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Parametric optimization of lactic acid production and its scale up using free and immobilized cells of *Lactobacillus amylovorus* NRRL B- 4542

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ABSTRACT

In the present paper, parametric optimization studies were performed to determine the most influential range of process parameters for maximum lactic acid production from Lactobacillus amylovorus NRRL B4542 under submerged fermentation conditions. Various parameters viz. agitation, pH of medium, inoculum size, temperature, buffering agents and different starchy sources were taken into consideration. The optimum conditions were agitation (200rpm), pH (8.5), temperature (40^{o}C), incubation time (30^{o}C) and inoculum (7%) for maximum lactic acid production. Also, scale up studies were conducted using corn starch from free and calcium alginate entrapped cells of L. amylovorous NRRL B- 4542 on 15 L fermenter scale using corn starch (60 g/l) as carbon source. Free cells of L. amylovorous NRRL B-4542 showed lactic acid production (55g/l) and immobilized cells produced lactic acid (60g/l) with the maximum yield 0.96g/g after 3 cycles thereafter showed declination in lactic acid yield.

Key words: *Lactic acid; Lactobacillus amylovorous NRRL B- 4542; Immobilized cells.*

INTRODUCTION

Lactic acid is a natural organic acid with a great variety of applications in pharmaceutical, chemical, food and health care industries[1,2]. It is also used in production of polypropylene oxide, biodegradable poly lactic acid (PLA), polypropylene glycol and acrylic fibers[3]. There has been a great interest in lactic acid production from biomass via fermentation[4,5]. Lactic acid has been reported from synthetic medium and beet molasses by free as well as immobilized bacterial cells using batch, fed-batch and continuous culture[6,7]. Monteagudo and his coworkers studied the effect of fermentation parameters (temperature, pH, inoculum and initial sugar concentration) on lactic acid production from beet molasses by *Lactobacillus delbrueckii* CECT 286. Submerged fermentation has proved several advantages in many ways, including easy scale up, high substrate utilization, less chances of contamination; easy down stream processing and most importantly less time requirement for metabolites production[8]. Lactic acid bacteria (LAB) are among the best studied microorganisms. The desirable characteristics of industrial microorganisms are their ability to rapidly and completely ferment cheap raw materials, requiring minimal amount of nitrogenous substances, providing high yields of preferred stereo specific lactic acid under conditions of low pH and high temperature, production of low amounts of cell mass and negligible amounts of other byproducts[9]. The choice of an organism primarily depends on the carbohydrate to be fermented[10].

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Over the years research has been carried out by exploiting a large number of carbohydrates and nitrogenous materials for production of lactic acid on the basis of high yields, optimum biomass production, negligible by product formation, fast fermentation rate, less pre-treatment, easy down stream processing, low cost, ease of availability etc. However the choice of the raw material depends on the microorganisms studied and also on the product of choice. Various starchy substrates[11], cellulosic substrates[12], woody materials[13] and alfalfa fibers[14] have been successfully exploited for lactic acid production.

Immobilized cell systems are now used industrially for production of different metabolites. There are several advantages of using immobilized cell system over to free cell system. First, a higher cell mass per unit fermentation volume can be achieved than with batch, continuous or cell recycle system, resulting in a corresponding increase in lactic acid production. Second, reduction in down stream processing costs as there is no need for cell removal making the product extraction is more economical[15]. Third, maintaining a specific growth rate and dilution rate in continuous free cell systems is not a factor in an immobilized system thus flow rates can be optimized for best system kinetics. Finally, the risk of contamination is reduced due to fast dilution rates and high cell densities[16].

The present study deals with the parametric optimization for maximum lactic acid production and its scale up by free and immobilized cells of *Lactobacillus amylovorous* B- 4542 under submerged fermentation by following the optimized parameters.

MATERIAL AND METHODS

Microorganism

The microorganism *Lactobacillus amylovorous* NRRL B- 4542 used in this study was obtained from the USDA Northern Regional Research Center, Peoria, Illinois. It was maintained on media containing (g/l) : FeS04 0.034, sodium acetate 1.0, MgS04.7H20 1.23, MnS04 0.034, KH2P04.3H20 0.65, KH2PO40.5, yeast extract 30, soluble starch 10g, Agar 15, pH 5.5. The culture was grown at 40 C for 24h, and was stored at 4 C for further use.

Inoculum Preparation

The inoculum was grown in 50 ml liquid medium in 250 ml conical flask containing 1% soluble starch by incubating at 40 C for 18h, to obtain the required cell concentration (O.D 660 nm, 1.7). The cells were centrifuged, washed, and used as inocula in fermentation experiments.

Study of fermentation parameters

Different carbon source

A series of fermentation experiments were performed in 500 ml Erlenmeyer flask to examine the effect of different carbon sources viz. corn flour, corn starch, cassava flour, potato starch, soluble starch, rice flour, wheat flour, barley flour, sorghum flour and commercial glucose were studied. Each substrate was taken in flask at a concentration (10-100 g/l) in the fermentation medium. This was autoclaved at 120^{o}C for 15 min. Each flask was inoculated with the prepared inoculum. These flasks were incubated for maximum 50h of incubation time under shake flask conditions at 40^{o}C.

Effect of pH, temperature, inoculum size, incubation time and agitation

The pH of fermentation medium was adjusted to be in the range of 2 to 9. To study the effect of incubation temperature on maximum lactic acid production, the flasks were incubated at various temperature ranges (20 to 60^{o}c). The effect of inoculum size was also studied by adding cell suspension/g of substrate ranging from 2 to 14% (w/v). To study the time course, fermentation experiments were performed at 40^{o}C for 50h with inoculating 10% v/v exponentially growing culture. The effect of agitation was also studied at different range from 50 to 350 rpm.

Effect of different buffering agents

Effect of different buffering agents viz. CaCO3, Ca(OH)2, NaOH, NaHCO3 and NH4OH were studied on lactic acid fermentation using the corn starch as carbon source.

Immobilization of cells

L. amylovorae NRRL B- 4542 cells were immobilized in calcium alginate using the method of Kierstan and Bucke (1977). Cells were harvested after 14 h of incubation in a culture medium containing 20 g/l of

soluble starch in medium. The cell suspension prepared in 0.85% saline was mixed with 2.5 % (w/v) sodium alginate solution. The beads were formed by pumping the mixture through a peristaltic pump at a flow rate of 50 ml/min and gently dropping the mixture into cold 0.2M CaCl2.2H2O solution under sterile conditions. the beads obtained were about 2mm in diameter as measured by a varnier caliper and contained approximately 2.0 10 6 cells / bead. The beads were stored in saline at 4C for further experiments.

Scale up of lactic acid production using free and immobilized cells of *L. amylovorous* NRRL B- 4542

Scale up of lactic acid production was carried out using corn starch (60 g/l) as carbon source using free and calcium alginate entrapped cells of *L. amylovorous* NRRL B- 4542 under submerged culture cultivation conditions using in situ sterilizable 15 L fermenter (NBS Co., Edison, NJ, USA) with a working volume of 12L. The fermentation was run at agitation 200 rpm, aeration 3.5 L/min, temperature 30^{o}C and pH 5.5.The liquid medium (11.5 L) was prepared according to Cheng et al. (1991). A 500 ml of inoculum was transferred to the fermented medium. Antifoam (Thomas Baker, Mumbai, India), 3.0 ml was added to the fermentation medium prior to sterilization. For cell recycling immobilized cells were aseptically removed by centrifugation after each 30h of fermentation, transferred to a fresh medium containing 60 g/l corn flour and incubated further. This procedure was repeated for 5 times after a regular time interval of 72h. Sterile syringes were used to take samples at regular time intervals during fermentation for estimation of lactic acid, biomass, residual starch and amylase enzyme in broth[17].

Analytical methods

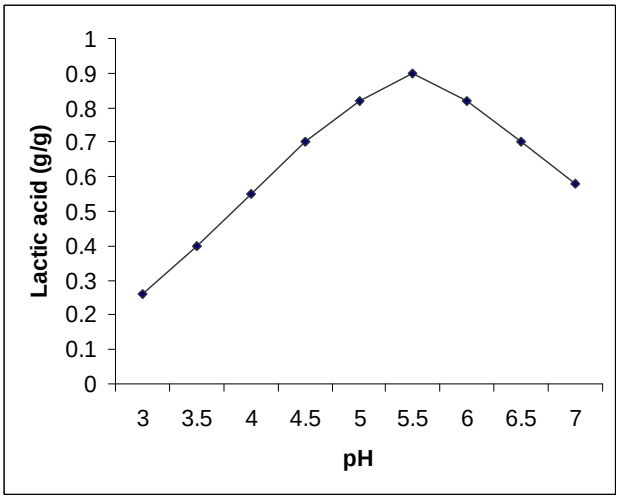
At specific intervals of time, as per the experimental design, the samples were recovered and the fermented broth was centrifuged at 11,200g for 15 min at 10^{o}C (Sigma, Osterodeam Harz, Germany) and the supernatant was analyzed for lactic acid according to the method of Kimberley and Taylor (1996). Amylase activity was estimated in reaction mixtures which consisted of 1ml of soluble starch in 0.1 M acetate buffer (pH 6.5) and appropriate concentration of enzyme (1 ml) and incubated at 37^{0} C for 30 minutes. The reaction was stopped by immediate cooling in an ice bath and the reducing sugars was measured by the DNSA method[18]

RESULTS AND DISCUSSION

Effect of initial pH

The effect of initial pH on lactic acid production by *L. amylovorous* NRRL B- 4542 was studied. The pH range was investigated between the ranges of 2-10 for the optimum lactic acid production. The pH was adjusted by either with 1N NaOH or 1N HCl. It is clear evident from the Fig. 1 that at pH 5.5 the lactic acid production was more favorable. Thereafter the production was showed declination. In general, low pH (less than 5.0) and high alkaline pH (more than 8.00) has adverse effect on growth of *Lactobacillius* sp. which eventually reflected in term of less metabolites production. A regular growth in biomass was observed, which in turn showed a concomitant increase in lactic acid yield.

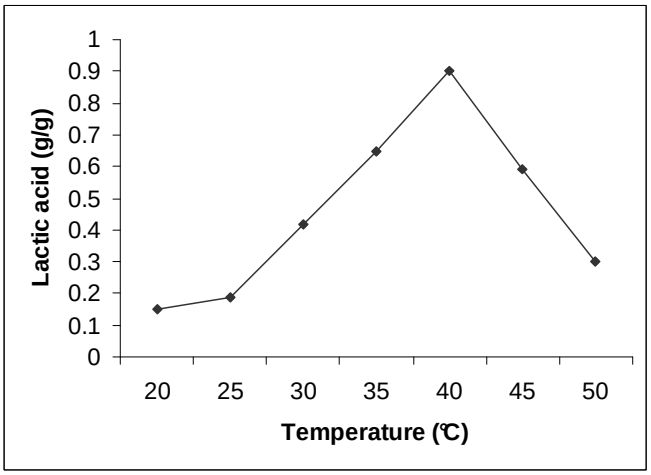
Fig. 1: Effect of pH on lactic acid production by *L. amylovorous* NRRL B- 4542



Effect of temperature

The effect of temperature on lactic acid production from *L. amylovorous* NRRL B- 4542 was investigated at the temperature range of 10-50 C. The results are summarized in Fig. 2. The optimum temperature for maximum lactic production was found at 40^{o}C. Thereafter lactic acid production was sharply decreased and finally at 50^{o}C a very negligible amount of lactic acid recorded. Generally mesophillic microorganisms exhibit decline in metabolites production was due to inactivation of cellular activities and at higher temperature the enzyme reactions in the cell are destroyed.

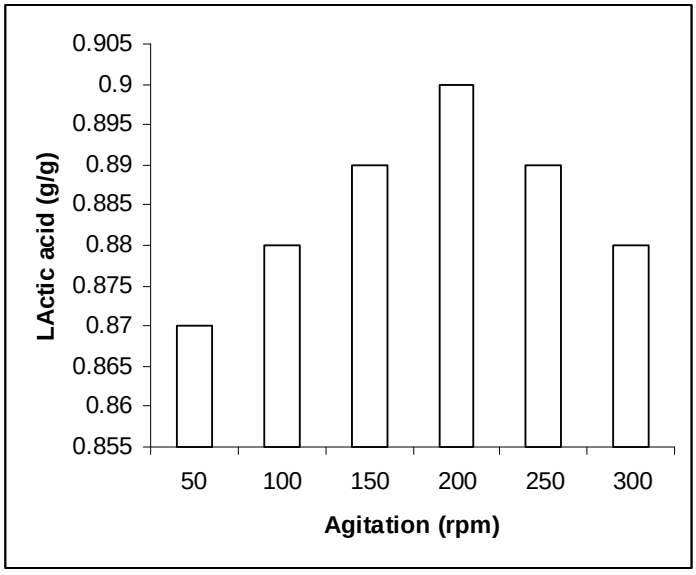
Fig. 2: Effect of temperature on lactic acid production by *L. amylovorous* NRRL B- 4542



Effect of agitation

Fig.3 shows the effect of agitation rates on lactic acid production, when *L. amylovorous* NRRL B- 4542 was grown under shake flask cultivation. The agitation rate kept between the ranges of 50-300 rpm. There was a regular increase in lactic acid production with the increase in agitation. The maximum lactic acid production was observed at the agitation rate of 200 rpm. Thereafter a fast declination in lactic acid production was found and almost 70% less lactic acid production yield was recorded when agitation was kept 300 rpm. The optimum level of agitation during submerged cultivation plays a crucial role for the production of desired metabolites. A high agitation leads to decrease substrate accessibility and inadequate oxygen supply to microorganism. Low agitation also causes poor accessibility of nutrients to microbial cultures resulting in poor culture.

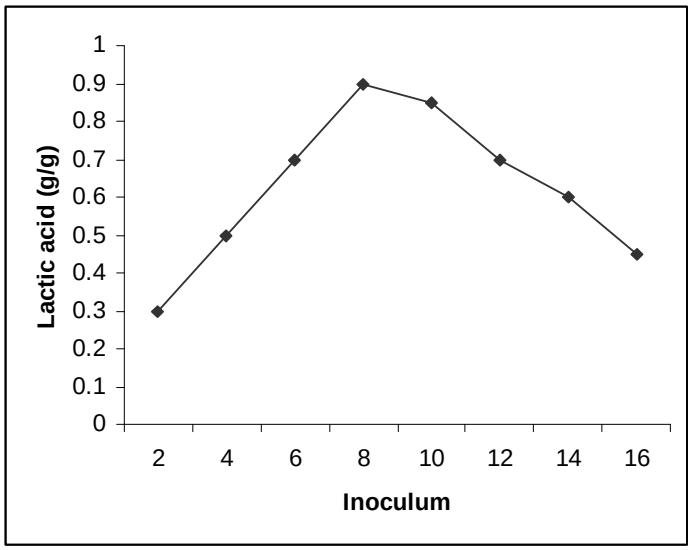
Fig. 3: Effect of agitation on lactic acid production by *L. amylovorous* NRRL B- 4542



Effect of inoculum size

The effect of inoculum size of *L. amylovorous* NRRL B- 4542 was studied to optimize the inoculum size for maximum lactic acid production under submerged fermentation. A different range of inoculum size (1-10%) was studied. A maximum of lactic acid production was noticed when inoculum size was 5% (Fig. 4). A linear increase in lactic acid production was found as inoculum size was increased from 1-5% thereafter a clear declination was noticed. In liquid fermentation, an appropriate size of inoculum is utmost important parameter for getting high product yield and productivities. At low inoculum size the substrate is utilized and prolongs incubation time. On the other hand high inoculum size will lead to competition of growth of microorganism over the limited substrate amount.

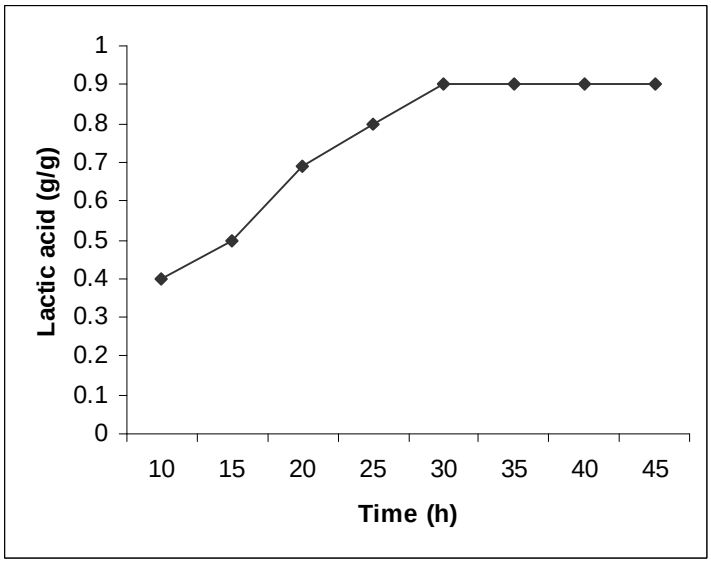
Fig. 4: Effect of inoculum size on lactic acid production by *L. amylovorous* NRRL B- 4542



Effect of incubation time

In lactic acid fermentation, an appropriate incubation period is important parameter. The results summarized in fig. 5 shows appreciable lactic acid production was increasing up to 30 h. However a continuous growth in biomass was up to 25 h and further showed no increase and remained almost same up to 48 h. There was no increase in lactic acid production was observed after 30h. It may be simply due to non availability of the substrate for the organism.

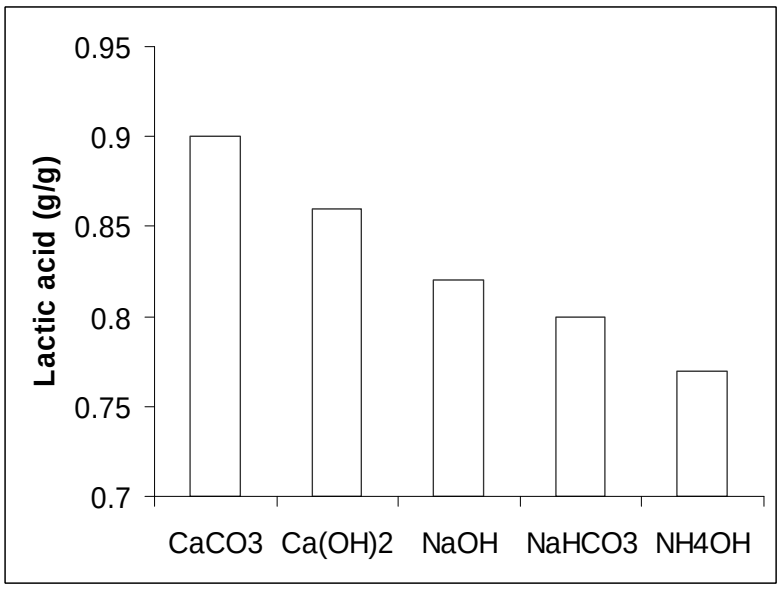
Fig. 5: Effect of incubation time on lactic acid production by *L. amylovorous* NRRL B- 4542



Effect of buffering agents

Effect of different buffering agents viz. $CaCO_3$, $Ca(OH)_2$, NaOH, $NaHCO_3$ and NH_4OH on lactic acid production was investigated. These buffering agents were added into the fermentation medium at the concentration of 4 %. The maximum lactic acid production was observed when calcium carbonate was used as a buffering agent followed by calcium hydroxide, sodium hydroxide, sodium bicarbonate and ammonium hydroxide respectively (Fig. 6). Among the different buffering agents tested calcium carbonate was found to be most efficient in controlling the fall in pH during the fermentation by *L. amylovorae* NRRL B- 4542. Earlier Kotzamanidis *et al.,* 2002 observed a maximum lactic acid yield using $CaCO_3$ at the concentration 5% (w/v) and remained constant between 5 to7 per cent and then decreased. The decrease in lactic acid yield at the concentration more than 7% (w/v) is due to the inhibition of enzyme activities which are responsible for the biosynthesis of lactic acid. A proper buffering of fermentation medium is required for the optimum production of desired metabolites. Microorganism needs a stable pH during the course of fermentation. The alteration in pH of medium normally affect on the production rate of lactic acid.

Fig. 6: Effect of different buffering agents (3.5% w/v) on lactic acid production by *L. amylovorous* NRRL B- 4542



Effect of different carbon sources

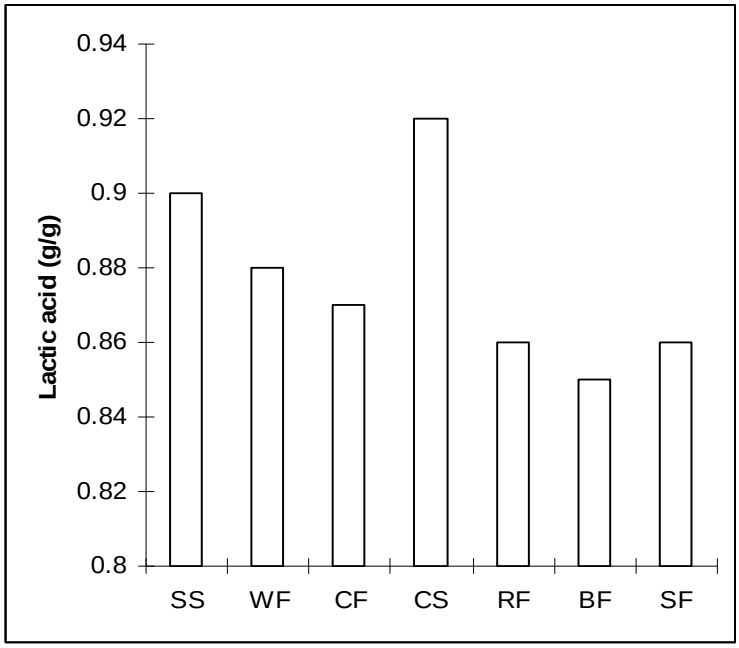
Different carbon sources viz. corn flour, corn starch, cassava flour, potato starch, rice flour, wheat flour, barley flour, sorghum flour, soluble starch and commercial glucose was studied for the maximum lactic acid production from *L. amylovorous* NRRL B- 4542 under submerged fermentation (Fig. 7). These substrates were tested under different concentration ranging from 10- 100 g/l at the optimized physiological parameters. Corn starch and soluble starch (60g/l substrate concentration) showed maximum lactic acid yield (0.94 g/g) after 30h of incubation. Upon increasing the substrate concentration from 10-60 g/l, lactic acid yield was found continuously increased thereafter production of lactic acid was found low and the incubation time was also prolonged. Cassava flour when used substrate in the concentration from 10-50 g/l showed, a regular decrease in lactic acid yield was found. A maximum lactic acid yield (0.87 g/g) was found at 50 g/l cassava flour concentration thereafter a clear cut declination in lactic acid yield was observed. More than 50% decrease in lactic acid yield was recorded at high substrate concentration (90 g/l) indicates that high substrate concentration did not enhanced more lactic acid production. Wheat flour (10-60 g/l) showed maximum production in lactic acid yield (0.88g/g). Further increase in wheat flour concentration did not improve the lactic acid yield. Rice flour gave maximum lactic acid yield (0.86 g/g) at the concentration (60 g/l of substrate).

Thereafter a clear declination in lactic acid yield was found. More than 50% decrease in production yield at high substrate concentration (80 g/l) shows that high substrate concentration did not positively influenced on lactic acid production.

Barley flour produced maximum lactic acid yield (0.85g/g) when used at the concentration (60 g/l) and showed declination thereafter while increasing substrate concentration. Sorghum flour showed maximum lactic acid yield (0.86 g/g) at the substrate concentration (20 and 40 g/kg) and decreased thereafter.

Soluble starch exhibited maximum lactic acid yield (0.94 g/g) when used at the concentration (60 g/l) thereafter it showed declination upon increase in substrate concentration and the incubation time. Potato starch produced a low lactic acid yield (0.87 g/g) at a substrate concentration (50 g/l) and showed a regular fall in lactic acid yield when used in the concentration in increase order. A maximum lactic acid yield (0.94g/g) was noticed when commercial glucose was supplemented as carbon source at the substrate concentration (50 g/l). A concomitant downfall in lactic acid yield was found as increase in substrate concentration. The less lactic acid yield at high substrate concentration may be due to the incomplete degradation of substrates. Exploitation of cheaper substrates is one of the key parameter to reduce the production cost of lactic acid. The results clearly show that starchy substrates are more favorable for more lactic acid yields rather than crude substrates. Our results are in close agreement with Hang, 1990 who reported same pattern of lactic acid production by using these complex carbohydrates by *Rhizopus oryzae*.

Fig. 7: Effect of different starchy substrates on lactic acid production by *L. amylovorous* NRRL B- 4542



SS(Soluble starch), WF (Wheat flour), CF (Cassava flour), CS (Corn starch), RF (Rice flour), BF (Barley Flour) and SF (Sorghum flour).

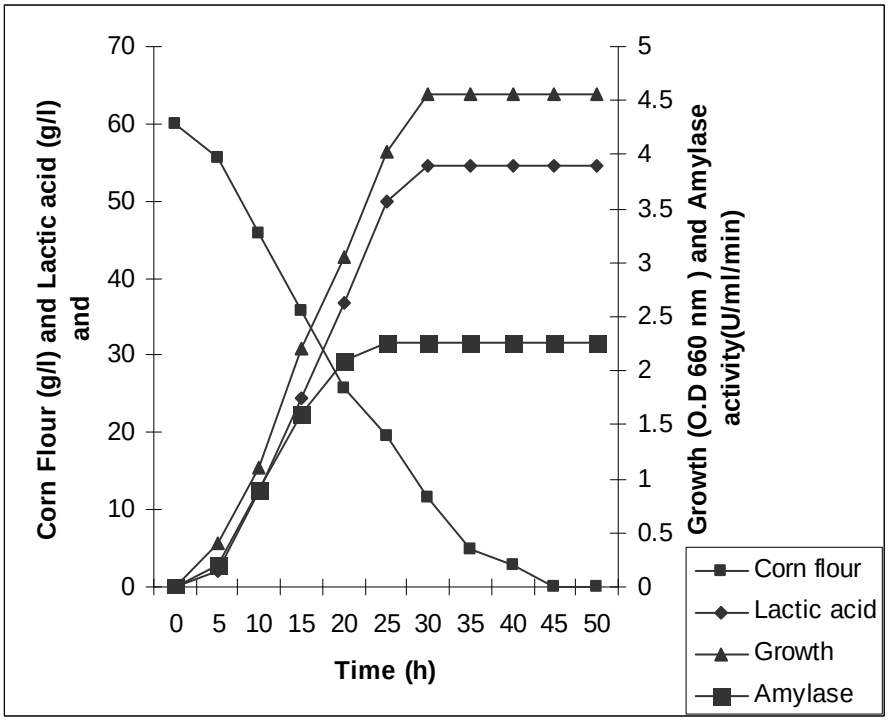
Scale up of lactic acid production from free and immobilized cells of *L. amylovorous* NRRL B- 4542

Free cells

Scale up studies for lactic acid production using free cells of *L. amylovorous* NRRL B- 4542 were conducted using the corn starch as carbon source at a concentration of 60 g/l and following the optimized physiological conditions. However during the optimization of different carbon source study the same lactic acid yield was observed when soluble starch was used. But on looking into cost economics, corn starch was preferred over to soluble starch. A maximum of lactic acid produced after h of incubation. The corn starch consumption rate, biomass production rate and lactic acid production rate is summarized in Fig. (8). Starch consumption rate was slow for initial 10h thereafter a fast consumption of starch was found up to 25h of incubation and furthermore a constant rate was recorded. Amylase activity was observed continuously increase as increasing of incubation time. A maximum of 45 U/ml/min amylase

activities was noticed after 100 h of incubation. A maximum lactic acid (38 g/l) was produced after 30 h of incubation and remained constant thereafter. Earlier Vishnu (2000) reported a maximum 0.96 g/g lactic acid yield utilizing 1% and 2% corn starch from *Lactobacillus ampylophilus* GV6 under submerged cultivation conditions.

Fig 8: Kinetics of batch fermentation for lactic acid production using corn starch (60 g/l) under submerged fermentation conditions [pH 5.5, Temp. 40°C, agitation 200 rpm, inoculum size 3.5% (v/v)] under the submerged culture conditions by *L. amylovorous* NRRL B- 4542 cells

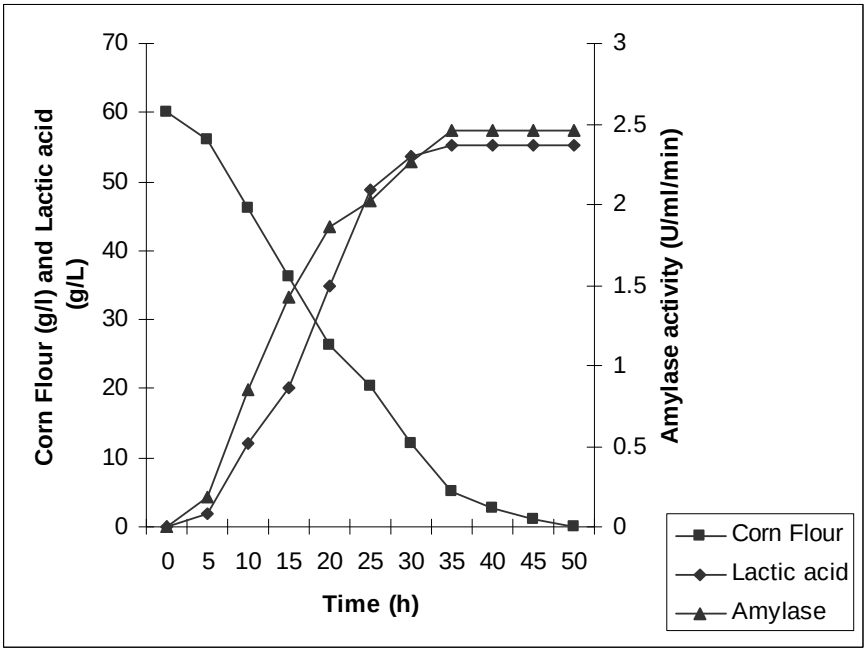


Immobilized cells

Fig. 9 shows the time course of lactic acid production by immobilized cells of *L. amylovorous* cells. It is evident with the results that the rate of soluble starch consumption was slightly lower than that of free cells, but higher lactic acid productivity was achieved. A maximum of lactic acid yield was achieved after 48h of fermentation at which % initial starch was consumed. When the microorganisms are attached to solid supports, fluid viscosity was lower which contributes to better mixing and mass transfer in the system. The formation of a dense membrane of very high alginate concentrations at the calcium alginate interface would slow the diffusion of sugars into the beads, particularly as more cells grow near the bead surface. This would cause more facilitation of starch to the centrally located cells to be more gradual allowing them to multiply normally and utilize large amount of starch. In an effort to increase the lactic acid productivity, while keeping the simplicity of batch process, cell recycling has been employed in many cases.

The immobilized cells were recycled into a fresh starch containing medium containing 60 g/l corn starch after every 30h of incubation. Although the cell recycling did not increase the efficiency of starch conversion into lactic acid, the time required for the fermentation to run to completion was reduced by as much as 60-70%. Wene and Antonopoulos (1988) found that complete recycling of *Fusarium oxysporum* cell mass has been shown to reduce fermentation time by 48h with equal ethanol yields. The immobilized cells of *L. amylovorous* could similarly be recycled four times to obtain an lactic acid yield of starch utilized (Fig. 9). The decrease in further lactic acid yield after 4 cell recycle may be because of oxygen and starch limitations as a result of increase in cell density.

Figure 9. Kinetics of batch fermentation using corn starch (60 g/l) under submerged fermentation conditions [pH 5.5, Temp. 40 C, agitation 200 rpm, inoculum size 3.5% (v/v)] under the submerged culture conditions by calcium alginate immobilized *L. amylovorous* NRRL B- 4542 cells. The culture medium containing 60 g/l corn starch was transferred after every 35 h of fermentation



CONCLUSION

In conclusion, the efficient fermentation production yields were obtained at optimum conditions like agitation, different carbon sources, temperature, pH, inoculum size and different buffering agents. The strain *L. amylovorous* NRRL B- 4542 was evaluated for its optimum fermentation conditions. The maximum lactic production was recorded at 40^{o}C, pH 8.5 in 48 h at rpm using corn starch (60 g/l) as carbon source at incubation time and with calcium carbonate as buffering agent. The scale up of lactic acid production was performed by free and calcium alginate entrapped cells using 15 L capacity. The maximum lactic acid production was observed 55g/l with the yield 0.96 g/g in 3rd cycle of fermentation with the immobilized cells. Efficient utilization of corn starch which reflected in terms of high lactic acid production by immobilized cells indicates the possibility of exploiting this strain for commercial scale.

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