Enhanced recovery of valuable metals from spent lithium-ion batteries through optimization of organic acids produced by *Aspergillus niger*

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Abstract

In the present study, spent medium bioleaching method was performed using organic acids produced by *Aspergillus niger* to dissolve Ni, Co, Mn, Li, Cu and Al from spent lithium-ion batteries (LIBs). Response surface methodology was used to investigate the effects and interactions between the effective factors of sucrose concentration, initial pH, and inoculum size to optimize organic acid production. Maximum citric acid, malic acid, and gluconic acid concentrations of 26478, 1832.53 and 8433.76 ppm, respectively, and a minimum oxalic acid concentration of 305.558 ppm were obtained under optimal conditions of 116.90 (g l⁻¹) sucrose concentration, 3.45% (v v⁻¹) inoculum size, and a pH value of 5.44. Biogenically-produced organic acids are used for leaching of spent LIBs at different pulp densities. The highest metal recovery of 100% Cu, 100% Li, 77% Mn, and 75% Al occurred at 2% (w v⁻¹) pulp density; 64% Co and 54% Ni recovery occurred at 1% (w v⁻¹) pulp density. The bioleaching of metals from spent LIBs can decrease the environmental impact of this waste. The results of this study suggest that the process can be used for large scale industrial purposes.

Keywords: Bioleaching; Valuable metals; Waste lithium-ion batteries; Organic acids; *Aspergillus niger*; Optimization

1. Introduction

Some solid wastes are rich in metal and at times are an even richer source of minerals than ore. Spent lithium-ion batteries (LIBs) are secondary sources of valuable metals (Xu et al., 2008). Methods are being developed to recycle to preserve resources and protect the environment (Chen et al., 2016), but no efficient recycling process for LIBs currently exists (Zeng et al., 2012). It is imperative to find an economical and eco-friendly method as well as optimizing the current strategies (Ferreira et al., 2009).

Bio-hydrometallurgical processes are powerful methods for recovering metals from waste. Bioleaching is a specialized bio-hydrometallurgical method (Anjum et al., 2012). It is based on the ability of microorganisms to transform insoluble solid compounds into soluble elements which can be recovered (Pant et al., 2012). The metabolites excreted by microbial activity help leach the metals from the waste (Mishra et al., 2008). Both bacteria and fungi have been reported for bioleaching of heavy metals from waste (Arshadi et al., 2016; Kim et al., 2016; Vakilchap et al., 2016). Fungi have advantages over bacterial leaching, including the ability to grow over a wide range of pH values, tolerate toxic materials and operate at a faster leaching rates (Santhiya and Ting, 2005).

Aspergillus niger is a haploid filamentous fungi found in mesophilic environments such as decaying vegetation and soil and can be used for waste management and bioleaching (Schuster et al., 2002). The bioleaching mechanism of *A. niger* is related to its secretion of low molecular weight metabolites, including organic acids (Deng et al., 2013). These organic acids act as lixivants for the leaching of heavy metals from solid waste (Wu and Ting, 2006) by way of two mechanisms which can occur simultaneously or separately. Firstly, hydrogen ions can replace metal cations from the waste matrix and induce metal solubilization. Secondly, organic acids can detach metals into soluble metal-ligand complexes by chelation (Ren et al., 2009).

Organic acids are much less toxic to many biological communities than inorganic acids, apparently because of the complexing capacity of organic acids which reduces toxic metal concentrations. Organic acids are much easier to dispose of (Burgstaller and Schinner, 1993) and are biologically degradable. Metal extraction by organic acids can also be carried out under mildly acidic conditions (pH 3-5) (Veeken and Hamelers, 1999). Chemical synthesis of organic acids is not suitable because of the expense of obtaining raw materials and the complicated processes with low yields. This makes microbiological production an important economic alternative to chemically-produced organic acids (Karaffa et al., 2001). *A. niger* exhibits good potential for production of an abundant concentration of organic acids (Burgstaller and Schinner, 1993) and has been proven useful for bioleaching of spent LIBs (Horeh et al., 2016).

The nature, type and the amount of organic acid produced by fungi is influenced by the physical, chemical and biological parameters of the system. These include the type and concentration of carbon source, the presence of trace elements, the pH of the culture medium, the temperature of the culture medium, aeration, the pre-culture period and inoculum used and the morphology of the producing microorganism (Max et al., 2010; Xu and Ting, 2004). The relationships between these parameters affect the amount of organic acid, so they must be optimized collectively (Arshadi and Mousavi, 2014). Carbon source, pH of the culture medium and inoculum size were considered in the present research.

The type and concentration of carbon source supports microbial growth and affects the production yield of organic acids. The presence of easily-metabolized carbohydrates that are taken up rapidly by microorganisms is essential for significant production of organic acids. In the case of *A. niger*, for instance, sucrose is the most favorable carbon source over sugars such as fructose, lactose and galactose (Soccol et al., 2006). *A. niger* has a potent

extracellular mycelium-bound invertase that is active at low pH values and hydrolyzes sucrose rapidly (Papagianni, 2007).

Although a larger inoculum size increases the density of the microbial population, production of organic acids does not directly depend on the total production of biomass. In fact, an excess of biomass inoculum may cause a decrease in the acid concentration; thus, inoculum size should be carefully selected and optimized (Xu and Ting, 2004).

Technologies based on microorganisms are attaining maturity and prominence in mineral processing; however, research on the recovery and recycling of spent LIBs is rare and more research is needed in this area. The present study investigated the effects and interactions of significant parameters on organic acid secreted by *A. niger* and on recovery of LIBs metals. This is the first report on an optimization strategy for bioleaching of metals from spent LIBs by *A. niger* using statistically designed experiments. Optimization was first carried out for sucrose concentration, inoculum size and initial pH using response surface methodology (RSM). Most metal oxalates precipitate and remain in the unleached battery powder residue, which decreases the recovery of metals such as Ni and Co. Optimization was, thus, carried out to maximize the concentration of citric acid, gluconic acid and malic acid and minimize the oxalic acid concentration to increase the recovery efficiency of the metals. Minimizing the sucrose concentration to lower costs is another goal of optimization. In the next step, bioleaching of spent LIBs for metal recovery was investigated at different pulp densities using spent medium bioleaching method.

2. Waste battery management and strategies

New technologies and portable electronic devices have increased the demand for rechargeable batteries (Jha et al., 2013). LIBs are now common power sources (Ferreira et al., 2009). Global LIB consumption was 4.49×10^9 units in 2011 (Niu et al., 2014) and 7 billion

1 in 2015 (Zhang et al., 2013). In 2020 in China, the weight and quantity of spent LIBs will
2 exceed 500 thousand tons and 25 billion units, respectively (Zeng et al., 2015). Spent LIBs
3 are a significant waste stream requiring adequate management. Reasonable management is
4 appropriate for at least two reasons. LIBs are sources of valuable metals; at least one-third of
5 the production cost for LIBs are for materials. LIBs contain toxic and flammable elements or
6 compounds that may become hazardous contaminants in landfill leachate, incinerator
7 emissions, incinerator ash and compost (Guo et al., 2016).

8 The most effective way to manage waste is through recycling, simultaneously saving
9 primary raw-materials, recovering valuable metals and minimizing the impact on the
10 environment (Nogueira and Margarido, 2015). Strict global regulations have been developed
11 on the production, use, collection, recycling and disposal of spent batteries. For example, the
12 US Environmental Protection Agency considers all spent batteries as hazardous waste (Zand
13 and Abduli, 2008). EU Battery Directive 2006/66/EC required that by 2011 at least 50% of
14 the average weight of spent batteries should be recycled into materials for their primary
15 application or for other applications besides energy recovery (Dewulf et al., 2010).

16 Before organizing of recycling processes, the separation and collection of spent batteries
17 must be well established (Zand and Abduli, 2008). The small amount of spent batteries
18 available to recycle does not match the huge amount of secondary batteries produced every
19 year. For example, less than 2% of waste batteries are recycled in China (Huang et al., 2010).
20 This may be due to the following reasons: (1) Long life time of secondary batteries and the
21 behavior of end users who keeps spent batteries for another period of time as “Hoarding
22 time” before disposing of them. (2) Systems for spent battery collection are inefficient
23 because of a lack of legislation and public awareness. Recent legislation has been
24 strengthened to ensure accessible collection points to make recycling free of charge for end
25 users. For instance, the EU required member states to achieve a minimum collection rate of

1 15% to 25% by 2015 with a minimum recycling efficiency of 45% to 50% (Al-Thyabat et al.,
2 2013).

3 Both physical and chemical processes are employed for recycling of LIBs. Physical
4 processes include mechanical and mechano-chemical separation, dissolution, thermal
5 treatment and chemical processes such as bioleaching, acid leaching, solvent extraction,
6 chemical precipitation and electrochemical processes (Ordoñez et al., 2016). Processes such
7 as thermal treatment and acid leaching have inherent drawbacks that include difficult
8 operating conditions, high energy consumption, stringent requirements for equipment, lack of
9 versatility, production of secondary pollution, risk and expense (Li et al., 2010; Nie et al.,
10 2015; Zeng et al., 2013). Technologies based on the use of microorganisms (bioleaching) are
11 low-cost (one-third to one-half the cost of conventional processes), leave a smaller
12 environmental footprint, are more efficient and offer lower energy consumption, few
13 industrial requirements and ease of management (Mishra et al., 2008; Xin et al., 2009; Zeng
14 et al., 2012).

16 3. Experimental

17 3.1. Spent Li-ion batteries

18 Spent Li-ion mobile phone batteries were the secondary source of heavy metals to be
19 recovered in this study. To avoid self-ignition and short-circuiting, the batteries were
20 discharged and separated manually into anodes, cathodes, plastic separators and metal cases.
21 All moisture was removed by drying the anodes and cathodes at 30°C for 24 h. The weight of
22 the sample before and after drying indicated that the sample contained about 11%
23 electrolytes. The cathodes and anodes were then ground using a ball mill (Fritsch; Germany)
24 into a fine homogeneous mixture and the powder was sieved to obtain a particle size of <75

1 μm (mesh #200). The sterile powder (autoclaved at 121°C for 15 min) was used in all
2 experiments.

3 4 3.2. *Microorganisms and culture media*

5 *A. niger* (PTCC 5210) was purchased from the Iranian Research Organization for Science
6 and Technology in Tehran, Iran. To prepare the inoculum, the fungus was cultured on potato
7 dextrose agar (PDA) plates and incubated for 7 days at 30°C. The mature conidia of *A. niger*
8 were washed from the surface of the PDA plates using sterile distilled water. A Neubauer
9 counting chamber (depth of 0.1 mm; area of 0.0025 mm²) was used to count the number of
10 spores under a phase contrast microscope (Standard 25; Zeiss; Germany) and the desired
11 number of spores (10⁷ spores ml⁻¹) was adjusted using sterilized distilled water. The spore
12 suspension was prepared as an inoculum and used for bioleaching.

13 14 3.3. *Experimental design of bioleaching experiment*

15 Bioleaching was performed through the spent medium approach. A specific volume of
16 spore suspension (defined by experimental design) containing approximately 10⁷ spores ml⁻¹
17 was added to 100 ml of sucrose medium in a 250 ml Erlenmeyer flask. The sucrose medium
18 contained NaNO₃ (1.5 g l⁻¹), KH₂PO₄ (0.5 g l⁻¹), KCl (0.025 g l⁻¹), MgSO₄.7H₂O (0.025 g l⁻¹),
19 yeast extract (1.6 g l⁻¹) and varying concentrations of sucrose for the purposes of
20 optimization. The sucrose concentration was determined by experimental design. The culture
21 medium was autoclaved at 121°C for 15 min. Prior to inoculation, the pH of the culture
22 medium was adjusted using sterile HCl and NaOH according to the experimental design. The
23 Erlenmeyer flasks were agitated in a shaker-incubator (WiseCube WIS-20; Daihan Scientific;
24 South Korea) at 130 rpm and 30 °C. After the desired pre-culture time (Horeh et al., 2016)
25 (day 14 of cultivation), the suspension of liquid medium and mycelia of each flask was

1 filtered and the organic acid concentrations (gluconic acid, oxalic acid, citric acid, and malic
2 acid) in the filtrate were determined. The pH and biomass concentration of each flask were
3 also analyzed.

4 Although oxalic acid has a high dissociation constant (see sec. 4.4), it has low dissolution
5 of most metals such as Co, Ni, and Cu. This can be attributed to the ability of oxalic acid to
6 form stable oxalate complex precipitates with dissolved metals (Saidan et al., 2012; Sun and
7 Qiu, 2012). In addition, citric acid plays a major role and gluconic acid and malic acid have
8 positive effects on bioleaching of spent LIBs (Horeh et al., 2016). Therefore, the goal of
9 experimental design was to maximize the concentration of citric acid, gluconic acid, and
10 malic acid and minimize that of oxalic acid. Minimizing the sucrose concentration to
11 decrease cost is another goal of optimization.

12 After determining the optimum conditions, a culture medium was incubated under those
13 conditions and filtered on day 14 of growth to obtain filtrate solution containing a maximum
14 concentration of citric acid, gluconic acid, and malic acid and a minimum concentration of
15 oxalic acid. The specified pulp density of the LIB powder (1% to 5% (w v⁻¹)) according to the
16 experimental design table was added to 100 ml of filtrate solution in 250 ml Erlenmeyer
17 flasks. The Erlenmeyer flasks were then shaken at 130 rpm and 30°C in a shaker-incubator.
18 After 8 days of incubation, the insoluble residue and leaching solution were separated by
19 filtration and the metal ion concentrations at different pulp densities were determined. Fig. 1
20 is a flowsheet of this process.

21 **Fig. 1**

22

23 *3.4. Biomass determination*

24 The dry weight of the fungal biomass was determined using the method of Aung and Ting
25 (Aung and Ting, 2005). Briefly, after filtering the suspension of each Erlenmeyer flask, the

resulting mycelium was put into a pre-weighed porcelain dish and dried in an oven (WiseVen; WON-50; Daihan Scientific; South Korea) at 80°C for 24 h. After drying and cooling in a desiccator, the porcelain dish was weighed again and the dry weight of the fungal biomass was calculated by the weight difference method.

3.5. Measurement and analysis

X-ray fluorescence (XRF) analysis was used to determine the spent LIB composition by XRF analyzer (PW2404; Philips; Netherland). X-ray diffraction (XRD) (X'Pert MPD; Philips; the Netherlands) was used to detect the component phases of the LIB powder with Co K α radiation, a tube voltage of 40 kV and 30 mA of current. The LIB powder was scanned from 5° to 90° using a step size of 0.04 and a scanning speed of 0.8 s step⁻¹.

An inductively-coupled plasma optical emission spectrometer (ICP-OES; Vista-pro; Varian; Australia) was used for metal ion analysis. The ICP multi-element standard (Merck) was used to prepare the calibration standards. The recovery of metals was calculated by following Eq. (1):

$$\text{Metal recovery} = \frac{C_S \times V_S}{C_F \times M_F} \times 100 \% \quad (1)$$

where C_S is the concentration of metal in leach liquor (mg l⁻¹), V_S is the volume of bioleaching solution (l), C_F is the amount of metal in the LIB powder (mg g⁻¹) and M_S is the mass of the LIB powder.

To quantify the organic acid concentration in the media (citric, oxalic, malic, and gluconic acids), high-performance liquid chromatography (HPLC) (Sykam; Germany) was used with a UV-VIS diode array detector at 210 nm. The HPLC column was Nucleodur C18ec (5 μ m, 250 mm $\times$ 4.6 mm; Macherey-Nagel; Germany). The operating temperature was 30°C and operating pressure was 5.7 MPa. The mobile phase was sulfuric acid (5 mM) with a flow rate of 0.5 ml min⁻¹ (Aung and Ting, 2005). Prior to injection (injection size of 20 μ l), the samples

were filtered through a 0.22-micron syringe filter to avoid blocking the chromatography column. Standards and samples were injected in triplicate.

Field emission scanning electron microscopy (FE-SEM) (S-4160; Hitachi; Japan) was used to observe the surface morphology of the LIB powder. The pH of the medium was measured by a digital pH meter (p25; Istek; South Korea). The loss of liquid through evaporation was estimated by the weight difference method and was compensated for by adding sterilized distilled water.

3.6. Design of experiments

RSM was used to plan and analyze experiments and optimize the parameter levels to attain the best system performance. It can reveal interactions among parameters, the importance of individual parameters and the sensitivity of the response to each parameter by fitting a polynomial equation to the data of experiments, that describes data set behavior to make statistical previsions (Bezerra et al., 2008; Xu and Ting, 2004).

Central composite design (CCD) is the most common RSM for estimating a second-degree polynomial (quadratic) model for the response variable in an optimal number of experiments (Amiri et al., 2011). CCD requires an optimal number of experiments according to $N = 2^k + 2k + c_p$, where k is the number of independent process variables, c_p is the number of center points (0, 0, 0, ..., 0) used to determine random error (standard deviation), 2^k is the factorial points (usually coded as ± 1 notation) and $2k$ is the axial points. The axial points are located at $(\pm\alpha, 0, 0, \dots, 0)$, $(0, 0, \pm\alpha, \dots, 0)$ and $(0, 0, 0, \dots, \pm\alpha)$, where α is the distance of the axial point from the center. All factors are studied at five levels $(-\alpha, -1, 0, +1, +\alpha)$ (Amiri et al., 2012).

Three independent factors (sucrose concentration, inoculum size, and initial pH), five levels and four center points were used in the present study and the alpha value was adjusted

to 2. A total of 18 runs (8 full factorial tests + 6 axial points + 4 center points) were performed to obtain the desired concentration of organic acids. The responses (organic acid concentration) were measured in three replicates and the mean values were used. In numerical optimization, a maximum and a minimum level of each parameter must be provided. Starting from an appropriate point in the design space increases the chance of finding the best local maximum or minimum (Motaghed et al., 2014). The critical range of factors was determined based on the literature review and preliminary experiments (Aghaie et al., 2009; Xu and Ting, 2004). The levels of the three test factors are given in Table 1.

Table 1

During optimization, the empirical linear or polynomial model can simply relate the responses to the independent factors. Eq. (2) gives the quadratic model, which also contains the linear model (Bezerra et al., 2008):

$$Y = \beta_0 + \sum_{i=1}^K \beta_i X_i + \sum_{i=1}^K \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

where Y is the predicted value of the response variable, k is the number of factors, ε is a random error, X_i and X_j are the coded input factors, β_0 is the constant term, β_i is the linear parameter coefficients, β_{ii} is the quadratic parameter coefficients and β_{ij} is the interaction parameter coefficient.

3.7. Software for experimental design

Design Expert 7.0.0 (State-Ease; USA) was used for statistical analysis of the data. A confidence threshold of 0.05 (confidence level of 95%) was used for all statistical models.

3.8. Validation experiments

Additional experiments were carried out under optimal conditions obtained by statistical experimentation to confirm agreement of the experimental results and the results from models for organic acid concentration.

4. Results and Discussion

Fungi produce organic acids through the Krebs cycle. These include malic, oxalic, gluconic, citric, lactic, fumaric, succinic and pyruvic acids. In the present study, only the first four organic acids were considered due to their ability to dissociate in solution and their higher solubility in water, which contributes to their acid strength (Saidan et al., 2012). Sucrose concentration, initial pH, and inoculum size were measured to optimize the concentration of citric acid, gluconic acid, malic acid, and oxalic acid by RSM to increase the recovery efficiency of LIB metals. Higher concentrations of organic acid have more effect on metal immobilization, indicating the importance of surface complexation reactions as a mechanism of metal release and complexation of metals into a solution (Kim et al., 2013).

The elemental composition of the spent LIBs was analyzed by XRF and showed that the spent LIBs contained Mn (22.0%), Co (17.1%), Al (9.4%), Cu (6.6%) and Ni (2.8%). Chemical digestion (Horeh et al., 2016) confirmed the results of XRF and also determined the amount of Li (2.2%). The crystalline phases of LiCoO_2 and $\text{LiNi}_{0.5}\text{Mn}_{1.1}\text{Ti}_{0.4}\text{O}_4$ of the LIB powder were clearly detected by XRD.

The parameter values for the 18 experiments are presented in Table 2. Data analysis for each response variable was carried out using the results listed in Table 2 and are explained in the following sections.

Table 2

4.1. Statistical analysis

Analysis of variance (ANOVA) was performed to evaluate the fit of the empirical statistical model (Aghaie et al., 2009). The terms in the equation models were calculated after excluding insignificant parameters and their interactions having the lowest F-values. A reduced third-order model was used to predict the concentration of citric acid and malic acid and a reduced quadratic model was used to determine the gluconic acid and oxalic acid concentrations. The final equation models in terms of coded factors are shown in Eqs. (3) to (6) as:

For gluconic acid concentration:

$$Y_{GA} = 5057.69 + 3856.00A - 315.88B + 1140.87C - 1266.00AB + 489.50AC + 1050.25BC + 959.97A^2 \quad (3)$$

For citric acid concentration:

$$Y_{CA} = 24311.17 + 5966.50A - 1521.50B - 590.75C + 3529.00AB + 538.50AC - 2031.25BC - 3132.83A^2 + 595.17B^2 - 3513.58C^2 + 404.25ABC + 2476.50A^2B - 2282.75AB^2 \quad (4)$$

For malic acid concentration:

$$Y_{MA} = 1636.03 + 376.00A - 45.25B - 135.50C + 106.38AB + 18.13BC - 130.12A^2 - 19.99B^2 + 63.13C^2 - 23.63ABC + 49.88A^2B + 88.13A^2C + 91.87AB^2 \quad (5)$$

For oxalic acid concentration:

$$Y_{OA} = 287.90 - 147.94A - 172.31B - 142.69C + 69.88AB + 156.63AC + 306.97A^2 + 117.97B^2 + 133.47C^2 \quad (6)$$

where Y is the predicted amount of organic acid as the response. The constant values of 5057.69, 24311.17, 1636.03 and 287.90 are the offset terms and A, B and C represent the sucrose concentration (g l^{-1}), inoculum size ($\% \text{v v}^{-1}$) and initial pH, respectively.

The results indicate that sucrose concentration (A), with the highest coefficient, had the most important effect on the production of all four acids. The sucrose concentration for citric,

malic and gluconic acid production had a positive effect and oxalic acid had a negative effect. A negative sign for the coefficients in the equation model indicated that the level of acid concentration increased as the levels of factors decreased. This means that, as the sucrose concentration increased, the production of citric, malic and gluconic acid increased and that a decrease in sucrose concentration increased oxalic acid production.

The results of ANOVA are presented in Tables 3 and 4. Because the significance threshold used for statistical models was 0.05 and values of "Prob > F" are less than 0.05 (< 0.0001 for Y_{GA} , Y_{CA} and Y_{MA} and 0.0038 for Y_{OA}), the models were significant at a 95% confidence level. The lack of fit compares the residual error with the pure error from replicated design points. P-values for lack of fit were not significant at $p > 0.05$, confirming the fitness of the model (Shahrabi-Farahani et al., 2014). The F-value compares factors or model variance with residual (error) variance. An F-value approaching unity indicates that none of the factors have a significant effect on the response. Higher F-values indicate increased importance of the factors (Arshadi and Mousavi, 2014). The high model F-values (17.53 for Y_{GA} , 92.87 for Y_{CA} , 132.78 for Y_{MA} and 7.26 for Y_{OA}) suggest that the models were significant and there was only a 0.01% chance for Y_{GA} , Y_{CA} and Y_{MA} and 0.38% for Y_{OA} that the "Model F-value" could occur because of noise.

The fit of polynomial equations Eqs. (3)- (6) were defined by the determination coefficient (R^2). In this case, R^2 equaled 0.9246 for Y_{GA} , 0.9955 for Y_{CA} , 0.9969 for Y_{MA} , and 0.8658 for Y_{OA} , which further indicates that only 7.54%, 0.45%, 0.31%, and 13.42% of the total variables were not explained by Y_{GA} , Y_{CA} , Y_{MA} , and Y_{OA} , respectively. The models were found to adequately represent the real relationships between factors (Amiri et al., 2011). A value for R^2 that approaches unity indicates that the model is stronger and can predict responses better.

The addition of variables to the model always increases R^2 whether or not the additional variables are significant. It is possible that models having a large R^2 will yield poor predictions of new observations. In the contrary, adjusted R^2 often decreases when unnecessary terms are added. Adjusted R^2 is preferable to R^2 (Amiri et al., 2012). Table 4 lists the values of the adjusted R^2 as 0.8719, 0.9848, 0.9894, and 0.7465 for Y_{GA} , Y_{CA} , Y_{MA} , and Y_{OA} , respectively, showing the high significance of the models. The adequate precision is a measure of the signal to noise ratio. Table 4 shows that the adequate precision of the models was greater than 4, which indicates an adequate signal (Amiri et al., 2011).

The predicted residual error sum of squares (PRESS) also gives a measure of how the model fits each point in the design (Aghaie et al., 2009). All results showed that using the CCD method, the model predictions for organic acid production can be used to navigate the space defined.

Table 3

Table 4

Fig. 2 shows the predicted data versus actual data. The actual data is the measured response data from experimental runs and the predicted data was derived using functions generated for the statistical models. The location of points around the diagonal 45° line show the correlation of the experimental and predicted data and indicate that the models are appropriate for response prediction (Arshadi and Mousavi, 2014).

Fig. 2

4.2. Response plots

4.2.1. Citric acid production

Citric acid is a tricarboxylic acid with a molecular weight of 210.14 g mol⁻¹. Its three carboxylic functional groups have pK_a values at pH 3.1, 4.7, and 6.4 (Max et al., 2010). In terms of tonnage, citric acid is the most important organic acid produced by fermentation and has a large number of applications. In chemical synthesis, citric acid is a reactive

intermediate. Its hydroxyl and carboxyl groups also allow formation of different complex molecules and reactive products of commercial interest (Soccol et al., 2006).

Biosynthesis of citric acid by *A. niger* occurs in the following way: At first, the sugar substrate is hydrolyzed to fructose and glucose and then absorbed. Early in fermentation, glucose oxidase forms and converts a significant amount of glucose into gluconic acid. Decreasing the pH to below 3.5 inactivates this enzyme. Then glycolytic catabolism of glucose produces two moles of pyruvate. Next, two moles of pyruvate are converted to oxaloacetate and acetyl-CoA as precursors of citrate. Citric acid synthesis occurs in the Krebs cycle and subsequently secretion of citric acid from mycelia and mitochondria occurs (Papagianni, 2007). The metabolic pathway for citric, malic, gluconic and oxalic acid synthesis in *A. niger* is shown in Fig. 3 (Kobayashi et al., 2014; Papagianni, 2007; Ramachandran et al., 2006).

Fig. 3

A previous study by the authors showed that citric acid is the most important organic acid from the malic, gluconic and oxalic acids for leaching metals from LIBs (Horeh et al., 2016). The related reactions between metal ions and citric acid are represented in Eqs. (7)-(12) in which M^{n+} denotes metal ions with a specific valence (Qu et al., 2013).

ANOVA showed that all three main variables (sucrose concentration, initial pH and inoculum size) and all interactions except that for AC (sucrose concentration versus initial pH) and ABC were significant. The high F-value of the sucrose concentration indicated that it was the most important parameter for the response. The interactions of A^2C , B^2C and C^3 were excluded to improve model adequacy.

Fig. 4(a) shows the sucrose concentration versus inoculum size at a constant initial pH of 4. It also shows that, at high concentrations of sucrose and high inoculum size, more citric acid was produced. This could result from the high sucrose concentrations, which repressed α -keto-glutarate dehydrogenase, allowing accumulation of citric acid in the culture medium. The effect of the sugar concentration can be explained in terms of enzyme repression. Additionally, the uptake of ammonia by germinating spores caused proton release, which decreased the pH level and increased citric acid production (Papagianni, 2007). At lower pH values, more citric acid was secreted (Ruijter et al., 1999).

Increasing the sucrose concentration and inoculum size are desirable up to optimum values. Past this, the high concentration of sucrose causes substrate inhibition and leads to the great amount of residual sugar, making the process uneconomical. It has been reported that increasing the sugar concentration to 20% (w/v) will produce a steady rise in citric acid production, but a further increase to above 20% (w/v) will decrease citric acid accumulation as a result of reduced water activity and plasmolysis. This prolongs the lag phase of the fungus, reducing fungal numbers and thereby the amount of citric acid secreted outside the hyphal walls (Addo et. al., 2016). Too low a concentration of sugar decreases citric acid production due to the accumulation of oxalic acid in the culture medium (Aghaie et al., 2009). A much higher inoculum size decreases the dissolved oxygen associated with slower ammonium ion uptake and significantly lowers glucosamine concentrations in the culture medium (Papagianni, 2007). The formation and secretion of glucosamine in high amounts

causes short inhibition of citrate synthesis, increases growth, glucosamine utilization, and citric acid production rates (Papagianni et al., 2005).

Fig. 4(b) shows the sucrose concentration versus initial pH at a constant inoculum size of 3.4% (v v⁻¹). The pH of the culture medium affects citric acid fermentation at two different times and can increase production yield. Firstly, a suitable pH is required when germinating spores. Spores are the first step in all fermentation, even if the culture is scaled up by seed fermenters before transfer to production fermenters. Secondly, lower pH is needed for citric acid production to reduce the risk of contamination with other microorganisms during fermentation and to inhibit the production of unwanted organic acids such as oxalic acid (Max et al., 2010). Fig. 4(b) indicates that the best initial pH for citric acid production is 4 to 6 (pH = 5 is optimal). This range is appropriate for spore germination; an initial pH outside this range will lower the citric acid concentration in the culture medium.

Table 2 shows that the highest citric acid concentration was 29969 ppm and was obtained at a sucrose concentration of 100 (g l⁻¹), spore concentration of 1% (v v⁻¹) and initial pH of 5 (Run 11). The lowest citric acid concentration was 81 ppm and was obtained at a sucrose concentration of 50 (g l⁻¹), spore concentration of 3% (v v⁻¹) and an initial pH of 5 (Run 9). These results correlate well with the pH values. Run 11 had a maximum citric acid concentration at minimum pH and run 9 had a minimum citric acid concentration at maximum pH. Table 2 also shows that a high biomass concentration was generated in run 6 (19.6 g l⁻¹), which did not record the maximum citric acid concentration; this was recorded in run 11 with a biomass of 16.0 (g l⁻¹). It is evident that citric acid production does not depend directly on total biomass production. In fact, excessive biomass may decrease the acid concentration.

The significance of these parameters for citric acid fermentation has been emphasized in most studies. The carbon source concentration is also critical to citric acid production.

Honecker et al. (Honecker et al., 1989) reported that increasing in initial sugar concentration increased the citric acid yield and the highest yields were usually achieved at 14% to 22% (w v⁻¹) sugar. Xu et al. (Xu et al., 1989) reported that when the sugar concentration increased from 1% to 14%, the duration of the lag phase of growth increased from 12 to 18 h and the growth rate decreased by around 20%. Also, in media that contained less than 2.5% sugar, no citric acid was produced.

Fig. 4

4.2.2. Malic acid production

Malic acid belongs to the C4-dicarboxylic acid family and is freely soluble in water. Fig. 3 shows that, in the Krebs cycle, malate forms with the addition of an OH group to fumarate. It can also form through anaplerotic reactions from pyruvate. The ionization constants of malic acid are $K_1 = 4 \times 10^{-4}$ and $K_2 = 9 \times 10^{-6}$ (Li et al., 2010). Wang et al. (Wang et al., 2005) used malic acid to dissolve kaolinite in soil. Malic acid degrades easily under anaerobic and aerobic conditions and the remaining malic acid can be recycled and reused for subsequent leaching. Li et al. (Li et al., 2010) used DL-malic acid (D and L are enantiomers of malic acid) as leachants to recover lithium and cobalt from the cathode active material of LIBs. In a previous study by the current authors, malic acid was found to have a positive effect on leaching of metal from LIBs (Horeh et al., 2016). The reactions of malic acid dissociation are shown in Eqs. (13) and (14) (Saidan et al., 2012) as:

ANOVA results show that the models including the three main variables (sucrose concentration, initial pH, and inoculum size) and all interactions except that for BC

(inoculum size versus initial pH), B^2 and A^2C were significant ($p < 0.05$). The interaction of AC was excluded to improve model adequacy. The effect of sucrose concentration ($p < 0.0001$) was determined to be more significant than the effects of the other parameters.

Maximizing malic acid production is less important than citric acid and gluconic acid because less is excreted. Fig. 5(a) shows the sucrose concentration versus inoculum size at a constant initial pH of 5.44. As seen, the highest malic acid production corresponds to the highest concentration of sucrose (125-150 g l⁻¹) and largest inoculum size (3% to 5% (v v⁻¹)).

Table 2 shows that the highest malic acid concentration was 2196 ppm and was obtained at a sucrose concentration of 125 (g l⁻¹), spore concentration of 4% (v v⁻¹) and initial pH of 4 (Run 4). The lowest malic acid concentration was 346 ppm and was obtained at a sucrose content of 50 (g l⁻¹), spore concentration of 3% (v v⁻¹) and initial pH of 5 (Run 9).

4.2.3. Oxalic acid production

Oxalic acid is a common organic acid that dissolves easily in warm water. Fig. 3 shows that pyruvate from glycolysis is transformed to oxaloacetate and the oxaloacetate is then hydrolyzed to oxalate and acetic acid by oxaloacetase. In the Krebs cycle, the formation of oxaloacetate occurs and is hydrolyzed to oxalate (Kobayashi et al., 2014). Oxalic acid used in hydrometallurgy as a leaching agent for metals from minerals such as bauxite, clay, and kaolin. It is also widely used as a precipitant of metals (Sun and Qiu, 2012).

In a previous study by the authors, it was found that oxalic acid decreases the yield of metal recovery, especially for Ni and Co, from LIBs; thus, minimizing oxalic acid concentration was a goal of the present study (Horeh et al., 2016). The reactions between metal ions and oxalic acid are presented in Eqs. (15)-(18) in which M^{n+} denotes the metal ions of a specific valence (Qu et al., 2013).

ANOVA of the optimization study indicated that the main independent variables of A and B, interaction variable AC and cubic variables A^2 , B^2 , C^2 , and A^2C are clearly significant ($p < 0.05$). The interaction between initial pH and inoculum size (BC) was removed to improve model adequacy. The high F-value for sucrose concentration indicates that it is the most important variable affecting the response.

Fig. 5(b) shows the sucrose concentration versus inoculum size at a constant initial pH of 5.44 for oxalic acid production. The highest oxalic acid production corresponded to the lowest sucrose concentration and inoculum size. Because of the precipitation of most metal oxalates, a minimum concentration of oxalic acid favors leaching of metals from LIBs. Fig. 5(b) suggests a sucrose concentration of 90-120 (g l^{-1}) and inoculum size of 3% to 5% (v v^{-1}) to decrease production of oxalic acid. At higher or lower values, the amount of oxalic acid produced increased. Decreasing the carbon source concentration decreases citric acid production through the accumulation of oxalic acid in the culture medium (Aghaie et al., 2009).

Table 2 indicates that the highest oxalic acid concentration was 1913 ppm and was obtained at a sucrose content of 50 (g l^{-1}), inoculum size of 3% (v v^{-1}) and initial pH of 5 (Run 9). The lowest oxalic acid concentration was 152 ppm and attained at a sucrose content of 100 (g l^{-1}), inoculum size of 3% (v v^{-1}) and initial pH of 5 (Run 17).

Fig. 5

4.2.4. Gluconic acid production

Gluconic acid is a noncorrosive, nonvolatile, non-toxic, mild organic acid (Ramachandran et al., 2006). In hydrometallurgy, it used to dissolve metals from mineral deposits, especially in alkaline solution (Zhou et al., 2011). In the starting phase of fermentation, glucose oxidase is formed by *A. niger* and converts a significant amount of glucose into gluconic acid. The excretion of this enzyme is induced by the presence of high concentrations of glucose and is directly influenced by the pH of the medium. In fact, gluconic acid production occurs at pH values higher than the amounts suitable for citric acid production and lower than the appropriate values for oxalic acid excretion. A decrease in pH to less than 3.5 inhibits the activity of glucose oxidase. As long as the pH of the medium is maintained in the appropriate range, gluconic acid will be excreted sufficiently and will gradually cease (Papagianni, 2007).

In a previous study by the authors (Horeh et al., 2016), gluconic acid was found to be effective for dissolving LIBs metals. The reactions between metal ions and gluconic acid are shown in Eqs. (19) and (20) in which M^{n+} denotes the metal ions of a specific valence (Qu et al., 2013):

ANOVA indicated that the important model independent variables A and C, interaction variable AB and cubic variable A^2 were significant ($p < 0.05$). The cubic variables B^2 and C^2 were excluded to improve model adequacy.

Fig. 6 shows the sucrose concentration versus inoculum size at initial pH values of 3, 5, and 7. Fig. 6(a) for a pH of 3 shows an improvement in fungal growth and consequently gluconic acid production with an increase in sucrose concentration and a decrease in

inoculum size. Increasing the pH from 3 to 5 (Fig. 6(b)) had a positive effect on the production of gluconic acid. Fig. 6(c) indicates that at a high pH (about 7) and high sucrose concentration (above 130 (g l⁻¹)), gluconic acid production does not depend on inoculum size. All inoculum sizes (1% to 5% v v⁻¹) produced gluconic acid at its maximum value.

Table 2 shows that the highest gluconic acid concentration was 15849 ppm and was obtained at a sucrose concentration of 150 (g l⁻¹), inoculum size of 3% (v v⁻¹) and initial pH of 5 (Run 10). The lowest gluconic acid concentration was 73 ppm and was obtained at a sucrose content of 50 (g l⁻¹), inoculum size of 3% (v v⁻¹) and initial pH of 5 (Run 9).

Fig. 6

4.3. Optimum condition and validation test

In the numerical optimization, the goals for each parameter and response are chosen from the menu. The possible goals are maximize, minimize, target, within range, equal to (for factors only) and none (for responses only). Maximize was set for citric acid, malic acid, and gluconic acid and a minimize for oxalic acid and sucrose concentration. The model predicted maximum production of 26478, 1832.53, and 8433.76 ppm for citric acid, malic acid and gluconic acid, respectively, and minimum production of 305.558 ppm for oxalic acid under optimal conditions of 116.90 (g l⁻¹) sucrose concentration, 3.45 (% v v⁻¹) inoculum size and a pH of 5.44. Three experiments were performed under optimal conditions to verify the results of the model prediction. The results are shown in Table 5 and show that the prediction values were in good agreement at a 95% confidence interval. The experimental values were quite close to model prediction values and confirmed the validity of the model.

Table 5

4.4. Leaching tests

Heterotrophic microorganisms produce metabolic products such as amino acids, exopolysaccharides and proteins with the ability to dissolve the metallic fractions from waste. Organic acids aid metal dissolution through their supply of protons and ligands (Saidan et al., 2012).

The effect of organic acids can be understood from their K_a values (acid dissociation constants), which are indicators of the degree of ionization. Organic acid is either monoprotic (gluconic acid: $K_a=1.38\times10^{-4}$), diprotic (malic acid: $K_a=3.98\times10^{-4}$ and 7.76×10^{-6} ; oxalic acid: $K_a=5.9\times10^{-2}$ and 6.4×10^{-5}) or triprotic (citric acid: $K_a=8.4\times10^{-4}$, 1.8×10^{-5} and 4.0×10^{-6}). A low K_a value indicates greater acid strength and a smaller conjugated base has been produced by the acid. These values are related to the solution ionic strength required for interaction with metal cations (Marafi et al., 2012). An organic acid structure with more carboxyl groups is beneficial for metal leaching to form stable ligands. Citric acid, with 3 carboxyl groups, is more suitable for leaching metals than oxalic acid and malic acid with 2 carboxyl groups. Gluconic acid with only one carboxyl group has the least ability to leach metals (Yan et al., 2014).

Selective leaching is based on the chemical reaction between acid/complexing agents and metals, which form soluble metal complexes as in Eqs. (21) and (22):

$$k = \frac{[MX][H_2O]}{[MO][H_2X]} \quad (22)$$

where MO is metal oxide and k is the stability constant (a higher k means higher selectivity). Complex ion stability is denoted by its formation constant, k . A complex ion contains a

central metal ion to which are bonded two, four or six ionic or neutral species (ligands) (Marafi et al., 2012).

The organic acids excreted under the optimal conditions were used to examine metal recovery at different pulp densities of LIB powder using spent medium bioleaching. Fungi metabolite solution (100 ml) was added to different pulp densities of spent battery powder (1% to 5% w v⁻¹). Bioleaching occurred in a shaker incubator at 130 rpm and 30°C. Fig. 7 shows metal recovery at the different pulp densities. A pulp density of 2% (w v⁻¹) gave the maximum extraction yield of 100% Cu, 100% Li, 77% Mn and 75% Al, but maximum recovery of 64% Co and 54% Ni were obtained at 1% (w v⁻¹) pulp density. A high pulp density produced higher yields of the desired metals within a short span of time. Fig. 7 indicates that, generally, metal recovery decreased as the pulp density increased because, at a low pulp density, the number of protons are sufficient to react completely with the metal compounds.

The main crystalline phase of LIB powder was LiCoO₂. Eqs. (23) and (24) represent the leaching reactions of LiCoO₂ with malic acid as an example (Li et al., 2010) as:

Leaching between malic acid and LiCoO₂ is a multiphase reaction. It occurs at the surface of the solid and liquid and the reaction rate is influenced by the malic acid concentration and other properties of the malic acid solution and the LiCoO₂ particles. The mechanism of a multiphase reaction is determined by ion transfer and chemical reactions in the solution. The

leaching reaction is governed by the chemical reaction rate or ion transfer rate, whichever is slowest (Li et al., 2010).

Oxalic acid had a negative effect on total metal dissolution of LIBs because of the precipitation of metal oxalates. Eqs. (25) and (26) show that the reaction of LiCoO_2 with oxalic acid forms solid CoC_2O_4 , which decreases cobalt leaching efficiency (Sun and Qiu, 2012).

Fig. 7

4.5. Surface morphology analysis

Significant differences can be seen in the surface morphology of LIB powder before and after leaching (Fig. 8). Before bioleaching, the powder has a smooth surface; after bioleaching, the powder surface is rather rough and contains holes. Analysis shows the progress of spent medium bioleaching and that the attack by the biometabolites, especially organic acids, resulted in metal dissolution.

Fig. 8

5. Conclusion

Spent LIBs are important raw materials and green recycling of them produces environmental benefits. The present work successfully optimized conditions for higher recovery of LIB metals and decreased the cost of the process. The variables of sucrose concentration, initial pH, and inoculum size were selected for optimization of citric acid,

gluconic acid, malic acid and oxalic acid to increase recovery of Co, Ni, Mn, Li, Cu, and Al using organic acids biogenically produced by *A. niger*.

The optimal values obtained by CCD experiments for sucrose concentration, initial pH, and inoculum size were, respectively, 116.90 (g l⁻¹), 5.44, and 3.45% (v v⁻¹). Under optimum conditions, maximum production of 26478, 1832.53, and 8433.76 ppm was predicted for citric acid, malic acid and gluconic acid, respectively, and minimum production of 305.558 ppm for oxalic acid. The spent medium bioleaching method using biogenically-produced organic acids at different pulp densities under the optimum conditions gave the highest metal recovery of 100% Cu, 100% Li, 77% Mn, and 75% Al at 2% (w v⁻¹) pulp density of LIB powder and 64% Co and 54% Ni at 1% (w v⁻¹) pulp density of LIB powder.

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Table and Figure Captions

Table 1: Levels and codes of factors for optimization

Table 2: CCD matrix of three test factors, four observed responses, final pH of the cultivated media and Biomass concentration

Table 3: Summary of the ANOVA for the response surface models

Table 4: ANOVA for fitted statistical models from 18 experimental runs

Table 5: Verification results for the optimum condition

Fig. 1: Illustrations of the bio-recovery process flowsheet.

Fig. 2: Predicted vs. actual data for (a) gluconic acid (b) citric acid (c) malic acid (d) oxalic acid production.

Fig. 3: Metabolic pathway for production of oxalic acid, malic acid, gluconic acid and citric acid in *A. niger*.

Fig. 4: Two-dimensional contour plots of interaction between (a) inoculum size and sucrose concentration at constant initial pH of 4.00, (b) sucrose concentration and initial pH at a constant inoculum size of 3.40 for citric acid production.

Fig. 5: Contour plot of sucrose concentration versus inoculum size that initial pH was kept at an optimal value for (a) malic acid (b) oxalic acid production.

Fig. 6: Two-dimensional contour plots of the relationship between sucrose concentration and inoculum size at constant initial pH of (a) 3, (b) 5 and (c) 7 for gluconic acid production.

Fig. 7: Metal recovery yields at various pulp densities.

Fig. 8: FE-SEM images of powder (a) before and (b) after bioleaching.

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Table 1

Factors	Units	Levels				
		-2	-1	0	+1	+2
		(Low Axial)	(Low Factorial)	(Center Point)	(High Factorial)	(High Axial)
A: Sucrose concentration	(g L ⁻¹)	50	75	100	125	150
B: inoculum size	%v v ⁻¹	1	2	3	4	5
C: Initial pH	-	3	4	5	6	7

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Table 2

Run no.	Symbol code: Factors (unit)			Responses (unit)				Final pH of the media	Biomass concentration (g L ⁻¹)
	A: Sucrose concentration (g L ⁻¹)	B: inoculum size (%v v ⁻¹)	C: Initial pH	Oxalic acid (ppm)	Gluconic acid (ppm)	Malic acid (ppm)	Citric acid (ppm)		
1	75	2	4	1627	2047	1292	15731	2.32	11.3
2	125	2	4	1065	13237	1963	15772	2.57	13.4
3	75	4	4	1202	3384	1005	15454	2.66	14.3
4	125	4	4	831	5308	2196	27994	2.40	17.4
5	75	2	6	753	2989	1109	18101	2.54	15.0
6	125	2	6	729	11935	1884	18679	2.45	19.6
7	75	4	6	275	4325	989	8082	3.14	14.0
8	125	4	6	619	12409	2095	24393	2.54	17.7
9	50	3	5	1913	73	346	81	3.35	10.6
10	150	3	5	1036	15849	1850	23947	2.41	18.9
11	100	1	5	1096	4610	1629	29969	2.39	16.0
12	100	5	5	341	4474	1448	23883	2.46	15.6
13	100	3	3	764	1698	2142	11430	2.74	14.9
14	100	3	7	797	6984	1600	9552	2.87	18.5
15	100	3	5	199	3616	1634	25094	2.45	13.0
16	100	3	5	250	3772	1613	24343	2.70	13.8
17	100	3	5	152	5142	1690	25373	2.44	13.4
18	100	3	5	468	4546	1572	22903	2.69	13.6

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Table 3

Response	Model	ANOVA					
		Source	Sum of Squares	df	Mean Square	F-value	p- value
Gluconic acid concentration (ppm)	Reduced quadratic	Model	3.076E+008	7	4.395E+007	17.53	< 0.0001
		Residual	2.508E+007	10	2.508E+006		
		Lack of Fit	2.356E+007	7	3.366E+006	6.68	0.0734
		Pure Error	1.512E+006	3	5.041E+005		
		Cor Total	3.327E+008	17			
Citric acid concentration (ppm)	Reduced cubic	Model	1.054E+009	12	8.780E+007	92.87	< 0.0001
		Residual	4.727E+006	5	9.454E+005		
		Lack of Fit	1.058E+006	2	5.289E+005	0.43	0.6839
		Pure Error	3.669E+006	3	1.223E+006		
		Cor Total	1.058E+009	17			
Malic acid concentration (ppm)	Reduced cubic	Model	3.796E+006	12	3.164E+005	132.78	< 0.0001
		Residual	11912.69	5	2382.54		
		Lack of Fit	4673.94	2	2336.97	0.97	0.4737
		Pure Error	7238.75	3	2412.92		
		Cor Total	3.808E+006	17			
Oxalic acid concentration (ppm)	Reduced quadratic	Model	3.477E+006	8	4.346E+005	7.26	0.0038
		Residual	5.389E+005	9	59875.00		
		Lack of Fit	4.803E+005	6	80056.05	4.10	0.1370
		Pure Error	58538.75	3	19512.92		
		Cor Total	4.016E+006	17			

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Table 4

	Responses			
	Gluconic acid	Citric acid	Malic acid	Oxalic acid
R-Squared	0.9246	0.9955	0.9969	0.8658
Adj R Squared	0.8719	0.9848	0.9894	0.7465
Adeq Precision	14.963	36.170	43.808	8.807
PRESS	1.437E+008	9.130E+007	4.979E+005	3.406E+006

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Table 5

Response (ppm)	Goal	Prediction	Confirmation Experiment	95% CI Low	95% CI High
Oxalic acid	Minimize	305.558	443.31	48.72	562.40
Gluconic acid	Maximize	8433.76	9098.57	7091.58	9775.95
Malic acid	Maximize	1832.53	1871.12	1773.48	1891.58
Citric acid	Maximize	26478	27348.07	25272.53	27683.49

Fig. 1

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Fig. 2

Fig. 3

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Fig. 4

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Fig. 5

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Fig. 6

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Fig. 7

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Fig. 8

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