Mannitol relieves substrate inhibition during glucose fermentation by L. delbrucekii due to shift in NADH/NAD+ ratio

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Citation

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Abstract

Co-fermentation kinetics of glucose and mannitol with Lactobacillus delbrueckii NCIM 2025 has been studied in MRS media in aim to enhance lactic acid production. It was found that 0.05mole mannitol increases both cell mass and lactic acid yield significantly. Cell yield increases up to 0.2 hrs^{-1} while total lactic acid concentration increases up to 22g/l. Mannitol in the medium also found to reduce the growth associated coefficient (□) from 8.3 to 6.3 and non-growth associated (maintenance energy) coefficient (□) from 0.3 to 0.07. Mannitol also reduces ethanol concentration up to 93g/l.

INTRODUCTION

Presently the world wide production of lactic acid is more than 100,000 metric tones per year and its demand is increasing rapidly because of its use in making bioplastic. Lactic acid is produced by variety of microorganisms (Litchfield et al. 1996) but particularly lactobacillus strains, have been exploited commercially due to high acid tolerance and for production of selective D-(-) or L-(+) optical isomers.

The major problems associated with lactic acid production are, substrate inhibition, end product inhibition and byproduct formation. There are different strategies to check the end product inhibition for example neutralization of lactic acid with suitable alkali, but there are fewer attempts to account for the substrate inhibition. In addition to this genetic alteration in pdc (pyruvate decorboxylase) gene of Saccharomyces cerevisiae (Porro et al.1999) and mutant R. oryzae (R1021) have been attempted previously to increase lactate formation by decreasing the byproduct ethanol (Skory et al. 2004). End product inhibition occurs due to rise in pH of extracellular medium during lactic acid production (Siegumfeldt et al. 2000) that results in disturbance of $NADH/NAD^+$ ratio. It has been evidenced that conversion of pyruvate to lactic acid requires high cytosolic NADH and a high $NADH/NAD^+$ ratio increases ldh activity (Garrigues et al. 1997).

Mannitol is well known osmolyte and acts as a protector of Lactococcus lactis cells (Efiuvwevwere et al. 1999). In spite of its physiological and biotechnological interest, mannitol metabolism has not been investigated in lactic acid bacteria, except in Lactobacillus acidophilus and Lactococcus lactis that utilizes mannitol as primary energy source for growth (Liong and Shah, 2005;Neves et al. 2000) and increases $NADH/NAD^+$ ratio (Neijessel et al. 1997). Therefore role of mannitol over lactic acid production were studied. As par from our knowledge this is the first report about reliving of substrate inhibition by using mannitol.

MATERIALS AND METHODS

MICROORGANISM AND CULTURE MEDIUM

The stock culture of bacterial strain Lactobacillus delbrueckii NCIM 2025 was obtained from NCL Pune, India. Stock culture was stored in MRS medium as 15% glycerol stock at -80°C (as directed). L. delbrueckii NCIM 2025 was grown on 2% w/v agar plates at 40°C for 24hrs, and then kept at 4°C. Preculture was prepared in the 100ml MRS culture medium Five ml of this culture was used for further inoculation in the 250ml screw capped shake flask containing 100ml MRS production medium with buffer at pH 7.0

BATCH FERMENTATION

Batch fermentation was carried out in 500ml flask with Lactobacillus delbrueckii NCIM 2025 in MRS medium maintaining fix concentration of glucose (10% w/v) with

different concentration of mannitol (0.05, 0.1, 0.5, and 0.3mole) or different concentration of glucose (9%, 10%, 12% w/v) with fix concentration of mannitol 0.05 mole. Batch fermentation was also carried out with different concentration of NAD^+ (2, 4, 6, 8 and 10mM) with fix concentration of glucose (10% w/v).

BATCH FERMENTATION PARAMETERS

In batch fermentation different kinetic parameter were taken into account like cell mass, lactic acid, specific growth rate and specific production rate. Cell mass was estimated after separating the cell by centrifuging the sample at 10,000X for five minutes and the cell obtained as pellet were washed with distilled water. The cells were dried weighted and finally diluted with distilled water in different range of concentration and the slope obtained was used for cell mass calculation at 660nm. Cell yield was calculated as g. cells/g. glucose.

Maximum specific growth rate was determined by Monod kinetic parameters as a function of initial substrate concentration (S) and cell density (X). The specific growth rate (μ) was extrapolated from linear regression analysis of the growth curve. For product, the growth associated coefficient (□) and the non growth associated coefficient (□) (also termed as maintenance energy) was calculated using equation of Luedeking and Piret (1958) .The plot was drawn between specific production rate ((1/x) / (dP / dT)) and specific growth rate () ((1/X) / (dX/dt)) (and from slope and intercept of the graph □ and □ parameters were determined.

LACTIC ACID ANALYSIS

Total lactic acid content was determined by colorimetric method of Barker Summerson, (1941) by UV-VIS spectrophotometer (ELICO SL164 double beam) from the supernatant. The lactic acid yield was calculated as g.LA /g.glucose consumed.

SUGAR ANALYSIS

The reducing sugar was determined by method of Miller et al. 1959. This method is specifically used for reducing sugar in the mixed substrate also.

ETHANOL DETECTION

Ethanol was detected by Gas Chromatography (AIMIL, Nucon India, series5765) equipped with chromosorb 101 column using nitrogen as the carrier gas along with a mixture of hydrogen and oxygen gases to sustain flame. Temperature of detector, injector and oven were maintained at 200°C, 195°C and 180°C respectively.

RESULTS

EFFECT OF GLUCOSE FERMENTATION ON CELL MASS, LACTIC ACID AND ETHANOL

Result of glucose fermentation by L. delbrueckii NCIM 2025 has been summarized in Table-1. Bacterium produces lactic acid (82g/l) and ethanol (101g/l) from 10% w/v glucose. The cell mass observed at 660 nm was 9g/l in 48h at pH 7.0. Similar finding were reported earlier from glucose fermentation by Lactobacillus intermedius B-3693 that also produced lactic acid and ethanol. Bacterium was reported to utilizes all the glucose provided (150g/l) and lactic acid and ethanol reported after fermentation was 70.4±0.6g/l , 36.2±1.0g/l respectively in 40h while maximum cell growth reported at 24h was A_{660} of 7.3±0.1. At 200g/l glucose, the bacterium reported to utilizes only 80% substrate and produces 70.4±2.1 g lactic acid and 38.0 ± 0.9 g ethanol in 64h with a lag in growth and maximum cell growth reported was A_{660} of 8.3±0.3 at 48h (Saha et al. 2003). The data clearly shows that glucose was not utilized in the metabolism completely and there was ethanol byproduct along with lactic acid that might be responsible for decrease in yield of lactic acid.

EFFECT OF MANNITOL AND GLUCOSE CO-FERMENTATION

Lactic acid production profile with fix concentration of glucose (10%w/v) having different concentration of mannitol (0.05, 0.1, 0.3 and 0.5mole) has been analized. It can be observed from the result that 0.05mole mannitol has a significant role in increasing lactic acid concentration, while higher mannitol concentration (0.1, 0.3 and 0.5mole) is insignificant in lactic acid production. Mannitol (~0.55mole) exclusively produces low amount of lactic acid 40g/l. Consequently, it was observed that on increasing glucose concentration (9%, 10% and 12 % w/v) with fix concentration of (0.055mol) mannitol increases both lactic acid concentration (96, 104 and 124g/l respectively) and cell yield (0.166, 0.180, 0.183 g.cell/g.glucose respectively. It was observed that low concentration of mannitol was utilized fully while mannitol above 0.05mole concentration, remains in the medium (data not shown) and is insignificant for lactic acid production. Therefore in further experiment only 0.5mole mannitol was used.

Result of cell yield estimated with glucose alone (10%w/v) was 0.09 g.cell/g.glucose, while keeping mannitol

concentration constant (0.05mole) along with different concentration of glucose (9%, 10%, 12% w/v. Result shows a clear shift in lag phase of cell after addition of mannitol. It can be evident from the result that with increase of glucose concentration from 9% to 12%w/v in presence of mannitol, results in increase in yeild 0.093 g.cell/g.glucose. It appears that enhancement of cell mass and decrease in ethanol after mannitol addition is responsible for increase in lactic acid as is evident from GC chromatogram. The data also support the fact that mannitol is not only utilized in cell mass formation but also play a major role in shift of metabolic flux that is diverting the pyruvate towards lactate formation.

EFFECT OF MANNITOL AND NAD CO-FERMENTATION ON CELL MASS AND LACTIC ACID

To determine relation between mannitol and NAD^+ production, different concentration of NAD^+ (2, 4, 6, 8, and 10mM) along with constant amount of glucose (10% w/v) has been studied. it was observed that with increase in NAD^+ concentration there was significant increase in lactic acid up to 4mM (from 2 and 4 mM NAD^+ LA was 68 and 90g/l) but thereafter a decrease in lactic acid was observed with higher concentration of NAD^+ (6, 8, and 10 mM NAD^+ LA was 78, 70 and 65g/l. Therefore from data it can be deducted that 4mM NAD^+ could significantly increase the lactic acid while higher concentration is inhibitory for lactic acid (may be diverting it to other pathway) and hence this concentration was used in further experiments.

Result of lactic acid production with NAD^+ (4mM) or mannitol (0.05) and mixture of NAD^+ (4mM) and mannitol (0.05) along with constant amount of glucose (10% w/v) has beenstudied. It is evident from the data that mixture of mannitol and NAD^+ is insignificant (LA,88g/l) in compared to NAD^+ (92g/l) or mannitol (104g/l) alone. This may be explained as over production of NAD^+ from mannitol catabolism might be responsible for decreases in lactic acid concentration, and the fact is supported by similar result where higher molar concentration of NAD^+ addition results in decrease of lactic acid. Molar yield of lactic acid calculated was 0.9mol, with 0.5mol glucose without mannitol or, 1.8mol of lactate from 1mol of glucose. Thus strain used was homofermentative which agrees with earlier reports (John et al. 2007; Postma et al 2003).

Cell yield was estimated after addition of NAD^+ (4mM) or mannitol (0.05mole) with constant amount of glucose (10% w/v). it was observed that mannitol addition results in more increase in cell yield in compared to NAD^+ and cell yield observed with NAD^+ or mannitol was 0.166, 0.18 g.cell/g.glucose respectively. Mixture of mannitol (0.055mole) and NAD^+ (4mM) results in more decrease in cell yield (0.15g cell/g.glucose) in compare to when they added separately.

It appears that mannitol catabolism produces NAD^+ and thus excess of mannitol is responsible for decrease in total lactic acid. This could also be evident from data shown where excess NAD^+ concentration results in decrease in lactic acid. It was reported earlier that mannitol catabolism produces $NADH^+$ in excess and thus shift in the ratio $NADH^+$ /NAD occurs which could activate the ldh and thus all the pyruvate may be directed for lactate production [9]. It means that if $NADH^+$ is actually produced by the mannitol catabolism then alternative electron acceptor ethanol must either be low in concentration or must be nil from the broth sample. Therefore for potential byproduct measurement such as ethanol, gas chromatography was employed as shown in fig.4 GC chromatogram. Ethanol observed with glucose (10% w/v) or with mixture of glucose (10% w/v) and mannitol (0.055mole) was 101g/l and 8g/l respectively, while ethanol was nil with mannitol 10% w/v exclusively.

Thus data exhibit that mannitol fermentation produces low amount of pure lactic acid with none of ethanol, while mannitol 0.05mole along with glucose 10% w/v reduces ethanol up to 93g/l that led to increases in lactic acid up to 22g/l. Further phase contrast microscopy of lactobacillus cell, in glucose and in glucose with mannitol show comparatively difference in cell size with mannitol treated cell. It might be due to mannitol that acts as metabolite and osmolyte as well as depicted in fig.5. Thus we can deduct from the data that mannitol is a poor substrate but could be exploited to reduce ethanol byproduct during glucose fermentation if used in low amount (0.055mole) and thus helps in increasing overall lactic acid. However this strategy has to be examined with other type of sugar at higher concentration.

EFFECT OF MANNITOL ON ETHANOL CONCENTRATION

Neijessel, 1997 reported that mannitol catabolism produces $NADH^+$ in excess [9] and thus shift in the ratio of NADH/NAD^+ occurs that could activate the ldh. It means that if $NADH^+$ is actually produced by the mannitol catabolism then alternative electron acceptor ethanol must either be low in concentration or must be nil from the broth

sample. Therefore for potential byproduct measurement such as ethanol, gas chromatography was employed. It was observed that glucose 10% w/v, with 0.05mole mannitol ethanol was only 8g/l against glucose that produces ethanol 101 g/l, while ethanol concentration was almost nil with mannitol 10% w/v exclusively.

Thus data exhibit that mannitol fermentation produces low amount of pure lactic acid with none of ethanol, while mannitol 0.05mole along with glucose 10% w/v reduces ethanol up to 93g/l that led to increases in lactic acid up to 22g/l. Thus we can deduct from the data that mannitol is a poor substrate but could be exploited to reduce ethanol byproduct during glucose fermentation if used in low amount (0.05mole) and thus helps in increasing overall lactic acid. However this strategy has to be examined with other type of sugar at higher concentration.

EFFECT OF MANNITOL AND NAD CO FERMENTATION ON SPECIFIC GROWTH RATE ()

Effect of mannitol on specific growth rate (μ_m) of L. delbrueckii has been studied. Maximum specific growth rate (μ_m) in glucose (10% w/v) was 0.21h^{-1}, while with glucose (9% w/v) and mannitol 0.055mole, the specific growth rate was 0.329 h^{-1}. Further at glucose concentration of 10% and 12%, specific growth rate was 0.377 and 0.428h^{-1} respectively. Consequently an increase in specific growth rate (μ_m) 0.167 h^{-1} was noticed after addition of mannitol 0.055 mole with 10% w/v glucose.

Specific growth rate with mannitol and NAD^+ has been studied. It can be observed that specific growth rate with mannitol and NAD^+ together was somewhat lower (0.317h^{-1}) than the specific growth rate with NAD^+ exclusively (0.379h^{-1}), but was higher in comparison to glucose (0.21h^{-1}). Therefore an increase of 0.167h^{-1} was noticed in compare to glucose alone. This implies the relation between the mannitol and NAD^+ production. Both NAD^+ and mannitol individually have advantageous effect upon lactic acid yield and cell mass at fixed concentration but has negative effect over ethanol byproduct.

EFFECT OF MANNITOL ON GROWTH ASSOCIATED AND NON-GROWTH ASSOCIATED COEFFICIENT

Luedeking and Piret (1958) defined lactic acid production as a function of the energy, necessary to form new bacterial protoplasm and the energy for the normal metabolic activity irrespective of growth. In their report, with Lactobacillus delbrueckii at pH ranging from 6.0 to 4.5, the growth-associated coefficient changes from 24.4mM lactic acid per OD unit at pH 6.0 to 39.4 mM/OD unit at pH 4.5 and the values of the non-growth-associated coefficient changes from 6.1mM increased to about 667mM when the substrate concentration was zero.

In our experiment the growth associated coefficient () in glucose was 8.28 and the non growth associated coefficient () was 0.3. For glucose 9%, 10%, 12% (w/v) with mannitol 1% (w/v), () calculated was, 7.0, 6.5, and 6.4 respectively, while () was 0.12, 0.07, and 0.07 respectively. In same way for Glucose 10% w/v and NAD^+ 4mM, with Mannitol 0.055mol and without mannitol () was 7.3 and 6.7 respectively and () was 0.07 and 0.06 respectively.

It can be observed from the result that maintenance energy requirement was higher for glucose in absence of mannitol but along with mannitol or NAD^+ this coefficient decreases drastically. It appears that increase of pH due to lactate and associated byproduct is responsible for increasing both the coefficient together in glucose containing MRS media, while mannitol acts as metabolite in small amount and provides necessary cofactor NADH for the conversion of pyruvate into lactic acid and thus decreases maintenance energy requirement. This fact evokes strongly the role of mannitol in relieving from substrate inhibition, yet its role in relieving from substrate inhibition with other carbon sources (sucrose and cane molasses) at industrial scale has yet to verify.

DISCUSSION

EFFECT OF MANNITOL ON LACTIC ACID, AND ETHANOL

For L. delbrueckii substrate inhibition is of major importance (Neves et al. 2002b.). Substrate inhibition kinetics was studied in lactic acid in the range of 50-340g/l and reported that lactic acid concentration increases with increasing initial glucose concentration up to 200g/l, and a max of 140g/l lactic acid was produced and 185g/l lactic acid was produced from 200g/l initial glucose concentration, resulting in 70% and 90% yield. Similar inhibition was found in our case with L. delbrueckii. In our case lactic acid yield was 82% w/v with 10%w/v glucose, while yield get decreases on increasing glucose concentration further (data not shown). After addition of mannitol 0.055mole, lactic acid yield was almost 100% (104g/l with 10% glucose, and 124g/l with 12% glucose). Thus we deduce from the result

that, decrease in lactic acid concentration was because of substrate inhibition (glucose not fully utilized) but after using mannitol 0.055mole glucose was completely utilized at higher glucose concentration also. We also noticed that transport and catabolism of glucose increases after addition of 0.055mole mannitol and most of the glucose was metabolized in the fermentation medium.

POSSIBLE ROUTE OF MANNITOL IN NAD PRODUCTION

Some bacteria like L. planterum (Postma et al. 1993) and Lactobacillus acidophilus (Liong and Shah 2005) employ mannitol as primary energy source for growth. It is well established that glucose, mannitol, sorbitol all are transported and phosphorylated via phosphoenolpyruvate PEP via sugar PTS mediated mechanism. Therefore mannitol metabolism can be hypothesized to follow following route. Mannitol might enters the cell in form of mannitol-1-phosphate which should subsequently converted to fructose 6-phosphate (F-6-P) by the activity of Mtl1P dehydrogenase (Mtl1PDH). Mtl1P dehydrogenase reported to be a key enzyme for mannitol utilization, and is reported to show enhanced activity by at least 34-fold activities in mannitol as compare to glucose-grown cells (Neijessel et al. 1997; Neves et al. 2002b.). Formation of an additional NADH molecule (during conversion of Mtl-1-P into f-6-p) might be responsible for more conversion into lactate due to more availability of pyruvate and less ethanol since electron acceptor is no more limiting (Neves et al. 2002b)

Therefore the potential mechanism can be represented by the following reactions.

1 glucose + 2 NAD + ADP + Pi 2 pyruvate + 2 ATP + 2H + 2 NADH

1 mannitol 1 mannitol 1 P + 3 NAD^+ 1 Fructose 6-P + 3 NADH

1 Pyruvate + 2 NADH 1 Lactate + 2 NAD^+

From the result obtained it can be concluded finally that only a fixed concentration of mannitol is involved in enhancement of cell growth and lactic acid. Mannitol is also helpful in rapid transport of glucose and thus relieves substrate inhibition. This fact was also supported by the decrease in values of growth associated and non growth associated parameters. Mannitol as a catabolite is utilized for cell growth and also minimizes the ethanol byproduct during lactate formation by regenerating additional molecule of NADH.

Thus the insight into mannitol and glucose co-fermentation gained from the present work discloses potential impact of mannitol in repressing substrate inhibition and ethanol leads to increase in cell mass and lactic acid and thus can be aimed toward scale up of lactic acid production at industrial scale by taking advantage of metabolism itself without undergoing major genetic change that are very costly and decreases lactic acid yield.

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