

## Arginine Specific Aminopeptidase from *Lactobacillus brevis*

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### ABSTRACT

The proteolytic system of lactic acid bacteria contribute to the development of flavor during the ripening of cheese through the generation of short peptides and free amino acids, which directly or indirectly act as flavor precursors. Newly isolated lactic acid bacteria (LAB) as well as those procured from culture collection centers were screened for the production of various substrate specific aminopeptidases. Among all the strains screened, *L. brevis* (NRRL B-1836) was found to produce quantifiable amount of intracellular arginine specific aminopeptidase (EC 3.4.11.6). The productivity of arginine aminopeptidase in 5 L fermentor was 36 IU/L/h. The Luedeking and Piret model was tested for intracellular production of aminopeptidase and the data seemed to fit well, as the correlation coefficient was 0.9964 for MRS. The  $\alpha_{AP}$  and  $\beta_{AP}$  was 0.4865 and 0.0046, respectively in MRS medium indicating that the yield was predominantly depended on growth. The culture produced lactic acid and also tolerated pH 2.0-3.0 and 0.3-0.5% bile salts, the most important probiotic features.

**Key words:** *Lactobacillus brevis*, aminopeptidases, arginine- *p*- nitroanilide

### INTRODUCTION

Aminopeptidases (EC 3.4.11) are ubiquitous enzymes widely produced in both prokaryotic and eukaryotic cells. As exopeptidases, they catalyze the cleavage of amino acids from the N-terminal position of peptides and proteins. They are involved in many biological functions such as protein maturation, protein turnover, hydrolysis of regulatory peptides, nitrogen nutrition, modulation of gene expression, etc. Consequently, they are considered as essential enzymes (McDonald and Barret 1986; Christensen et al., 1999).

Lactic acid bacteria (LAB) are characterized by their high demand for essential growth factors such as peptides and amino acids. They require additional free amino acids if a sustained maximum growth rate is to be assured, as they

cannot synthesize several amino acids (Tsakaudou et al., 1993). Aminopeptidases, and more generally the proteolytic system of LAB are used as starters in food fermentation processes and are essential for the nitrogen assimilation in LAB and also for the development of the organoleptic properties of dairy products such as the texture and flavor (Macedo et al., 2000). In general, the concentration of free amino acids in dairy milk is insufficient to support the optimal LAB growth, and hence, the bacteria have developed complex enzyme systems such as aminopeptidases, dipeptidases and carboxy peptidases, which bring about production of small peptides and release of free amino acids starting from the large peptides in their immediate environment (Wilhelm et al., 1995; Pritchard and Coolbea, 1993; Tan, 1993). Free amino acids have direct (although probably

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limited) contribution to cheese flavor development, as well as an indirect contribution via formation of the afore mentioned catabolic metabolites such as amines, thiols, thioesters, aldehydes and ketones (Bibal et al., 1989). Arginine aminopeptidase (AP) could be involved both in bacterial growths by supplying amino acids, and in the development of dairy product's flavour, by hydrolyzing bitter peptides and liberating aromatic amino acids which are important precursors of aroma compounds (Yolanda and Fidel, 2002).

The modeling of microbial system is an important tool in the understanding how the system operates and how it would perform under different sets of conditions (Mohammed et al., 2007). The most widely used kinetic model for product formation in biological processes is the Luedeking–Piret model (Zeng, 1995). This model assumes that the product formation may be attributed to growth-associated and/or non growth-associated mechanisms. The non growth-associated mechanism is often considered to be conditional on maintaining the functions of cells. In the present study, attempts were made to screen LAB strains having substrate specific aminopeptidase activities. The study includes medium formulation and production optimization for arginine aminopeptidase using *L. brevis*.

## MATERIALS AND METHODS

### Materials

Substrate specific *p*-Nitroanilides for L-Arginine, L-Glycine, L-Leucine-, L-Methionine, L-Proline, L-Valine and Arg-Pro *p*NA and the chromogenic substrate, *p*-nitroaniline were procured from Sigma, USA. MRS (broth/ agar) medium was from Hi-media (Mumbai, India) and all other analytical grade reagents were purchased from either Merck (Mumbai, India) or SD Fine Chemicals (Mumbai, India).

### Microorganisms, maintenance and inoculum preparation

*Enterococcus faecalis* RKY 1, *Lactobacillus brevis* NRRL B-1836, *Lactobacillus casei* ATCC 11-443, *Lactobacillus delbrueckii* NCIM 2025, *Lactobacillus halotol* ATCC 35410 8909, *Lactobacillus plantarum* ATCC 8014 8802, *Lactobacillus pentosus* ATCC 8041 and *Streptococcus faecium* TUB B590 were obtained

from the American Type Culture collection (ATCC). Thirteen lactobacilli cultures were isolated from various sources and labeled as W3 (isolate from whey), SD1, SD2, SD3 (isolate from Sour Dough), SG1, SG2 (isolate from Snake guard), G1, G2, G3 (isolate from goat excreta) and CB1, CB2, CB3, CB5 (isolated from cabbage). All the cultures were screened for aminopeptidase activity.

The cultures were preserved by making stock solutions in 50% (v/v) sterile aqueous glycerol and were stored at -80 °C. From this glycerol stock, the stab cultures were prepared by inoculating the cultures in MRS agar medium and incubating at 37°C for 48 h. The fully grown stabs were stored at 4°C for routine purposes. To obtain a working culture, a loopful of stab culture was inoculated into MRS medium and grown at 37° C for 24 h. The resulting culture was then transferred to fresh MRS medium and incubated for 18 h at 37° C. This culture which contained  $2.2 \times 10^9$  CFU/ml was used as an inoculum. Generally 2% (v/v) inoculum was used for fermentation studies.

### Screening for extra and intracellular aminopeptidases

Fermentation was carried out in 250-ml Erlenmeyer flasks with a working volume of 100ml MRS medium autoclaved at 121° C for 15 min. After cooling, the flasks were inoculated with 2 % (v/v) inoculum and incubated at 37° C in static condition. After the desired intervals of incubation, the samples were withdrawn as whole flasks. The fermented broths were centrifuged at 12000x g for 15 min at 4° C (Himac CR 22G, Hitachi, Japan) and both the clear supernatant and the pellets were collected. The enzymatic assay was carried out with the supernatant to determine the extra-cellular APs activity (Choi et al., 1996). The pellet was collected and washed twice with 0.1 M potassium phosphate buffer of pH 7.0. After washing, the cells were dissolved in a minimum amount of the same buffer and lysozyme was added to the final concentration of 1mg/ml and incubated at room temperature (30° C) for 1 h. The cells were then sonicated (Sonics ,USA) for 5 minutes with an active time of 30 seconds cycles at 42 % amplitude and the crude cell lysate was centrifuged at 12000xg for 15 minutes at 4°C (Tsakaudou et al., 1993). The clear cell free supernatant was collected and enzymatic assay was done to check the intra cellular enzyme activity. All the sets of experiments were carried

out in duplicates and the average value has been reported.

### Aminopeptidases activity

The aminopeptidase activities were measured using the following substrates, Arg-*p*NA, Gly-*p*NA, Leu-*p*NA, Met-*p*NA, Pro-*p*NA and Val-*p*NA. These were dissolved to a concentration of 2.5 mM in 50 mM Tris-HCl buffer (pH7.6). Dipeptidyl aminopeptidase activity was determined using Arg-Pro-*p*NA dissolved to the concentration of 1mM in the same buffer. The aminopeptidase assay was carried out as described earlier by Tan and Konings (1990) with slight modifications. The reaction mixture contained 100µl of 2.5 mM selected substrate, 100µL of 50 mM Tris-HCl buffer (pH 7.6) and 50 µL of the properly diluted crude enzyme extract. The well-mixed solution was incubated at 37° C for 15 min (Fernandez-Esplá et al., 1997). The reaction was stopped by the addition of 100 µL (30%) glacial acetic acid and the absorbance was measured at 405 nm in a microplate reader (Yolanda and Fidel, 2002) (Bio-Rad, Model 680 XR). The concentration of *p*-Nitroaniline released was calculated from a calibration curve. One International Unit (IU) of enzyme activity was defined as the amount of enzyme that releases 1 µmol of *p*-nitroaniline from 4-Nitroanilide derivatives of different substrates per minute per ml under the specified assay conditions. The protein concentration was determined either by Lowry method using BSA as standard (Lowry et al., 1951) or in Nanodrop spectrophotometer (ND-1000) at A280 nm.

### Medium formulation and optimization

Initially, the growth of the culture was monitored in MRS medium using 2 % (v/v) inoculum ( $2.2 \times 10^9$  CFU/ml.) of 18 h old culture. At desired interval of time, samples were removed and plated after serial dilution. Growth was recorded as CFU/ml. By keeping the dextrose (2% w/v) originally present in the MRS medium as control, the different carbon sources such as fructose, maltose, sucrose, lactose, starch, cellulose and glycerol were supplemented individually in MRS medium to study their effect on enzyme production in *L. brevis*. Similarly, by keeping the ammonium citrate (0.2% w/v) used in the basal medium as a control and 0.2% (w/v) of nitrogen sources such as ammonium sulphate, ammonium

nitrate, ammonium dihydrogen ortho-phosphate, ammonium molybdate, ammonium chloride, ammonium bicarbonate, sodium citrate and urea were tried to find the efficacy of each nitrogen source for enhanced arginine-AP production in *L. brevis*. To study the effect of different inoculum size, the size was varied (0.5, 1, 2, 3 and 4 % v/v). The age of the inoculum was maintained as 18 h. A time course study (12, 15, 16, 18 and 21h) was conducted with *L. brevis* to find out the optimum incubation period for maximum arginine aminopeptidase production.

### Batch Fermentation in 5 L bioreactor

For scale-up studies, the medium was sterilized at 121°C for 15 min in a 5 L stirred tank bioreactor (Biostat B-5; B. Braun Biotech-Sartorius) with 3 L working volume. *L. brevis* 2% (v/v), 18 h old was used as inoculum. The culture was agitated at 60 rpm. The initial pH was set at 6.2 and temperature was maintained at 37°C. The flow rate of sparged air was 0.5 vvm. The fermentation was carried out for 24 h. The samples were withdrawn at regular intervals and the lactic acid production, utilization of sucrose and arginine aminopeptidase production was determined. The growth was monitored by determining the dry weight of the biomass and the cell density was counted by haemocytometer. A kinetic model for the product formation rate was described by Luedeking and Piret (Luedeking and Piret, 1959). The model illustrated an empirical relationship between the rate of product formation and the rate of cell growth measured by optical density, which was originally applied to lactic acid production from *Lactobacillus delbrueckii* growing in MRS medium.

$$dP/dt = \alpha dX/dt + \beta X$$

Where, X was the bacterial concentration, P represented the product concentration,  $\alpha$  and  $\beta$  were empirical constants were determined from the plot of specific rate of lactic acid synthesis as a function of the specific rate of bacterial growth during batch fermentations at the given pH, and t (fermentation time). The term ( $\alpha dX/dt$ ) was referred to as the product formation rate associated with growth and describes the additional product formation by the growing organism;  $\beta X$  was the product formation rate associated with non-growth.

### Probiotic features of *L. brevis*

Few probiotic features were evaluated by checking the tolerance of the cultures to varying concentrations of salt, phenol and bile salt in MRS broth. Growth at 37°C at different medium pH (2.5, 3, 4 and 5), NaCl concentration (4, 5, 8 and 12% (w/v), phenol level (0.2–0.5 g phenol/100 ml) and bile salt concentrations (0.3, 0.5 and 0.8 g sodium taurocholate/100 ml) was monitored at 620 nm after 24 h.

## RESULTS AND DISCUSSION

### Screening of LAB for aminopeptidases activity

The strains were tested for the extracellular

aminopeptidases activity with different substrate specificity as describe above. Most of these strains did not produce extracellular enzyme activity (data not shown). However, as shown in Table 1, there were considerable amount of intracellular enzyme activity in many of them. Although there are some studies on intracellular and extracellular aminopeptidase activities (Shihata et al., 2000), several reports (El Soda et al., 1978, Kolstad and Law, 1985) have described that aminopeptidases were intracellular enzymes that were released or leaked when the cells were lysed or damaged. Extracellular arginine aminopeptidase from *Streptococcus gordonii* has been reported by Goldstein et al, (2002).

**Table 1** - Specific activity (IU/mg) of different intracellular aminopeptidases from LAB

Cultures	Arg-pNA	Pro-pNA	Leu-pNA	Met-pNA	Gly-pNA	Val-pNA	Arg-Pro-pNA
<i>L. brevis</i>							
NRRL B-1836	0.0366	0.0007	0.0003	0	0	0	0
<i>L. casei</i>							
ATCC 11-443	0.0248	0.0002	0.0948	0.0248	0	0.0135	0.0173
<i>L. delbrueckii</i>							
NCIM 2025	0.0159	0	0.0062	0	0	0	0.0011
<i>L. plantarum</i> ATCC							
8014 8802	0.0428	0	0.0086	0.0023	0	0	0.0062
<i>L. pentosus</i> ATCC							
8041	0.0226	0	0.0041	0	0.0003	0	0.0022
<i>L. halotol</i>							
ATCC 35 410	0.0273	0	0.0058	0.0021	0	0	0.0019
<i>E. faecalis</i>							
RKY 1	0.0347	0	0.0082	0	0	0	0
<i>S. faecium</i>							
TUB B 590	0.0221	0	0.0035	0.0009	0	0	0
W 3	0.0289	0.0012	0.0618	0.0183	0	0.0093	0.0096
MC 1	0.0124	0	0.0041	0.0012	0	0	0.0003
CB 1	0.0095	0	0.0042	0	0	0	0
SG 2	0.011	0	0.0031	0	0	0	0

The results indicated that among different LAB bacteria showing APs activity, *L. brevis* and *L. plantarum* showed comparatively higher specific activity for arginine-AP but since all other APs (leucine, proline, methionine, glycine, valine and the dipeptidase arg-pro) activity were either less or negligible in *L. brevis* than *L. plantarum* and it could make the downstream processing of the arginine-AP easier and it could also improve the substrate specificity. Therefore *L. brevis* was selected for further study.

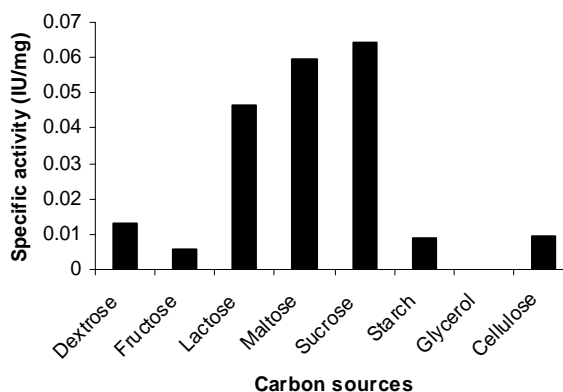
Macedo et al., (2003) showed that Arg pNA, Leu pNA and Lys pNA were the substrates hydrolysed at the highest rate (under the condition of saturation by substrate) by cell free extracts (CFE) of *Lactobacillus paracasei* ESB 230 and *Leuconostoc mesenteroides* ESB 136, whereas Met pNA and Arg pNA were the substrates more rapidly taken up by CFE of *Lactobacillus lactis* ESB 117 and *Enterococcus faecium* ESB 50. Intracellular aminopeptidase activities from *L. brevis* ATCC 14869 cells for pro pNA substrate

has been reported by Miia et al., (2002).

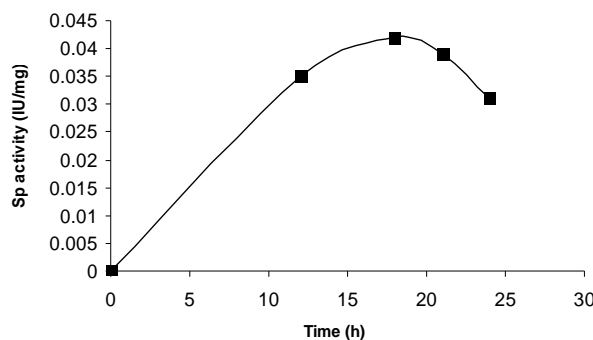
#### Effect of carbon source and other parameters on Arginine-aminopeptidase activity

As shown in Fig.1, the specific activity for arginine-AP was maximum (0.064) using sucrose as carbon source. The enzyme activity was relatively low in the case of cellulose and no activity in case of glycerol. In the case of nitrogen sources, the maximum enzyme activity was with ammonium citrate which was the nitrogen source

used in the control as shown in Table 2. There were reports dealing with inorganic nitrogen sources such as ammonium sulphate and ammonium citrate giving better protease yields in some organisms (Wellington and Martins, 2004). Regarding the inoculum size, there was not much difference in specific activity of enzyme while using different inoculum sizes as shown in Table 2. Hence, 2% (v/v) was used for further studies.



**Figure 1** - Effect of different carbon sources on arginine aminopeptidase production by *L. brevis*



**Figure 2** - Time course study on arginine aminopeptidase production.

**Table 2** - Effect of different nitrogen sources and inoculum size on arginine-AP production by *L. brevis*

Nitrogen source (0.2%, w/v)	Specific activity	Inoculum size (% v/v)	Specific activity
Ammonium citrate	<b>0.0569</b>	0.50	0.0585
Ammonium sulphate	0.0512	1	0.0441
Ammonium nitrate	0.0377	2	0.0456
Ammonium dihydrogen ortho- phosphate	0.0513	3	0.0469
Ammonium molybdate,	0.0468	4	0.0429
Sodium nitrate	0.0388		
Urea	0.0463		
Ammonium bicarbonate	0.0353		
Ammonium chloride	0.0472		

During the time course study to monitor the enzyme activity, the maximum specific activity was recorded after 16 h of growth (0.042 IU/mg protein) (Fig.2).

### Batch fermentation in 5-L fermenter

The growth pattern of *L. brevis* in MRS medium is shown in Fig. 3. The distinct feature of cells grown in MRS media was a short lag phase of about 3 to 6 h (Fig. 3). The culture almost immediately entered the exponential phase and reached the stationary phase in 18 h at a biomass concentration of 2.285 g/L and a cell count of  $32 \times 10^8$  CFU/ml (Fig. 3).

The fermentation profile of *L. brevis* grown in

MRS medium in 5 L fermenter is shown in Fig. 4. The pattern of biomass change was comparable with that of cell density count. Lactic acid concentration of 20.42 g/L was obtained in 24 h (Fig. 4). The initial sucrose concentration in the medium was 20 g/L. At the onset of the stationary phase, sucrose was almost utilized and the lactic acid production was about to cease. Lactic acid has been reported to inhibit the cell growth, but other environmental factors could also involve in growth inhibition (Christensen et al., 1999). The cell counts were maximum after 24 h as shown in Fig. 3 ( $54 \times 10^8$  CFU/ml) and biomass concentration was 2.45 g/L (Fig. 4).

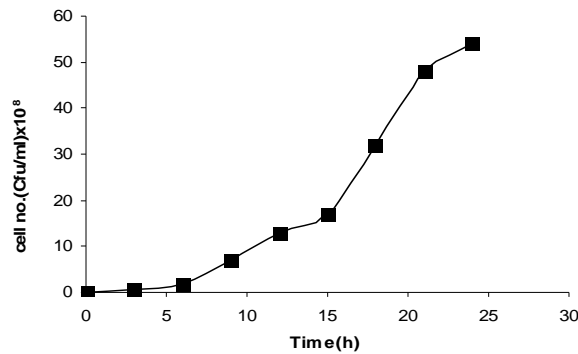


Figure 3 - Growth pattern of *L. brevis* in MRS medium.

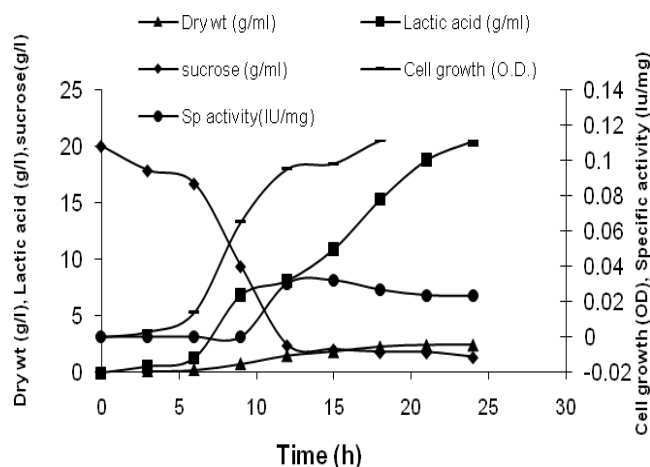


Figure 4 - Fermentation profile in 5-L batch fermenter for arginine-aminopeptidase production by *L. brevis*.

The productivity of arginine-aminopeptidase in fermentor was 36 IU/L/h. The maximum specific growth rate of the culture grown in MRS was 0.046 per h and this was reached in 18 h, indicating fast growth in MRS medium. The maximal specific rate of substrate utilization in MRS medium was 0.033 / h. The biomass yield coefficient on substrate was 0.131

The kinetics of growth and lactic acid production for *Lactobacillus* sp. has been described earlier by Luedeking and Piret (1959). The coefficients given by the Luedeking and Piret model were determined from the plot of specific rate of lactic acid synthesis as a function of the specific rate of bacterial growth during batch fermentations at the given pH 4.2. In the present study for the estimation of parameters, only data from the exponential and stationary phases were included because no meaningful information was obtained from the lag phase data. For MRS medium in lactic acid production, the  $\alpha_{LA}$  was 1.3045 and  $\beta_{LA}$  was 0.0138; correlation was 0.9989. The value of  $\alpha_{LA}$  was higher than  $\beta_{LA}$  as lactic acid production was an energy dependent pathway and thus associated with growth. Kemp et al., (1989) have also reported high value for  $\alpha_{LA}$  than  $\beta_{LA}$ . Luedeking and Piret had reported that for *Lactobacillus* grown in MRS medium,  $\alpha$  and  $\beta$  values were also dependent on the pH of the growth medium. The Luedeking and Piret model was also tested for intracellular production of aminopeptidase. The data seemed to fit well, as the correlation coefficient was 0.9964 for MRS. The  $\alpha_{AP}$  and  $\beta_{AP}$  were 0.4865 and 0.0046, respectively in MRS medium. The aminopeptidase production was predominantly associated with growth although a small portion could be attributed to metabolism not associated with the growth. The increased production of aminopeptidase led to the hydrolysis of the available peptides into amino acids, which supply the cells with sufficient amounts of amino acids for cell synthesis.

#### Probiotic characteristics

*L. brevis* culture was checked for the tolerance to NaCl, low pH, bile salt, and phenol. The strain had tolerance towards NaCl (4 %, OD 1.634 at 600nm), pH (2-3, OD 0.03 at 660nm), low phenol level (0.3%, OD. 1.359 at 660 nm) and bile salt (0.5 %, OD 0.998 at 660nm). According to Pancheniak and Soccol (2005), the strains that showed 0.3% tolerance to bile could be used as

probiotic for swine. This was found to be an added advantage of this strain. In a study by Ronka et al., (2003) concluded that *L. brevis* was claimed as a promising candidate as a probiotic supplement in dairy products.

#### CONCLUSION

An over view of the aminopeptidase profiles of selected strains of lactic acid bacteria showed the presence of substrate specific intracellular aminopeptidase activity, including arginine amino peptidases. Using *L. brevis* NRRL B-1836 the maximum production of this enzyme was obtained at a late exponential phase. This knowledge and the screening of new LAB strains which perform better may provide healthy food with appealing texture and flavor.

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