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Screening of Inexpensive Nitrogen Sources for Production of L(+) Lactic Acid from Starch by Amylolytic *Lactobacillus amylophilus* GV6 in Single Step Fermentation

*Mohammad Altaf, Basa Janakiram Naveena and Gopal Reddy**

Department of Microbiology, Osmania University, Hyderabad-500007, A.P., India

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Summary

L. amylophilus GV6 was studied for production of L(+) lactic acid in single step fermentation using starchy substrates. Seven types of inexpensive organic nitrogen supplements (flour of pigeon pea, red lentil gram, black gram, bengal gram, green gram, soya bean and baker's yeast) were evaluated for their potential to replace more expensive commercial nitrogen sources, peptone and yeast extract. Red lentil and baker's yeast cells were found to be the best alternative nutrient sources of peptone and yeast extract for lactic acid production. L(+) lactic acid yield was about 92 % m(lactic acid)/m(starch) utilized in this study.

Key words: nitrogen nutrition, pulse flour, baker's yeast, L(+) lactic acid, fermentation, *Lactobacillus amylophilus* GV6

Introduction

Lactic acid is one of the most important organic acids, discovered by Swedish scientist C. W. Scheele in 1780 from sour milk (*1*). It is a product of natural fermentation occurring in buttermilk, cheese, beer, sourdough, *etc*. Lactic acid exists in two optically active stereoisomers, the L(+) and the D(–). Racemic mixture (DL) results from chemical synthesis or through some lactic acid bacteria possessing both D and L lactate dehydrogenases. Different stereoisomeric forms of lactic acid are produced by microbial fermentation using a specific microbial strain. L(+) isomer is used by human metabolism due to the presence of L-lactate dehydrogenase and is preferred in food. More than 85 % of lactic acid produced is used as acidulant, flavour enhancer, pH regulator, as a source for mineral fortification and as buffering agent having antimicrobial effect useful to increase the shelf life of processed foods (*2,3*). Leo Hepner of L. Hepner and Associates, a UK based management consultancy for food ingredients and biotechnology industries, rates worldwide consumption of lactic acid at 130 000 to 150 000 tonnes per year (*4*). By the end of year 2011, global demand for lactic acid is expected to shoot up to 200 000 t and domestic demand for lactic acid in India is expected to touch 2000 t from the present demand of 560 t (*5*). New applications of L(+) lactic acid, such as in biodegradable polylactate (PLA) synthesis or as an intermediate in synthesis of high-volume oxygenated chemicals, have the potential to greatly expand the market for lactic acid.

The widely used choice substrates for lactic acid fermentation are refined sugars, which are expensive. Lactic acid can also be produced from abundantly available cheaper substrates like starch in a two-step process of saccharification followed by *Lactobacillus* fermentation (*6,7*). Direct conversion of starch to lactic acid by bacteria which show amylolytic activity and have the ability to produce lactic acid will eliminate the two-step process and make it more economical. In this direction we have reported a single-step fermentation of starch to L(+) lactic acid by an amylolytic *Lactobacillus amylophilus* GV6

*Corresponding author; E-mail: gopalred@hotmail.com, altaf_nizams@yahoo.co.in

with high efficiency (*8–14*). Studies of this organism were significant because of the use of various low cost starchy substrates for lactic acid fermentation. Particular attention has to be made also towards the nitrogen sources, as high supplementation of expensive peptide nitrogen and growth factors (B vitamins) have great effect on lactic acid production. In an economic analysis for lactic acid fermentation, the largest contributor was found to be yeast extract, accounting for about 38 % of medium cost (*15*). There is a need for investigation of the use of cheap renewable nitrogenous materials like industrial by-products and agricultural wastes/by-products. The lysate of yeast cells is a valuable source of various substances used as ingredients in fermentation media (*16*). Yeast extract rich in vitamins B is known to enhance lactic acid production (*17*) and is relatively expensive material for large-scale fermentations (*18*). The success in our earlier studies with cheap carbon substrates (*8–14*) lead us to investigate the possibility of replacing nitrogen sources, peptone and yeast extract, with cheaper agricultural products and baker's yeast. In this paper we report the use of various types of pulse flour and baker's yeast cells as alternatives to peptone and yeast extract respectively, for single step fermentation of starch to lactic acid by *L. amylophilus* GV6.

Materials and Methods

Organism and growth media

Lactobacillus amylophilus GV6, an amylolytic lactic acid producing organism, isolated from cornstarch processing industrial wastes using MRS medium, was used in the present study. The organism was maintained in prereduced modified MRS medium in a refrigerator and subcultured every 30 days (*11–14*).

Nitrogen sources

Flour from pigeon pea, red gram, black gram, bengal gram, green gram and whole yeast cells (baker's yeast) was tested as substitutes for peptone and yeast extract. Different types of flour were obtained from local milling industries and total starch content in flour was determined by standard acid hydrolysis methods (*19*).

Experimental methods

A volume of 1 mL of stock culture was inoculated into 20 mL of modified MRS medium (pH=6.5) containing 1 % soluble starch in 120-mL serum vials and incubated at 37 °C. The strain was activated by a few subcultures and active culture grown for 24 h with 10^9 colony forming units (CFU)/mL was used as inoculum. The modified MRS medium had the following concentration of each nutrient (g/L): peptone 10, yeast extract 5, soluble starch 10, sodium acetate 5, triammonium citrate 2, $Na_2HPO_4·2H_2O$ 2, $MgSO_4·7H_2O$ 0.1, $MnSO_4·4H_2O$ 0.05, Tween 80 1 mL, resazurin 0.002. The serum vials were sealed under nitrogen atmosphere with headspace gas to maintain anaerobic conditions. Experiments were carried out for lactic acid production by substituting the peptone and yeast extract in MRS medium individually with various concentrations of six different types of flour mentioned above and baker's yeast cells ranging from 0.25–2 %. Lactic acid production from different types of flour dispensed in distilled water and in MRS medium devoid of YE and peptone was studied. Lactic acid production from different types of flour in MRS medium lacking soluble starch was studied for comparative observations. The results presented are mean values of each experiment in triplicates conducted three times on different occasions.

Analytical method

Fermented broth was cold centrifuged (8000 rpm for 20 min) and supernatant was taken for estimating lactic acid by the method of Kimberley and Taylor (*20*).

Results and Discussion

Lactic acid production by L. amylophilus GV6 without supplementation of nutrients

Various types of flour (pigeon pea, red gram, black gram, bengal gram, green gram) were found to contain starch in the range of 31–66 % (Table 1). Out of these types of flour tested, bengal gram and red lentil gram flour contained high starch content. Lactic acid production was higher with green gram flour at 1 and 2 % fraction compared to other types of flour (Fig. 1). This may be due to unavailability of certain minerals in the flour to the extent required by the organism as the flour was dispensed only in distilled water devoid of nutrients. This explains the requirement for vitamins and certain specific amino acids for organism's growth, which may not be present in the flour.

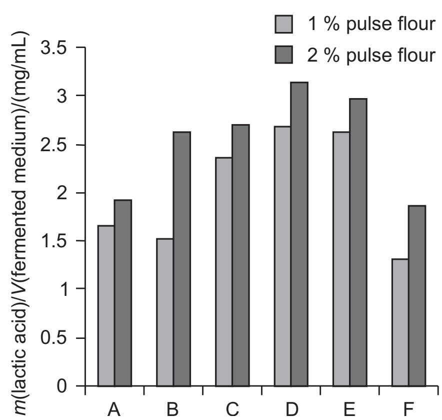

Fig. 1. Lactic acid production by *Lactobacillus amylophilus* GV6 from different types of flour from various kinds of pulse in distilled water without the addition of other nutrients
A – bengal gram, B – red lentil, C – pigeon pea, D – green gram, E – black gram, F – soya bean

Lactic acid production in MRS medium in presence of different types of flour from various kinds of pulse but without soluble starch

Increase in lactic acid production was observed with increase of the fraction of all tested types of flour from 1 to 2 % when supplemented in MRS media (Fig. 2). Lactic acid produced is from the starch present in different types of flour from various kinds of pulse. High lactic

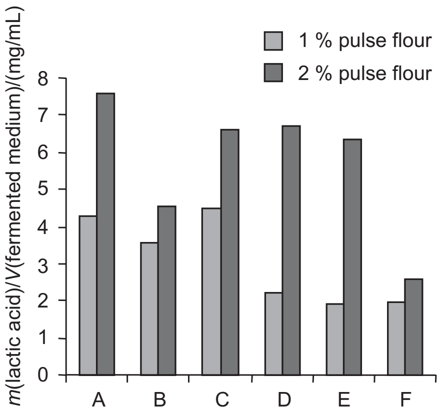

Fig. 2. Lactic acid production by *Lactobacillus amylophilus* GV6 from different types of flour from various kinds of pulse in MRS medium without soluble starch
A – bengal gram, B – red lentil, C – pigeon pea, D – green gram, E – black gram, F – soya bean

acid production in bengal gram compared to other types of flour (Fig. 2) may be due to the presence of more carbohydrates in it (Table 1) (*21*). However, lactic acid production was low in red lentil flour although it has high starch content. This may be due to the inhibitory action of high total protein content in MRS medium with peptone and yeast extract in addition to the protein of red lentil flour. On the other hand, lactic acid production was higher with green gram and black gram than red lentil. This may be due to inadequate amino acids or their lower amount and vitamins in green gram and black gram whose loss is filled by peptone and yeast extract (*17,22*). Similarly, lactic acid production was observed to be the lowest in soya bean flour, containing 43 % of protein with less starch content (Table 1 and Fig. 2). These results suggest that the nutrient supplementation enhances lactic acid production compared to the results obtained without supplementation of various media components (Figs. 1 and 2).

Table 1. Starch and protein fractions of different types of flour from various kinds of pulse

Type of flour	*w*(starch)/%	*w*(protein)*/%
Pigeon pea	48.4	22.0
Red lentil	66.4	25.1
Black gram	48.0	24.0
Bengal gram	66.0	20.8
Green gram	46.5	24.5
Soya bean	31.4	43.2

*according to M.K. Ismail (*21*)

Lactic acid production in MRS medium devoid of peptone and/or yeast extract in presence of different types of flour from various kinds of pulse

Red lentil flour was observed to give high lactic acid in absence of peptone and yeast extract in MRS medium compared to other types of flour from various kinds of pulse (Fig. 3). Red lentil flour contains 25 % of protein (Table 1) (*21*), which supplements the required nitrogen in fermentation medium. Lactic acid production was studied by replacing only yeast extract with these types of flour in MRS medium to verify their effect. Red lentil flour (1 %) showed more lactic acid production followed by bengal gram flour compared to other types of flour (Fig. 4). Lower production of lactic acid with other types of flour may be due to lower or higher nitrogen and carbon contents than optimum required for the growth of the organism. The organism produced a large amount of lactic acid in the presence of yeast extract and red lentil flour, which indicates that yeast extract could effectively replace peptone in MRS medium (Figs. 4 and 5). Effect of nitrogen concentration for the production of lactic acid by *L. casei* has been reported by Hujanen and Linko (*22*). The present study explains that red lentil is the best among all types of flour tested for the replacement of peptone. The folic acid, pantothenic acid and pyridoxine are essential vita-

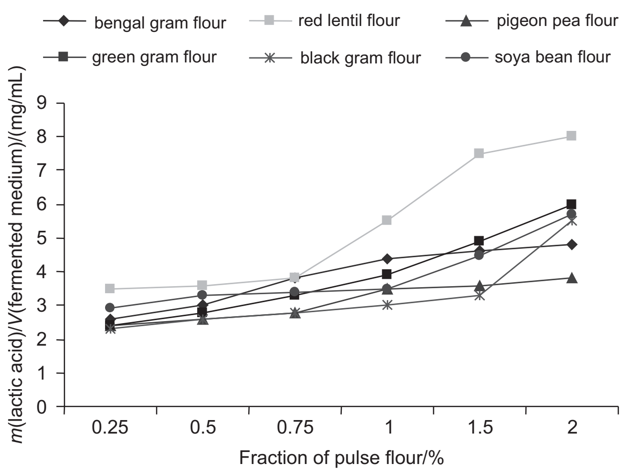

Fig. 3. Lactic acid production by *Lactobacillus amylophilus* GV6 from different kinds of flour from various kinds of pulse in MRS medium containing 1 % soluble starch and devoid of peptone and yeast extract

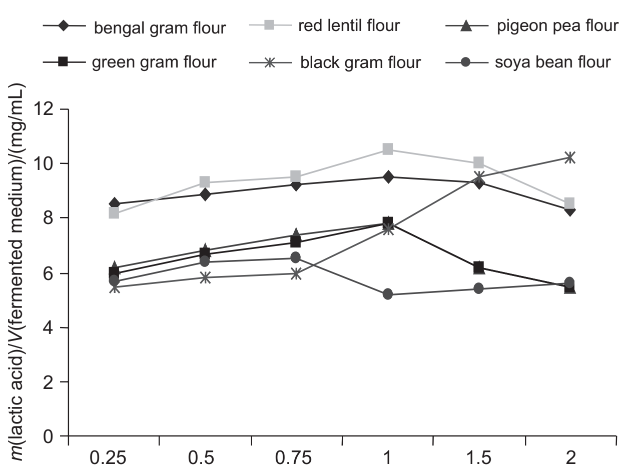

Fig. 4. Lactic acid production by *Lactobacillus amylophilus* GV6 from different types of flour from various kinds of pulse in MRS medium containing 1 % soluble starch and devoid of yeast extract

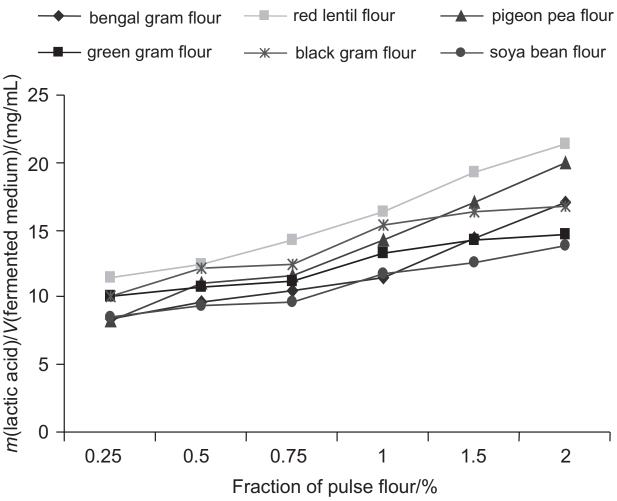

Fig. 5. Lactic acid production by *Lactobacillus amylophilus* GV6 from different types of flour from various kinds of pulse in MRS medium containing 1 % soluble starch and devoid of peptone

mins B for the growth of *L. amylophilus* GV6, which are absent from red lentil as it contains only riboflavin and niacin (*21*). Since the objective of the present study was also to replace yeast extract with cheaper source for vitamins and nitrogen, baker's yeast, which contains higher nitrogen content than brewer's yeast (*23*), was further tested for lactic acid production.

Lactic acid production using baker's yeast to replace yeast extract

The experimental results show significant production of lactic acid with high concentrations of yeast cells substituting yeast extract in MRS medium (Fig. 6). The organism assimilates specific growth factors (amino acids, vitamins, purine and pyrimidine bases) and peptides, which are made available by the lysates of baker's yeast cells (due to autoclaving) provided in the medium. Berg *et al*. (*24*) suggest that the presence of peptides in yeast extract enhances growth of *Lactobacillus*. The nutritional requirements have been discussed by Rogosa *et al*. (*25*), who emphasized the importance of amino acids

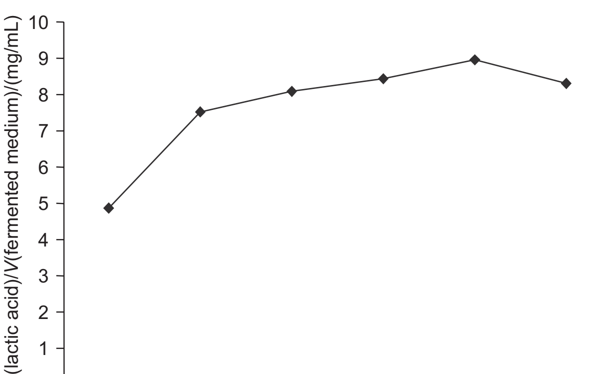

Fig. 6. Lactic acid production by *Lactobacillus amylophilus* GV6 in MRS medium with yeast cells (baker's yeast) to replace yeast extract

and vitamins for the growth of *Lactobacillus*. Amrane and Prigent (*26*) and Nancib *et al.* (*27*) explained the contributions of B vitamins, purine and pyrimidine bases in the medium for the growth of *Lactobacillus*. Our observations explain that the lysate of baker's yeast provides all vital requirements for growth and lactic acid production by *L. amylophilus* GV6. Based on the above observations, an experiment was run with 2 % of red lentil and 1 % of yeast cells, as substitutes for peptone and yeast extract in MRS medium with 1 % of soluble starch, which resulted in *m*(lactic acid)/*V*(fermented medium)=12.2 mg/mL. In this initial screening the lactic acid yield by the organism was 92 % (*m*(lactic acid)/*m* (starch consumed)). Baker's yeast cells and red lentil flour could successfully replace the yeast extract and peptone to reduce the cost of fermentation medium.

Conclusions

Inexpensive nitrogen sources were screened for direct fermentation of starch to L(+) lactic acid by *L. amylophilus* GV6. Of all the nitrogen sources tested, red lentil and baker's yeast could replace peptone and yeast extract in MRS medium. L(+) lactic acid could be produced with low cost carbon and nitrogen substrates in fermentation medium with high efficiency.

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Preispitivanje jeftinih izvora dušika za proizvodnju L(+) mliječne kiseline iz škroba s pomoću amilolitske bakterije *Lactobacillus amylophilus* GV6 jednostupanjskom fermentacijom

Sažetak

Proučena je bakterija *L. amylophilus* za proizvodnju L(+) mliječne kiseline jednostupanjskom fermentacijom koristeći škrobne supstrate. Ispitano je sedam tipova jeftinih organskih izvora dušika (brašno dobiveno od biljke *Cajanus cajan* – engl. pigeon pea, crvene leće, crnog, bengalskog i zelenog slanutka te pekarskoga kvasca) kao mogućnost nadomjestka skupljih komercijalnih izvora dušika, kao što su pepton i ekstrakt kvasca. Nađeno je da su crveni slanutak i stanice pekarskoga kvasca najbolja zamjena za pepton i kvaščev ekstrakt u proizvodnji mliječne kiseline. Postignuto iskorištenje na L(+) mliječnoj kiselini iznosilo je oko 92 % (*m*(mliječna kiselina)/*m*(škrob)).