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# Simultaneous saccharification and fermentation (SSF) of jackfruit seed powder (JFSP) to L-lactic acid and to polylactide polymer

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## HIGHLIGHTS

- Isolation of amylolytic latic acid bacteria.
- Simultaneous saccharification and fermentation of jackfruit seed to lactic acid (LA).
- Recovery and purification of LA using a week anion exchange resin.
- Polymerisation of L-lactic acid to poly (L) lactic acid by polycondensation method.

## GRAPHICAL ABSTRACT


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## ABSTRACT

A newly isolated amylolytic lactic acid bacterium, *Streptococcus equinus*, was used for the production of L-lactic acid from jackfruit seed powder (JFSP) by simultaneous saccharification and fermentation (SSF). After optimization of shake flask fermentation by a response surface box-behnken design, the maximum lactate titer was 109 g/L from 200 g/L jackfruit seed powder. Amberlite IRA67, a weak base resin, was used to recover pure lactic acid from fermented broth and subsequently used for the synthesis of poly-lactic acid by direct condensation polymerization method with a yield of 62%.



## 1. Introduction

A wide variety of petroleum-based synthetic polymers are produced worldwide to the extent of approximately 140 million tons per year and remarkable amounts of these polymers are introduced in the ecosystem as industrial waste products (Shah et al., 2008; Nampoothiri et al., 2010). Therefore, high expectations are being placed on the biodegradable polymers gained from the renewable raw materials. There was a notable increase in the number of publication citations on bio-based polymers in recent years (Chen and Martin, 2012; Ramesh et al., 2013). Among the biopolymers, the aliphatic polysters such as polylactide has the potential to replace traditional polymers such as PET, PS, and PC for packaging to electronic and automotive applications (Majid et al., 2010; Lasprilla et al., 2012; Masamichi Ando et al., 2013; Guan et al., 2015).

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Lactic acid can be produced by either microbial route or by chemical synthesis and the former route has received considerable interest due to environmental concerns and other advantageous such as racemic specificity. Traditionally, glucose, sucrose from molasses and lactose from whey are widely used for lactic acid production. (Abdel-Rahman et al., 2011; Dusselier et al., 2013; Probst et al., 2015). Utilization of biomass also has gained substantial attention due to the impending scarcity of fossil fuels and also due to the requisite of increasing world food and feed supplies. Starch has also been used in a two-stage industrial production of lactic acid. The starch material is, first, chemically and/or enzymatically hydrolyzed to glucose, which is then fermented by LAB in the second stage (Otto, 2008; Pagana et al., 2014).

Two important methods for preparing PLA have been known; ring opening polymerization and direct polycondensation (Gupta et al., 2007). Many catalyst systems have been evaluated for the polymerization of lactide including strong bases such as metal alkoxides, tin compounds, especially tin (II) bis-2-ethylhexanoic acid (tin octoate), Sn (II) Lewis acid catalysts such as SnO, $SnCl_2$-$\cdot 2H_2O$ etc., and different reaction conditions for the direct dehydropolycondensation of L-lactic acid to make PLA are testified (Smith et al., 2001; Kim and Woo, 2002; Wang and Yan-zhi, 2008).

The ability of amylolytic lactic acid bacteria (ALAB) to hydrolyze starch and then to ferment maltose and glucose has earned much attention and has been explored for one-step one-pot lactic acid production. ALAB produce amylases, enzymes capable of cleaving a-1, 4-glycosidic linkages between a-D-glucopyranosyl residues in the molecules of a complex carbohydrate and, thus, can directly produce lactic acid from starch and its derivatives (Naveena et al., 2005; Vishnu et al., 2006; Akerberg and Zacchi, 2000; Reddy et al., 2008). Lactic acid bacteria have the conversion efficiency of 1 M glucose to 2 M lactic acid, but fungus like *Rhizopus* which was used for direct conversion has only 1.5 M/M glucose (Naveena et al., 2004). The main economic concern in starch utilization by non amylolytic LAB, however, is the starch saccharification, a step that could be performed separately.

In jackfruit seeds, a large amount of starch is present in the fibrous matrix of the seeds that can be used for the production of many value added products. The relatively high starch content of these seeds made us to choose it as the substrate for lactic acid production in the present study. Jackfruit (*Artocarpus heterophyllus Lam.*) is one of the most popular tropical fruits grown in Asia. It is a monoecious evergreen tree that is popular in several tropical countries that have 100 or up to 300 seeds in a single fruit. Seeds make up around 10–15% of the total fruit mass and have high carbohydrate and protein contents. Reports say that the amylose content of jackfruit seed starch was 32%, higher than the mean value found in tapioca starch (17%) and corn starch (26%) (Kumar et al., 1988; Marta et al., 2014). Seeds are normally discarded or steamed and eaten as a snack or used in some local dishes.

Response Surface Methodology (RSM) not only allows quick screening of large experimental domain, but also reflects the role of each of the components and has already been successfully applied for optimization of the media and culture conditions in many cultivation processes for the production of primary and secondary metabolites (Sivaramakrishan et al., 2006; Swain and Ray, 2007).

The entire bioprocess of PLA synthesis starting from the raw material, fermentation to produce its monomer and subsequent polymerization is described in this work.

## 2. Materials and methods

All microbial culture media like MRS (broth and agar), lactic acid bacteria differential broth, ISP-2, salts such as ammonium chloride, ammonium sulphate, ammonium dihydrogen phosphate, ammonium nitrate and sodium nitrate and organic nitrogen sources like yeast, beef and malt extracts, peptone, tryptone, were procured from Hi-media Laboratories (India). The software Design-Expert (Version 9.0.9, Stat-Ease Inc., (USA) was used for statistical optimizations. Basic anion exchange resin IRA-67, used in this study was procured from Fluka (Switzerland).

### 2.1. Raw materials

Jackfruit (*Artocarpus heterophyllus Lam*) seeds, the raw material used for lactic acid production in this study were obtained from local sources. Jackfruit arils were peeled off manually and the cotyledons were washed with water, drained, and washed thrice with distilled water and dried in a hot air drier at 50 °C for 8 h. The cotyledons were ground to fine particles and sifted through a 0.5 mm mesh sieve.

### 2.2. Microorganisms

A lactic acid bacterium with amylolytic activity was isolated from Milma diary waste and identified as a *Streptococcus* with 99% similarity to *Streptococcus equinus* and was used for lactic acid fermentation studies. The culture was maintained in MRS agar medium and sub cultured fortnightly.

### 2.3. Simultaneous saccharification and fermentation

The inoculum for the experiments was prepared from fresh MRS stab of *S. equinus*. Under the standard condition, production medium consisted of 20% w/v jack fruit seed powder in distilled water enriched with (% w/v) yeast extract, 0.5 and $(NH_4)_2SO_4$, 0.25. $CaCO_3$ (4%w/v) was added for buffering unless otherwise stated. The medium was autoclaved at 121 °C for 15 min. The medium was then cooled to 50 °C and sufficient amount of enzymes (α-Amylase (5000 IU/mL) and glucoamylase (200 IU/mL) were added and kept in shaking incubator at 50 °C for an hour and cooled to room temperature. The inoculum ($4 \times 10^8$ CFU/mL of 18 h old culture) was added and further incubated at 37 °C for 120 hunder static condition.

Samples were withdrawn at desired intervals and treated with 1 M $H_2SO_4$ to release lactic acid from medium as it is formed as calcium lactate with buffering agent, $CaCO_3$. Lactic acid extracted out by squeezing from medium and the extract was diluted to the required level with distilled water. The amount of total lactic acid, reducing sugar and starch was estimated as per standard protocols (Barker and Summerson, 1941; Miller, 1959; Nampoothiri et al., 2003). All the represented values are means of three replicates ± SD.

### 2.4. Statistical design

The Design Expert software (Version 9.0.0, Stat-Ease Inc., and Minneapolis, USA) was used for the experimental design and the analysis of variance (ANOVA) for the data.

### 2.5. Plackett–Burman (PB) experimental design

The Plackett–Burman design provides an efficient way of a large number of variables and identifies the most important ones. Table 1 illustrates the factors under investigations as well as levels of each factor used in the experimental design. Based on the Plackett–Burman factorial design, each factor was examined in two levels: (−1) for a low level and (+1) for a high level. This design is practical especially when the investigator is faced with a large number of factors and is unsure which settings are likely to be nearer to

**Table 1**
Plackett–Burman design matrix: media components and test levels of experiment.

| Sl No: | Variable | Variable code | Minimum selected value (−1) | Maximum selected value (+1) |
|---|---|---|---|---|
| 1 | Substrate concentration | *A* | 10% | 20% |
| 2 | pH | *B* | 4 | 9 |
| 3 | Temperature | *C* | 28 | 42 |
| 4 | Inoculum size | *D* | 2 mL | 8 mL |
| 5 | Inoculum age | *E* | 6 h | 24 h |
| 6 | Inorganic $N_2$ source | *F* | 0.25% | 2% |
| 7 | Organic $N_2$ source | *G* | 0.25% | 2% |
| 8 | Agitation | *H* | +(150 rpm) | static |
| 9 | Surfactant (Tween 60) | *I* | 100 mg | 1000 mg |
| 10 | Incubation period | *J* | 24 h | 120 h |
| 11 | $CaCO_3$ conc. | *K* | 1% | 5% |

optimum responses (Plackett and Burman, 1946). Plackett–Burman experimental design is based on the first-order polynomial equation: $Y = \beta^{\circ} + \Sigma\beta ixi$, Where $Y$ is the response (enzyme activity), $\beta$ is the model coefficient and $\beta i$ is the linear coefficient, and $xi$ is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response.

A predictive model was developed to screen the most important variables influencing lactic acid production by submerged fermentation of jackfruit seeds and analyzed by PBD in twelve experimental designs (Table 2). All experiments were carried out in triplicates.

### 2.6. Box Behnken design (BBD) matrix for bioprocess evaluation of lactic acid

Based on the PB results, three factors namely incubation period, calcium carbonate concentration and inoculum age were selected as independent variables in the Box-Behnken response surface design. A three-factor and three-level BBD of RSM was chosen to evaluate the combined effect of three independent variables. The incubation period, calcium carbonate concentration and inoculum age were coded as *A*, *B*, and *C* respectively. The levels of each variable and the design matrix is given in Table 3. The response surface graphs were obtained using the software to understand the effect of variables individually and in combination, and to determine their optimum levels.

### 2.7. Purification by ion exchange resins

Fermented media with *S. equinus* obtained through simultaneous saccharification and fermentation of jackfruit seed starch based medium was used for lactic acid recovery using weak base resin column. In order to purify lactic acid from the fermentation broth, a packed bed column chromatography was performed respectively on a column (length, 27 cm; i.d., 1.2 cm, bed volume, ~28 $cm^3$) packed with 14.5 g of resin (Amberlite IRA 67). After steeped for 24 h in distilled water at room temperature, removed the floating resin, packed into the column and conditioned using distilled water of three column volumes (CV). Lactic acid solution containing 43 g lactic acid/L at pH 5 was used for this purpose and chromatographic separations were carried out using a peristaltic pump at a flow rate of 0.3 mL/min at all time. Fractions of the effluent were collected and analysed for lactic acid. The resin was considered to be saturated with lactic acid when the lactic acid concentration in the effluent decreased and reached the value corresponding to the feed. The interstitial solution was removed by pumping distilled water until the lactic acid concentration of the effluent was below 0.1 g/L. Lactic acid recovery was achieved by pumping 1 N HCl at a flow rate of 0.3 mL/min through the column. Samples of the effluent were collected and analyzed for lactic acid until an outlet concentration below 0.1 g/L was reached. Finally, a washing step was carried out using distilled water to remove the HCl contained in the interstitial space and the column became ready for a new cycle. Fermentation broth was used along with the standard lactic acid solution.

### 2.8. Lactic acid polymerization

PLA polymerization was carried out in two steps. In the first part of synthesis purified lactic acid obtained from fermented broth was boiled at 100 °C on a thermostat attached magnetic stirrer to remove water generated by the union of the monomer molecules. Second part was the addition of catalyst (0.3% w/v) and reacted for 3 h in which it promotes the esterification reaction to form the polymer. Then the viscous suspension was kept in vacuum dried oven (180 °C) for 12 h and at the end of the reaction, the samples were allowed to cool to room temperature. Synthesis was performed by direct condensation reaction at specific temperature and reaction time. The samples were then dissolved with acetone and precipitated with water in order to remove unreacted lactic acid and other water soluble components from PLA. Then samples were filtered and then dried in desiccator or in oven at 50°.

**Table 2**
Plackett–Burman experimental design with the response.

| Run | Substrate con. (%) | pH | Organic $N_2$ (g) | Inorganic $N_2$ (g) | Inoculum size (mL) | Inoculam age (h) | Incubation period (h) | Surfactant | $CaCO_3$ (g) | Agitation (rpm) | Temperature (°C) | *R*1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10 | 4 | 2 | 0.25 | 8 | 24 | 120 | 0.1 | 5 | 1 | 28 | 36.3 |
| 2 | 10 | 9 | 2 | 0.25 | 8 | 24 | 24 | 1 | 1 | 0 | 42 | 27.4 |
| 3 | 20 | 9 | 2 | 0.25 | 2 | 6 | 120 | 1 | 1 | 1 | 28 | 32.7 |
| 4 | 20 | 4 | 2 | 2 | 8 | 6 | 120 | 0.10 | 1 | 0 | 42 | 45.3 |
| 5 | 10 | 4 | 0.25 | 2 | 2 | 24 | 120 | 1 | 1 | 1 | 42 | 34.5 |
| 6 | 20 | 4 | 2 | 2 | 2 | 24 | 24 | 1 | 5 | 0 | 28 | 31.5 |
| 7 | 20 | 9 | 0.25 | 0.25 | 2 | 24 | 120 | 0.10 | 5 | 0 | 42 | 44.1 |
| 8 | 20 | 9 | 0.25 | 2 | 8 | 24 | 24 | 0.10 | 1 | 1 | 28 | 39.4 |
| 9 | 10 | 9 | 2 | 2 | 2 | 6 | 24 | 0.10 | 5 | 1 | 42 | 25 |
| 10 | 20 | 4 | 0/25 | 0.25 | 8 | 6 | 24 | 1 | 5 | 1 | 42 | 56.8 |
| 11 | 10 | 4 | 0.25 | 0.25 | 2 | 6 | 24 | 0.10 | 1 | 0 | 28 | 24.4 |
| 12 | 10 | 9 | 0.25 | 2 | 8 | 6 | 120 | 1 | 5 | 0 | 28 | 38.9 |

**Table 3**
Factors and their levels studied by Box-Behnken design.

| Factor | Name | Low Level | Level | High level |
|---|---|---|---|---|
| A | Incubation period (h) | 48 | 72 | 96 |
| B | $CaCO_3$ conc. (g/100 mL) | 1 | 3 | 5 |
| C | Inoculum Age (h) | 6 | 18 | 24 |

## 3. Results and discussion

### 3.1. Simultaneous saccharification and fermentation

In the current study, a 20% w/v concentration of jackfruit seed powder was selected as the higher substrate level since any further increase in concentration resulted in an undesirable increase of viscosity, causing it to solidify readily and negatively affecting the lactic acid production and inoculum distribution. The starch concentration of the jackfruit seed powder used for the current study was estimated to be 79%. A starch hydrolysis zone of around 12 ± 5 mm was observed for *S. equinus* in starch agar plates on primary screening and was capable of producing extracellular alpha amylase.

### 3.2. Plackett–Burman (PB) experimental design

Analysis of variance (ANOVA) of main effects of factors indicated that the model *F* value of 59.69 implies that the model is significant. There is only a 0.32% chance that a "Model *F* Value" this large could occur due to noise. Values of "Prob > *F*" less than 0.0500 indicated that the model terms are significant. In this case *A*, *B*, *C*, *D*, *E*, *G*, *H*, *I*, *J*, *K*, are the most significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Pred R-Squared" of 0.9979 is in very good agreement with the "Adj R-Squared" of 0.9998.

**Table 4**
Box-Behnken design arrangements and responses.

| Run | Incubation period (h) | $CaCO_3$ Conc. (g) | Inoculum age (h) | Lactic acid (g/L) |
|---|---|---|---|---|
| 1 | 48 | 1 | 18 | 46 |
| 2 | 48 | 5 | 18 | 56 |
| 3 | 96 | 5 | 18 | 91 |
| 4 | 48 | 3 | 8 | 42 |
| 5 | 72 | 3 | 18 | 105 |
| 6 | 72 | 5 | 8 | 55 |
| 7 | 72 | 3 | 18 | 105 |
| 8 | 96 | 3 | 8 | 46 |
| 9 | 72 | 5 | 24 | 79 |
| 10 | 48 | 3 | 24 | 33 |
| 11 | 96 | 1 | 18 | 49 |
| 12 | 72 | 1 | 8 | 36 |
| 13 | 96 | 3 | 24 | 63 |
| 14 | 72 | 3 | 18 | 105 |
| 15 | 72 | 3 | 18 | 105 |
| 16 | 72 | 1 | 24 | 35 |
| 17 | 72 | 3 | 18 | 105 |

### 3.3. BBD matrix for bioprocess evaluation of lactic acid

There were a total of 17 runs for optimizing the three individual parameters in the current Box-Behnken design and results were as in Table 4. According to the analysis of variance (ANOVA) for RSM (Table 5), the regression coefficient ($R^2$) of the model was 0.99, which indicated that the model had adequately represented the real relationships between the parameters chosen. For any of the terms in the models, a large *F*-value and a small *P*-value indicate a more significant effect on the respective response variables. The Model *F*-value implies the model is significant and there is only 0.01% chance that a "Model *F*-Value" this large could occur due to noise. Values of "Prob > *F*" less than 0.0500 indicate model terms are significant. In this case *A*, *B*, *C*, *AB*, *AC*, *BC*, $A^2$, $B^2$, $C^2$ are significant model terms and they were found to interact with each other and among themselves. The "Pred*R*-Squared" of 0.9451 is in reasonable agreement with the "Adj*R*-Squared" of 0.9922.

The final polynomial equation derived from regression analysis in terms of coded factors follows:

$$R1 = 105 + 9A + 14.375B + 3.875C + 8AB + 6.5AC + 6.25BC - 24.875A^2 - 19.625B^2 - 34.125C^2 \quad (A.1)$$

The final polynomial equation derived from regression analysis in terms of actual factors.

$$R2 = -262.094 + 5.552A + 18.375B + 13.9375C + 0.1667AB + 0.034AC + 0.391BC - 0.043A^2 - 4.906B^2 - 0.533C^2 \quad (A.2)$$

The graphical representation provides a method to visualize the relation between the response and experimental levels of each variable, and the type of interactions between test variables (Rahulan et al., 2009). The optimum value of each variable was located based on the hump in the three-dimensional plot, or from the central point of the corresponding contour plot.

From Fig. 1, it is evident that as the $CaCO_3$ concentration increases, initially the lactic acid production also increases. It is similar with incubation period also. But, beyond the mid value, an increase in incubation period and $CaCO_3$ level negatively

**Table 5**
ANOVA for response surface quadratic model analysis of variance table.

| Source | Sum of squares | df | Mean square | *F* value | *P*-Value Prob > F | |
|---|---|---|---|---|---|---|
| Model | 13130.75 | 9 | 1458.97222 | 225.6973603 | 8.75991E-08 | Significant |
| A-Incubation period | 648 | 1 | 648 | 100.2430939 | 2.12237E-05 | |
| B-$CaCO_3$ conc. | 1653.125 | 1 | 1653.125 | 255.7320442 | 9.07884E-07 | |
| C-Inoculum age | 120.125 | 1 | 120.125 | 18.58287293 | 0.003519711 | |
| AB | 256 | 1 | 256 | 39.60220994 | 0.000406888 | |
| AC | 169 | 1 | 169 | 26.14364641 | 0.001379295 | |
| BC | 156.25 | 1 | 156.25 | 24.17127072 | 0.001720619 | |
| A^2 | 2605.329 | 1 | 2605.32895 | 403.0343123 | 1.90462E-07 | |
| B^2 | 1621.645 | 1 | 1621.64474 | 250.8621692 | 9.69519E-07 | |
| C^2 | 4903.224 | 1 | 4903.22368 | 758.5097412 | 2.13549E-08 | |
| Residual | 45.25 | 7 | 6.46428571 | | | |
| Lack of Fit | 45.25 | 3 | 15.0833333 | | | |
| Pure Error | 0 | 4 | 0 | | | |
| Cor Total | 13,176 | 16 | | | | |

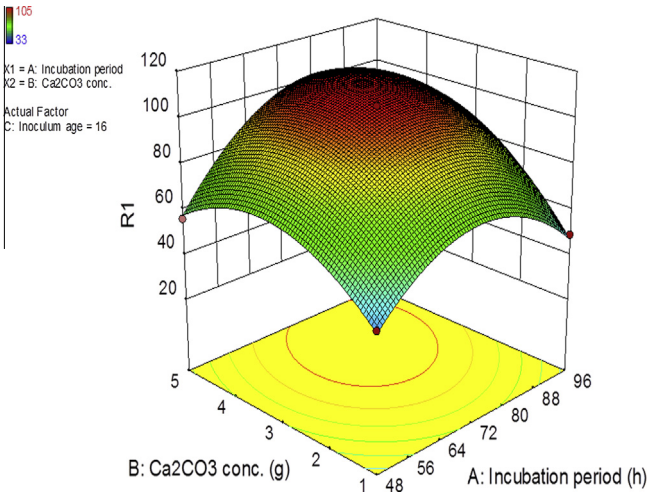


**Fig. 1.** Three dimensional surface plot showing the response from the interaction between (A) Incubation period and (B) $CaCO_3$ concentration.

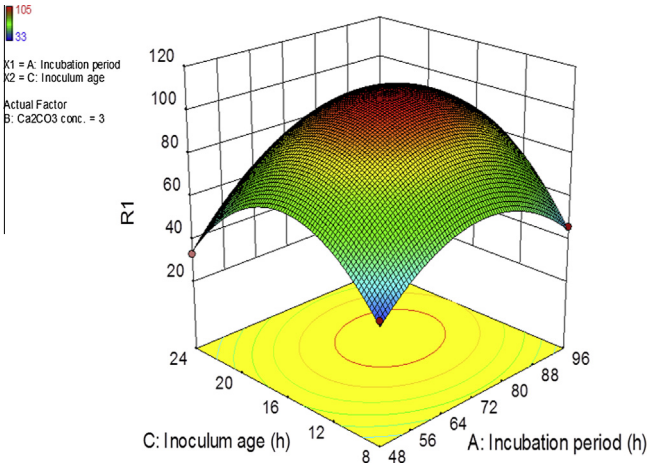


**Fig. 2.** Three dimensional surface plot showing the response from the interaction between (A) Incubation period and (C) inoculum age.

impacts the lactic acid yield. Hence the optimum conditions are within the range 3–4% $CaCO_3$ and 72–80 h incubation.

Lactic acid concentration increases initially with increase in inoculum age and incubation period (Fig. 2). But increase in these factors beyond the midlevel value was found to have a detrimental effect on the lactic acid yield. The optimum conditions for production are an inoculum age of 18–20 h and 72–80 h incubation.

As with previous interactions, the lactic acid concentration increased with increasing $CaCO_3$ concentration and inoculum age till the mid value. But increase beyond 18–20 h inoculum age and 3–4 g $CaCO_3$ reduces the product (Fig. 3). Hence the optimum conditions for the production of lactic acid could be an inoculum age within 18–20 h and incubation period of 72–80 and buffering with $CaCO_3$ in the range of and 3–4% w/v.

### 3.4. Validation of the optimized condition

Based on the quadratic model, the optimal values of each test variables in coded levels were as follows: *A* (Incubation period)-72 h, *B* ($CaCO_3$ concentration)-3% and *C* (inoculum age)-18 h, the model predicted that the production of lactic acid could reach 105 g/L under the optimal conditions. To verify the predicted result, validation experiment was carried out in triplicate test with these optimized conditions such as 20% (w/v) jackfruit seed

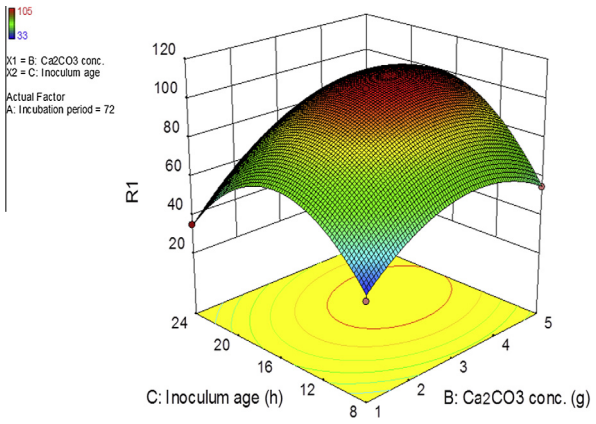


**Fig. 3.** Three dimensional surface plot showing the response from the interaction between (B) $CaCO_3$ concentration (C) and inoculum age.

powder, 15 mL saccharifying enzyme mixture, 0.5% yeast extract and 0.25% $(NH)_2SO_4$ together with 3% 18 h inoculum ($4 \times 10^8$ CFU) incubated in static conditions resulted in the production of 109 g/L lactic acid which was closer to the predicted response.

The costs of recovery and purification account for almost 50% of the overall production costs in lactic acid fermentation. Like in other reports, due to the presence of nutrients and ions other than lactate, the binding capacity was slightly lesser while using fermented media (86%) instead of aqueous lactic acid solutions (~88%). Data on binding capacities and recovery proved that weak base resin was the most favorable one for lactic acid recovery from solutions. So it can be concluded that Amberlite IRA 67 showed a constructive performance for lactic acid recovery from fermented samples in terms of affinity, capacity and susceptibility to regeneration with 1 N HCl as elutant.

### 3.5. Polycondensation of lactic acid

Polycondensation of lactic acid proceeds stepwise in a similar manner to the esterification of a diacid with a diol. One of the key points in the overall process is to reduce the viscosity of the reaction system as much as possible and to choose a method to remove the water formed effectively from the reaction system. Since the molecular weight of the polymer was influenced by water content in the reaction mixture, an apparatus that can efficiently overcome this limitation was used and the study focused on the optimization of different conditions for polymer synthesis. $SnCl_2$ $H_2O$ was selected as the catalyst for the polymerization process. The polymer can be melt processed into transparent films.

## 4. Conclusion

A viable process based on a low cost production medium is desired to improve the techno economic aspects of L-lactic acid fermentation. To the best of our knowledge, there were no reports on one step production of lactic acid from jackfruit seed (JFS) powder. The process offer advantageous such as no need of hydrolysate preparation, use of relatively high substrate concentration and controlled release of sugar etc. Thus, the value addition of a less explored agro residual waste material by biotechnological intervention is well depicted in this study.

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