive effect, were glucose, $MgSO_4$, K_2HPO_4, peptone, $FeSO_4$, and KH_2PO_4. Similarly, the factors with the greatest to the least negative effect on the production of biobutanol were yeast extract, Na_2CO_3, tryptone, NaCl, and ammonium acetate. Increasing the concentrations of the factors that had a positive effect and decreasing the concentrations of the factors that had a negative effect should lead to an increase in the production of biobutanol.

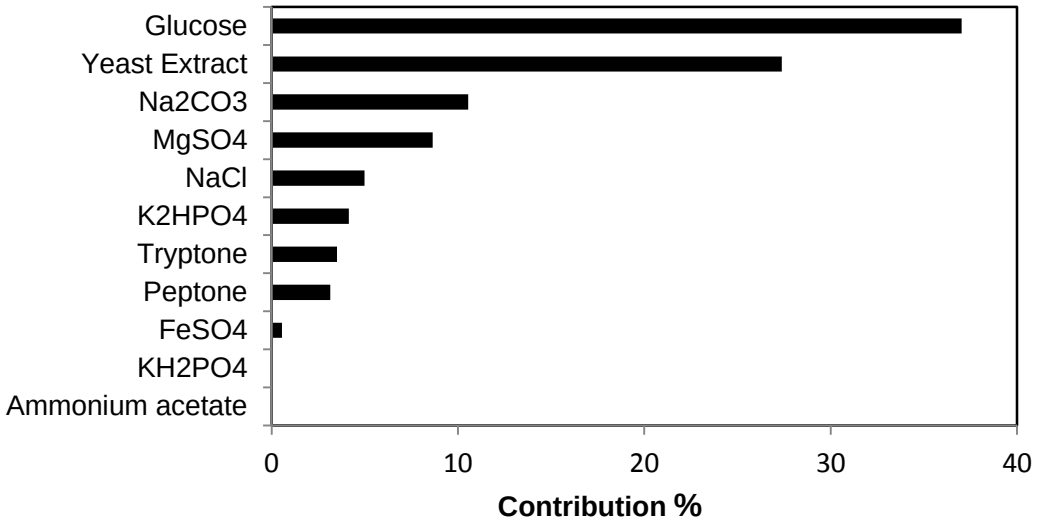

Fig. 1. Pareto plot of the Plackett-Burman design for parameter estimation of butanol production by YM1isolate

Supplementing the biobutanol fermentation medium with yeast extract is a common practice, as reported in the literature (Fontaine *et al.* 2002; Yan *et al.* 1988;Yu *et al.* 2011). The most significant factor in this study affecting butanol production was the concentration of glucose ($p = 0.0115$), which was used as a carbon source. The presence of an excess concentration of glucose (60 g/L) in the fermentation medium has been reported as a typical concentration that is essential for the maintenance of ABE production (Jones and Woods 1986). Glucose had the highest confidence level, at 98.9%, followed by yeast extract, K_2HPO_4, and Na_2CO_3, which had a positive and extensive influence on butanol production. This phenomenon can be attributed to the requirements of butanol fermentation and its metabolic nature (Table 5).

Yeast extract was used as a nitrogen source for cell culture and fermentation processes (besides peptone, tryptone, and ammonium acetate), which is enriched with proteins, amino acids, minerals, vitamins, and growth factors that promote the growth of microorganisms (Tran *et al.* 2011). It was found that yeast extract has a strong effect on the production of biobutanol and sugar utilization during biobutanol fermentation from spoilage date fruits; the addition of yeast extract significantly increased the production of biobutanol (Abd-Alla and Elsadek El-Enany 2012). Chua *et al.* (2012) investigated the effect of yeast extract on biobutanol production using *Clostridium* G117 and found that increasing the yeast extract addition from 0.4% to 1% enhanced the production of butanol from 8.52 to 8.61 g/L. This study also found that using 0.1% yeast extract reduced the production of biobutanol (Chua *et al.* 2012). Tryptone, peptone, or hydrolyzed casein

have also been used as nitrogen sources in fermentation medium in different quantities in addition to yeast extract (Fontaine *et al.* 2002; Yan *et al.* 1988).

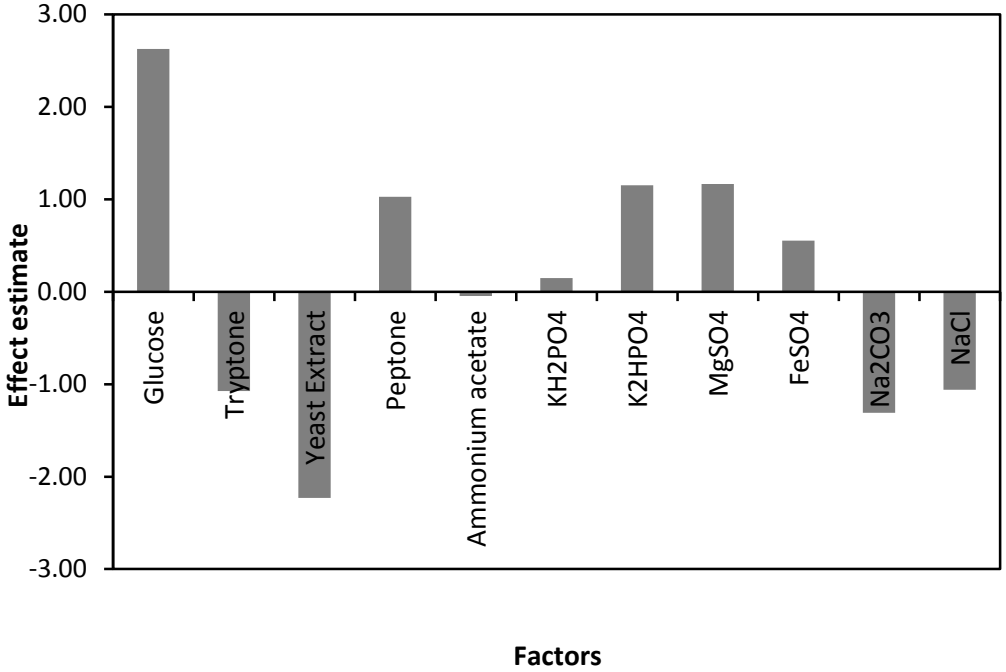

Fig. 2. Estimate of the effect of factors on biobutanol production by YM1 isolate

K_2HPO_4 is the source of phosphate in the medium and it has a buffering effect that maintains the pH during fermentation. In ABE fermentation, pH is decreased during the log phase (acidogenic phase) due to the production of acids (butyric and acetic acids) and then increased in the stationary phase (solventogenic phase) due to the reassimilation of acids to produce solvents. It is believed that pH is responsible for the initiation of the solventogenic enzymes (Nair *et al.* 1999). Also, it was reported that pH has a main effect on the production of biobutanol from sago starch (Salleh *et al.* 2008). Carbonate salt (Na_2CO_3) also has a buffering effect on pH. It was reported that carbonate salt has the ability to enhance the production of butanol and increase the *Clostridium*'s tolerance against the accumulation of solvent (Richmond *et al.* 2011).

Applying the optimized medium obtained from the PBD, which contained glucose (50 g/L), yeast extract (1.09 g/L), tryptone (3.01 g/L), ammonium acetate (4.06 g/L), K_2HPO_4 (0.99 g/L), $MgSO_4$ (0.86 g/L), peptone (6.62 g/L), Na_2CO_3 (1.86 g/L), NaCl (0.1g/L), $FeSO_4$ (0.001 g/L), and KH_2PO_4 (0.62 g/L), biobutanol production was 10.93 g/L with total ABE of 16.85 g/L, which was more than the predicted value by PBD. This indicated the strength of the model in this study as well as the potential value of this strain in biobutanol production.

It was reported that the wild-type of solvent-producing *Clostridium* strains were able to produce 9 to 12 g/L of butanol in a batch culture in the presence of 40 to 60 g/L of glucose in the medium (Chua *et al.* 2012; Formanek *et al.* 1997; Monot *et al.* 1982).

The results of this study suggested that higher glucose concentration, lower yeast extract concentration, lower tryptone concentration, and higher K_2HPO_4 concentration are able to increase the production of biobutanol using a new isolate of *Clostridium* in

batch fermentation. Moreover, the analysis exhibited that there is a probability of interaction among the significant variables that will affect the production of biobutanol. Hence, the interactions among these significant factors will be considered further in medium optimization using response surface methodology (RSM) in order to improve biobutanol fermentation by the new isolate of *Clostridium.*

CONCLUSIONS

1. The pre-optimization of medium composition for biobutanol production in batch culture by a novel isolate of a solvent-producing strain was successfully conducted through screening of significant nutrient factors using the PBD design.
2. Among the 11 factors tested, glucose, tryptone, yeast extract, peptone, K_2HPO_4, Na_2CO_3, and $MgSO_4$ were found to be significant parameters affecting biobutanol production.

ACKNOWLEDGMENTS

This study was funded by Universiti Kebangsaan Malaysia under grant UKM/DLP 2012-007.

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Article submitted: November 13, 2012; Peer review completed: January 11, 2013; Revised version received and accepted: January 27, 2013; Published: January 30, 2013.