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Factors Affecting Precipitation of Calcium Lactate from Fermentation Broths and from Aqueous Solution

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Abstract
Lactic acid is one of the most important organic acids which is being extensively used around the globe in a range of industrial and biotechnological applications. Lactic acid can be produced either by fermentation or by chemical synthesis but the biotechnological fermentation process has several advantages compared to the other one. However fermentation broth contains a number of impurities which must be removed from the broth in order to achieve more pure lactic acid. Efficiency of recovery is crucial to the economy of the whole process as well since the costs of separation and recovery are responsible for more than half of the entire cost of production. In the traditional procedure, the heated and filtered fermentation broth is concentrated to allow crystallization or precipitation of calcium lactate, followed by addition of sulphuric acid to remove the calcium in form of calcium sulphate. The disadvantage of this procedure is the relatively high solubility of calcium lactate which causes product loss in the crystallization step. Therefore we investigated the effects of four operating parameters of the crystallization/precipitation process from two different fermentation broths and from an aqueous solution. Thus we applied three central composite statistical designs, in which the examined parameters were the temperature of the solution, the duration of the process, the effect of ethanol addition to the solution as well as the effect of multi-cycle precipitation after separation of the precipitated calcium lactate. According to the results, more than 50 percent calcium lactate precipitation increase can be obtained from fermentation broth by adding sufficient amount of ethanol and adjusting the proper temperature of the process.

Keywords
calcium lactate precipitation, central composite statistical design, processing fermentation broth, downstream, solubility

1 Introduction

Lactic acid is a valuable industrial chemical used in food, pharmaceutical, leather, and textile industries, as well as a chemical feedstock [1–5]. One of the most promising applications of lactic acid is its use for biodegradable and biocompatible lactate polymers, called polylactic acid [6–9]. Recent trends showed that lactic acid can be produced either by fermentation or by chemical synthesis but the biotechnological fermentation process has received significant importance due to environmental concerns, use of renewable resources instead of petrochemicals, low production temperature, low energy requirements and high optical purity [10, 11]. Furthermore the biotechnological process depending on the applied microorganism may yield either D(+) or L(+) isomers alone, or in a racemic mixture, whereas the chemical production can only results in racemic mixture of the two isomers [12]. The eco-friendly processing and fermentable capability of many agricultural and agro-industrial based wastes or by-products respectively makes these wastes attractive candidates for raw materials of fermentation biotechnology in formation of value added products with multiple applications [12]. Fermentation broth contains a number of impurities such as residual sugars, pigments, nutrients and other organic acids, as well as part of cell mass. These impurities must be removed from the broth in order to achieve pure lactic acid [6]. Also, efficiency of recovery is crucial to the economy of the whole process since the costs of separation and recovery are responsible for more than half of the entire cost of production [13].

Lactic acid has an inhibitory effect on microbial growth, especially at low pH values [14, 15]. There are several strategies to handle pH changes: 1) the pH of the fermentation

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is either set at the beginning and then left to decrease due to acid production, 2) or it is controlled due titration with alkali 3) or by product removal via extraction, or adsorption. The traditional optimal pH range for lactic acid production is between 5.0 and 7.0 [12]. To maintain the pH of the fermentation in the optimal range $Ca(OH)_2$ is generally added [16]. Therefore calcium lactate is the initial product which is generally converted into lactic acid during the downstream process by addition of H_2SO_4 [14, 17].

Downstream processing of lactic acid currently in use include solvent extraction [18], electrodialysis [19], ion exchange [15], nanofiltration [13], and reverse osmosis [7] [13]. These methods can be effective in separation, purification or even concentration of fermented lactic acid but their combination is required to reach high purity product.

Lactic acid is commercially available at different grades (qualities). These are technical grade lactic acid (20–80 %), food grade lactic acid (80 %), pharmacopoeia grade lactic acid (90 %), and plastic grade lactic acid. Pharmaceutical and food grade lactic acids are considerable to be of most important. For the recovery of lactic acid, additional calcium carbonate is added to the medium, the pH is adjusted to approximately 10, and the fermentation broth is heated and then filtered. This procedure converts all of the lactic acid to calcium lactate, kills bacteria, coagulates protein of the medium, removes excess calcium carbonate and aggregates and finally helps to decompose any residual sugar in the medium. Various procedures are employed for the recovery and purification of the lactic acid [12]. In the traditional route, the heated and filtered fermentation broth is concentrated to allow crystallization of calcium lactate, followed by addition of sulphuric acid to remove the calcium in form of calcium sulphate. The lactic acid is then re-crystallized as calcium lactate, and activated carbon is used to remove colored impurities [7]. As an alternative to the latter step, the zinc salts of lactic acid are sometimes prepared because of the relatively lower solubility of zinc lactate [20]. The resulted grade of lactic acid both from recrystallized calcium lactate and zinc-lactate still contains many impurities from fermentation medium, but is already appropriate for industrial use where purity of the product is not essential, like in the deliming of hides in the leather industry [12].

The disadvantage of this procedure is the high solubility of calcium lactate which causes product loss in the crystallization step. Therefore we investigated the effects of four operating parameters of the crystallization/precipitation process from two different compositions of fermentation broths and from an aqueous solution. The examined parameters were the temperature of the solution, the duration of the process, the effect of ethanol addition to the solution as well as the effect of multi-cycle precipitation after separation of the precipitated calcium lactate. The experiments were designed and evaluated statistically, using the toolbars of central composite design and response surface methodology (RSM).

2 Materials and methods

2.1 Preparation of fermentation broths for precipitation tests

Lactobacillus sp. MKT878 (NCAIM B.02375) was used in fermentations to produce lactic acid.

In this study two fermentation broths were used to investigate process parameters of calcium lactate precipitation. The only difference between the fermentation broths were the form of carbon source. The broth 1 contained 120 $g*L^{-1}$ saccharose, broth 2 contained diluted molasses providing the same saccharose content. Other nutrients were 6 $g*L^{-1}$ yeast extract, 14 $g*L^{-1}$ corn germ flour and 70 $g*L^{-1}$ $CaCO_3$. While the carbon sources were solved and sterilized in 200-200 mL distilled water in a 500 mL Erlenmeyer flask, the nitrogen sources were solved in 300 mL distilled water in a 1 L Erlenmeyer flask and finally $CaCO_3$ was sterilized in solid form. Fermentations were carried out in 500 mL broth at 37 °C and at 250 RPM in a shaking incubator (New Brunswick Scientific Excella E24).

Cells and particulars were separated in a centrifuge (MLW K23D) at 6000 RPM and at 80 °C for 20 min.

2.2 Analysis, Software

Sugars and organic acids were quantified using high performance liquid chromatography (Waters Breeze System, Waters Co. USA) with an analysis column of Biorad Aminex HPX-87H, (Bio-Rad, USA) operated at 65 °C eluted by 0.5 mM H_2SO_4 as mobile phase with a flow rate of 0.5 $mL*min^{-1}$. The detection of compounds was performed with RI Detector at 40 °C. Statistica 13.1 software (StatSoft, Inc, Tulsa, USA) was used for statistical and graphical evaluation of the experimental results.

2.3 Experimental design

Face centered central composite design with four factors was used as experimental design to gain data, which could be used to perform comprehensive statistical evaluation. The process temperature, the duration of one precipitation cycle, the number of cycle of the precipitation and the volume of added ethanol volume were treated as fixed factors. The cycle means the repeats of the precipitation process from the centrifugation. Every factor had three levels (Table 1).

The runs of this experimental design were prepared on different days, therefore two blocks were applied. The output of the experiments was the precipitation ratio: dry mass of precipitated calcium lactate to initially total solved calcium lactate in percentage. ANOVA was used to gain preliminary information about the effect of the factors.

The applied polynomial quadratic response model was given by this equation:

$$\hat{Y} = \beta_0 + \sum_{i=1}^{k}\beta_i X_i + \sum_{i=1}^{k}\beta_{ii} X_i^2 + \sum_{i=1}^{k}\sum_{j=1}^{k}\beta_{ij} X_i \beta_{ii} X_j + \varepsilon$$

where $\hat{Y}$ is the predicted response variable (precipitated calcium lactate in ratio to total soluted calcium lactate), X_i is the independent variable (temperature, cycle, time, ethanol), β_0, β_i, β_{ii}, β_{ij} are the regression coefficients and ε is the random error.

2.4 Precipitation

7.5 mL of cell-free fermentation broth samples were placed into 15 mL centrifuge (Falcon) tubes and were supplemented with ethanol, corresponding to Table 1. Based on preliminary experience seed crystals (1 mg) were added to the tubes to facilitate nucleation. Agitation also facilitates precipitation by producing smaller nodules. Therefore, samples were rotated by a rotospin test tube rotator with adjustable temperature, (Rotomix, Kutesz, Hungary). After the desired time has passed the tubes were centrifuged (HERMLE Z 200 A) at 6000 RPM for 20 min. The supernatant was removed and, precipitate was dried and measured by Sartorius MA35 Moisture Analyzer (Sartorious, Germany). Supernatant was taken back to the thermostat if the cycle number was higher than 1.

3 Results and discussion

The results of face centered central composite experimental design formed a data matrix. For studying the effect of the different factors (temperature, time, cycle, ethanol) on the precipitation process ANOVA design was applied. The output of the experiments was the precipitation ratio: dry mass of precipitated calcium lactate to initially total solved calcium lactate in percentage.

Table 1 Examined factors and levels

Factors name	Low value	Center value	High value
T (°C)	5	10	15
Cycle	1	2	3
Time (h)	24	36	48
Ethanol (%)	0	25	50

3.1 Calcium lactate precipitation from molasses based fermentation broth

Precipitated calcium lactate from molasses based fermentation broth had only a little amount and was a brown precipitate in the centrifuge tube after centrifugation, generally. Fig. 1 showed which factors may have significant effect (based on p value) on calcium lactate precipitation from molasses based fermentation broth. While temperature and time had significant linear effect, ethanol showed linear and quadratic significant effects as well, furthermore the interaction of cycle and temperature were also found significant. The R^2 is 0.9498, which means that the applied model fits well on the experimental data and 94.98 % of the variability can be explained with it. Residues were inspected and showed approximately normal distribution and there was no anomaly and regularity among them, therefore the statistical model is considered adequate (Fig. 2).

The factors effects are illustrated with RSM (response surface methodology), but hence of too many variations, the significant ones were selected (based on p value) and illustrated. While the first combination of factor's effect (Fig. 3) showed an optimal ethanol amount of 40%, in term of cycle time no optimum was observed, but longer cycles resulted higher amount of precipitate. Furthermore, remarkable difference was found when comparing lowest and highest levels: while the difference of precipitation ratio between the lowest and highest level of cycle time at

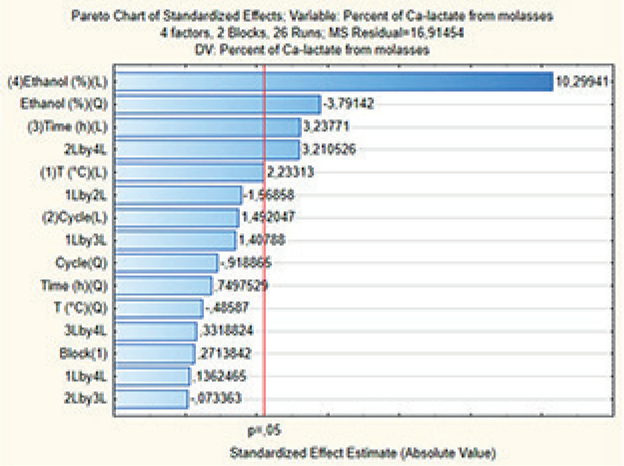

Fig. 1 Pareto chart for standardized effects for calcium lactate precipitation from broth 1 (molasses)

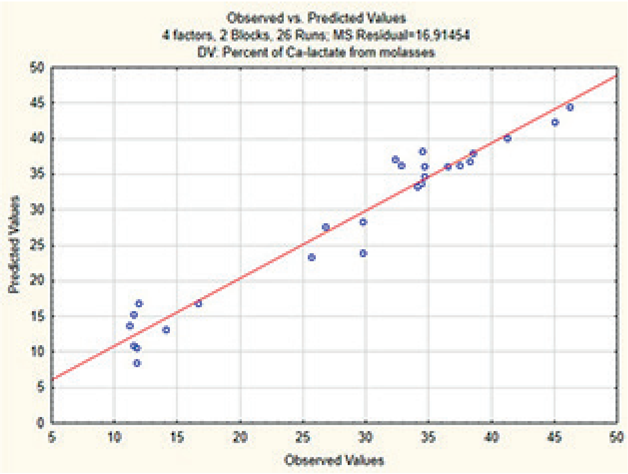

Fig. 2 Scatterplots of residues for calcium lactate precipitation from broth 1 (molasses)

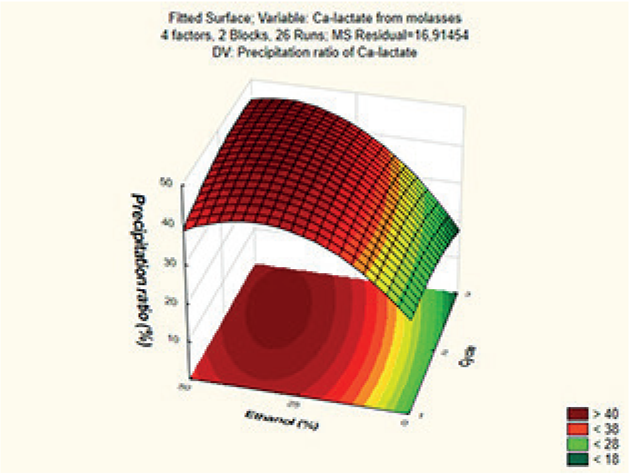

Fig. 4 Fitted response surface and contour plot of calcium lactate precipitation from broth 1 (molasses). The effects of cycle and ethanol were illustrated.

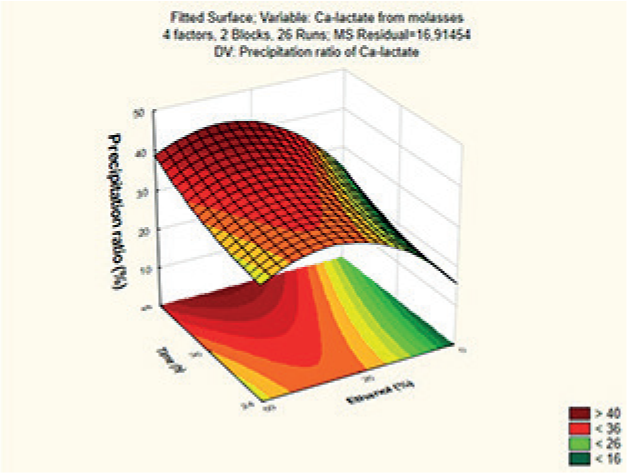

Fig. 3 Fitted response surface and contour plot of calcium lactate precipitation from broth 1 (molasses). The effects of time and ethanol were illustrated.

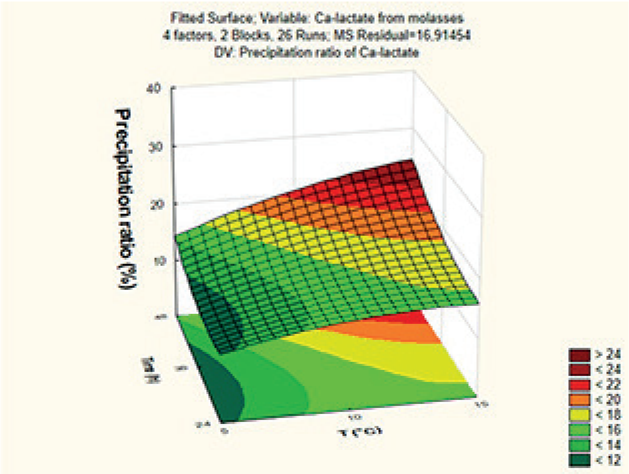

Fig. 5 Fitted response surface and contour plot of calcium lactate precipitation from broth 1 (molasses). The effects of time and temperature were illustrated.

40 % ethanol was only 6 %, the same difference between the lowest and highest level of ethanol at 48 h was 15 %. This also demonstrates well, that ethanol addition had higher impact on precipitation than cycle time.

Depicting the effect of ethanol and number of cycle (Fig. 4), an optimum can be seen for both factors. The center of the optimum was at 40 % of ethanol and between cycles 2 and 3. The highest result's range overlap with second level of cycle as factor, therefore two cycles can be enough to achieve the desired product quantity. It is important to be noted that any repeats (i.e. more cycle) always provided some new precipitates, but their amount were not enough to be significant. The difference of precipitation ratio between the lowest and highest level of cycle period at 40 % ethanol was only 3 %.

Surprisingly there was not a huge difference in precipitated calcium lactate versus examined temperature range at short cycle time (24 h), showed on Fig. 5. However there was great difference in the amount of the precipitate between 5 °C and 15 °C at long cycle time (48 h). While Cao et al. [20] reported that the solubility of calcium lactate is only 1.5 % higher at 15 °C compared to 5 °C, we observed that higher temperature resulted more precipitate versus lower temperature. Probably the nucleation rate was faster at higher temperature resulting more precipitate thus it compensated the solubility increment. It can be clearly seen in the figure that simultaneous high level of factors gave the best result which means quantitatively 13 % more efficiency in precipitation ratio.

3.2 Calcium lactate precipitation from saccharose based fermentation broth

Precipitated calcium lactate from broth 2 (saccharose) had yellowish white color which almost filled the total volume of the centrifuge tube after centrifugation. The effect estimates on calcium lactate precipitation from fermentation broth 2 (saccharose) are showed on Fig. 6. The significant effects were gone beyond the line, where p value is less than 0.05. The temperature, the addition of ethanol and the cycle of the precipitation showed significant effects on the amount of the precipitate. While linear correlation were significant for all significant three factors, all quadratic correlations of these were insignificant. These three factors had only linear significant effect but no one of the factors had quadratic significant effect. It is worth to note that while the effect estimation were similar for both molasses and saccharose based fermentation broths in term of significance of temperature and ethanol, there was also a difference in term of cycle and time: cycle was significant in molasses case and insignificant in saccharose case, in contrary time was insignificant in molasses case and significant in saccharose case. The R^2 is 0.8711, which means that the applied model fits roughly on the experimental data and the 87.11 % of the variability can be explained with it. Residues were inspected and showed approximately normal distribution and there was no anomaly and regularity among them, therefore the statistical model is considered adequate (Fig. 7).

Similar to the broth 1 (molasses), ethanol addition to the solution and repeat of the precipitation process greatly increased the amount of the precipitate from broth 2 as well, which has been shown on Fig. 8. In this case optimum was not observed, but remarkable differences in precipitation ratio were obtained. In detail, the difference between the lowest and the highest precipitation ratio was 40 %. Interestingly, there was not a huge difference in precipitated calcium lactate versus examined cycle range at high ethanol level. However at lower ethanol level there was about 20 % difference between the amounts of the precipitate.

In contrast to the molasses case, the precipitation reversed proportionately depended on temperature, which has been shown on Fig. 9. It illustrated the significant effect of temperature and the insignificance of time confirming Fig. 6. This can also be illustrated with the difference of precipitation ratio between the lowest and the highest level of temperature at 24 h which was 20 %. Interestingly time was not a significant effect within the examined range, therefore it seems not to be economic to run the process more than one day.

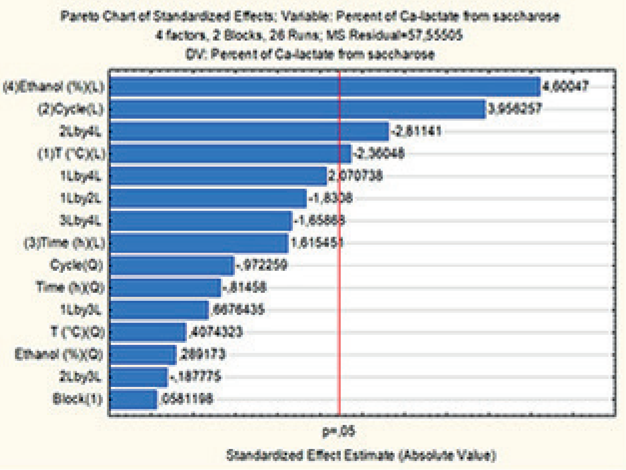

Fig. 6 Pareto chart of effects of calcium lactate precipitation from broth 2 (saccharose).

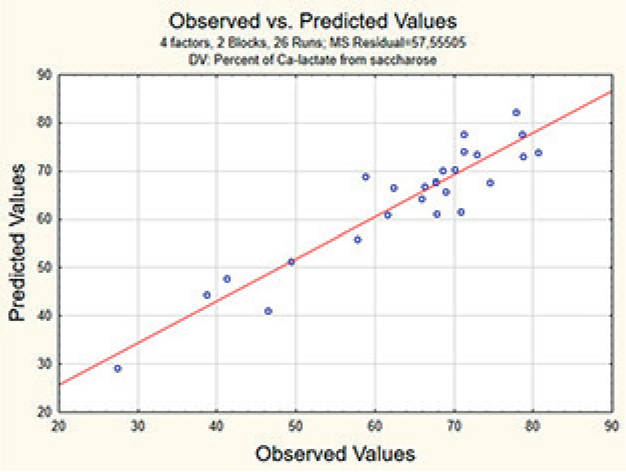

Fig. 7 Scatterplots of residues for calcium lactate precipitation from broth 2 (saccharose)

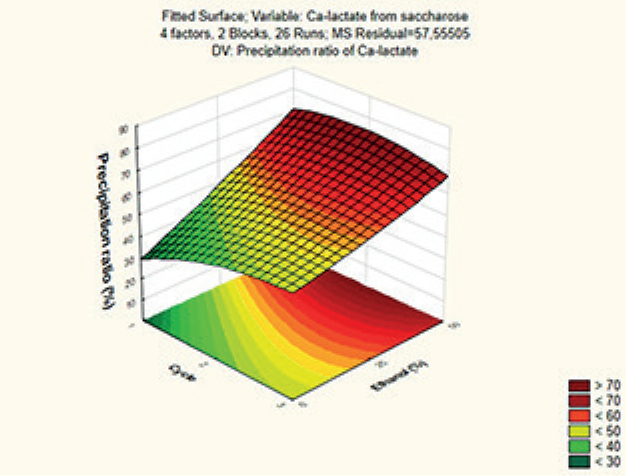

Fig. 8 Fitted response surface and contour plot of calcium lactate precipitation from broth 2 (saccharose). The effects of cycle and ethanol were illustrated.

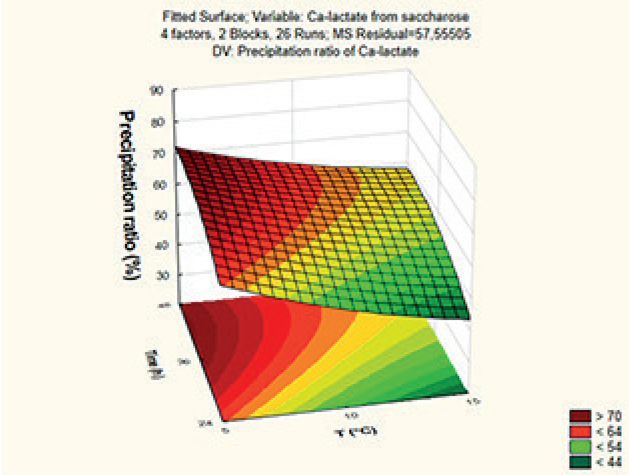

Fig. 9 Fitted response surface and contour plot of calcium lactate precipitation from broth 2 (saccharose). The effects of time and temperature were illustrated.

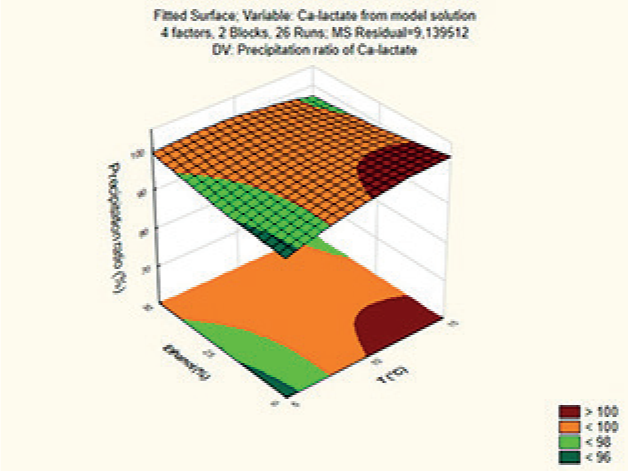

Fig. 10 Fitted response surface and contour plot of calcium lactate precipitation from model solution. The effects of time and temperature were illustrated.

3.3 Calcium lactate precipitation from model solution

Precipitated calcium lactate from its pure aqueous solution was clear white precipitate and filled the total amount of the centrifuge tubes even after centrifugation i.e. it could not be compacted. Therefore the precipitate could not be separated from liquid phase, consequently the cyclic process could not be repeated and effect of cycle could not be studied. Furthermore, because the two phases could not be separated, both precipitated and dissolved calcium lactate were also measured in the dry weight measurement. Fig. 10. showed the response surface about temperature versus time plane, indicating almost no difference between the precipitation ratio. There was less than 5 % difference between the between the lowest and highest value of precipitation ratio. Probably the effect of the factors could have been measured in a shorter time, where less calcium lactate is precipitated resulting better phase separation, but for best comparison the three experiments (molasses based, saccharose based and model solution) were carried out simultaneously and with the same level of factors (i.e. long time). Thus certainly no significance could be observed in this case. However, these concluded, that pure calcium lactate precipitates very quickly with high precipitation ratio, and any accompanying compounds in broths decrease both precipitation rate and efficiency.

4 Discussion

The effects of four factor of calcium lactate precipitation were examined from two fermentation broths and from aqueous solution. The effect of ethanol addition to the solution and the effect of temperature were both significant for calcium lactate precipitation from all examined fermentation broths. The effect of ethanol addition to the solutions achieved the most impressive impact. The repeat of the precipitation process from fermentation broth containing molasses had no significant effect but was significant from fermentation broth containing saccharose. Conversely, the effect of the duration of the process was vice versa (Table 2).

A number of conclusions can be drawn from these precipitation experiments. Firstly, the calcium lactate precipitation from aqueous solution was faster and probably more effective than from fermentation broths, so the components of the fermentation broth in some way obstructed the formation of the precipitate. Secondly, the precipitate absorbed lot of water and carried away dissolved components as

Table 2 The effect of the four examined factor and their values on the best result setting

	Molasses based broth	Setting on best result	Saccharose based broth	Settings on best result
Temperature	significant	10 °C	significant	5 °C
Cycle	significant	2	not significant	3
Time	not significant	48 h	significant	40 h
Ethanol	significant	39.5 %	significant	50 %
Best precipitation ratio	47 %		86.5 %	

well, contaminating the precipitated calcium lactate product. These problems can be solved by pressing and alcoholic washing of the precipitate. Finally, the amounts of the precipitates from fermentation broth containing saccharose were almost twice as much as the ones from the fermentation broth containing molasses. Based on this, it can be concluded that molasses contain a component that inhibit the process of calcium lactate precipitation. Therefore in a next study we are going to make process simulations involving economic evaluations to make a decision between the following scenarios: cheap fermentation molasses with difficult product recovery versus more expensive sugar based fermentation with easy one step product recovery. In that study we will also both economically and technologically compare this ethanol mediated precipitation process to other conventional lactic acid recoveries.

In summary, more than 40 % (in the molasses based broth) and 80 % (in the saccharose based broth) of the calcium lactate can be obtained directly from fermentation broth in a multi-cycle precipitation process by adding sufficient amount of ethanol and adjusting the proper temperature of the process. Therefore both the loss can be reduced during downstream processing, as well the volume to be processed resulting lower downstream costs. Furthermore, the reuse and regeneration of the used ethanol can be easily achieved, resulting a sustainable process.

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