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RESEARCH ARTICLE :

Studies on Amylase activity of probiotic lactic acid bacteria *Enterococcus durans*

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SUMMARY : The aim of this research was to study the amylase activity of probiotic lactic acid bacteria *Enterococcus durans* Amf 50. The strain *E.durans* Amf 50 originally isolated from finger millet. The amylase activity of the strain was determined in MRS starch medium and at different concentrations of soluble starch, rice flour and various pH levels. Kinetic parameters of amylase enzyme were also determined. Maximum amylase activity (9.17±0.01 U min^{-1}) exhibited at 48 h when the cell population also at its maximum (9.31±0.04 log cfu ml^{-1}). The amylase enzyme activity and stability were observed in pH range of 5 to 6.5. The highest amylase activity was recorded at pH 5.5.which was the optimal pH. The amylase activity of *E.durans* Afm50 displayed in starch and rice flour at concentration of 1 to 10 % and exhibited the maximum activity at 6 % of pure starch and in 6 % rice flour concentration. Nevertheless, the activity in rice flour was less than in soluble starch. The Km values of amylase of *E. durans* Afm50 were 0.7 and 6.92 mg ml^{-1} for soluble starch and rice flour, respectively.

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KEY WORDS :
Amylase activity, Probiotic, Lactic acid bacteria, *Enterococcus durans*

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BACKGROUND AND OBJECTIVES

Lactic acid bacteria (LAB) are widespread micro-organisms which can be found in many environment rich mainly in carbohydrates, such as plants, fermented foods and the mucosal surfaces of humans, terrestrial and marine animals. In the human and animal bodies, LAB are part of the normal micro-biota or micro-flora, the ecosystem that naturally inhabitats the gastrointestinal tract which is comprised by a large number of different bacterial species with a diverse amount of strains (Stiles and Holzapfel, 1997). Various strains of LAB can be found in dairy products (yogurt, cheese), fermented meats (salami), fermented vegetables (olives, sauerkraut), sourdough bread, etc (Korhonen, 2010). The lactic acid bacterial fermentations provide foods that have a variety of flavours, aromas, textures in addition to the foods being safe and having a long shelf life.

LAB possess an extensive collection of starch-modifying enzymes, many of which have the potential to influence the composition and the processing, organoleptic properties and quality of foods and feeds.Inspite that lactic acid bacteria are used for production of fermented foods and drinks for millennia, their

ability to grow using starch as a sole carbon source was noticed in the last 30 years. The amylolytic lactic acid bacteria (ALAB) are distributed in tropical fermented foods, prepared from cassava, maize, sorghum, rice, millet, wheat and rye. The lactobacilli group is that most efficiently utilizes starch as a carbon and energy source among all ALAB (Petrova *et al.*, 2013). According to Vishnu *et al.* (2002) some LAB possess amylase activity. They degrade the starch present in food to lactic acid and fermentable monosaccharides that can be easily assimilated in the body, thus improving utilization of dietary starch and enhancing digestion. The amylase enzymes of *Lactobacillus amylovorus* and *L. amylophilus* were purified for the first time by Pompeyo *et al.* (1993).The maximum amylase activity was displayed at pH 5.5 and 40° C in *L. amylophilus* whereas in *L.amylovorus*, it was at 63° C and pH 5.0. The optimum amylase production for the strain *L.plantarum* was achieved at a pH of 5.5, temperature of 65° C and at Km value of 2.38 g L^{-1} with soluble starch as substrate (Giraud *et al.*, 1993). Nguyen *et al.* (2007) added that amylolytic LAB are now implicated in preparing high energy density cereal-based foods for improving utilization of dietary starch in infants and children. This study describes the effect of soluble starch, rice flour and pH on amylase activity of probiotic lactic acid bacteria *Enterococcus durans*

RESOURCES AND METHODS

Micro-organism and growth conditions :

The amylolytic LAB strain *Enterococcus durans* Afm50 used in this study was obtained from the culture bank of Department of Agricultural Microbiology, TamilNadu Agricultural University, Coimbatore. The strain *E.durans* Afm50 was originally isolated from fermented finger millet and developed for functional properties. LAB culture was kept at -20°C in de Man Rogosa Sharpe (MRS) broth containing 25% (v/v) glycerol. The strain was sub cultured twice in MRS broth at 37°C for 48 h. The amylolytic strain *E. durans* Afm50 was grown in modified MRS medium containing 20 g L^{-1} of starch instead of glucose.

Determination of growth :

The growth of the amylolytic strain of *E. durans* Afm50 was determined in modified MRS broth containing 20 g L^{-1} of starch instead of glucose. There after it was designated as MRS-Starch broth. The growth of *E.durans* Afm50 was determined by recording the OD (optical density) at 660 nm in UV-VIS spectrophotometer (Varian, Cary® 50 Bio, Australia) against uninoculated MRS-starch broth as blank. The viable cell count was determined by plating serially diluted samples in MRS medium and the plates were kept for incubation at 37° C for 48 h. The growth of the cells was expressed in cfu ml^{-1}. Viable cell count was taken at 0, 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h of incubation.

Culturing of *E. durans* Afm50 for amylase production :

Before the experiment, the culture was activated by two transfers in MRS-Starch broth with 1 % and 2 % of starch, respectively. The activated culture was again inoculated with 2 % starch containing MRS- Starch broth. After activation of the culture, it was cultured in 100 ml of MRS-Starch broth. The cultures were incubated in static condition at 37° C in an incubator (New Brunswick, USA) for 48, 60 and 72 h. The culture broth grown at different time intervals were centrifuged (7000 rpm, 20 min, 4° C) to obtain the cell free extract and was as enzyme source.

Assay of extracellular amylase activity :

Soluble starch solution (1 %) prepared in acetate buffer (pH 6.5, 0.1M) was used as the substrate for the assay. For estimation of the enzyme activity, 1 ml of the substrate solution was mixed with 1 ml of enzyme broth and incubated at 37° C for 30 min. The reaction was stopped by cooling in ice bath and addition of 1 ml of dinitrosalicyclic acid reagent. The amount of reducing sugars released at the end of reaction was determined by dinitrosalicyclic acid method (Miller, 1959) as described in section 3.3.6. One unit of enzyme activity was defined as the amount of enzyme required to liberate one μ (μ denotes micro) mole of glucose per minute and designated as U min^{-1}.

Determination of pH on amylase activity and stability :

The extra cellular amylase activity was determined at various pH values using different buffers such as glycine-HCl (2.0-3.0), sodium acetate (4.0-5.0), sodium phosphate (6.0-7.0), Tris-HCl (8.0-9.0) at 50 mM concentrations with 6 % soluble starch as substrate. The enzyme solution was maintained at 37°C in each buffer

and the enzyme activity was measured by standard assay conditions. Stability of extracellular amylase enzyme was studied by incubating the amylase enzyme solution in a water bath at 60° C at varying pH buffers (equal volume) for 30 min. The residual activity was detected under the same conditions and expressed as percentage of the activity and untreated control taken as 100 percentage.

Effect of starch and rice flour concentration on amylase activity :

Soluble starch solution and rice flour concentrations of 1, 2, 4, 6, 8 and 10 % concentration prepared in acetate buffer (pH 6.5, 0.1 M) was used as the substrate for the assay. The extra cellular amylase enzyme activity was measured by the standard assay conditions as described above. The kinetic parameters of extra cellular amylase enzyme Vmax (maximum rate achieved at maximum substrate concentration) and the Michaelis constant Km (substrate concentration at which the reaction rate is half of Vmax) were analyzed using Line weaver-Burk plot in which reaction rates (U min^{-1}) were plotted against substrate concentration.

Statistical analysis :

All the experiments were carried out in triplicate. All the data were subjected to statistical analysis using analysis of variance (ANOVA) (Gomez and Gomez, 1984) at $p<0.05$ levels which considered as statistically significant.

OBSERVATIONS AND ANALYSIS

Among the LAB, amylolytic strains are commonly found in members of lactobacilli (Blandino *et al.*, 2003). In enterococci, amylolytic representatives of the *Enterococcus sulfureus* from maize pozol (Diaz-Ruiz *et al.*, 2003) and *E.faecium* from starchy beverages were reported (Muyanja *et al.*, 2003). In our previous studies, *E.durans* Amf50 possess probiotic properties (Karthikadevi and Vijila, 2013) The strain *E.durans* Afm50 used in this study grew and exhibited extracellular amylase production in MRS-Starch culture medium at 37° C (Table 1). The amylase production pattern in *E.durans* Afm50 indicated that the induction of amylase took place after 4 h of incubation in the presence of starch. The level of amylase production increased significantly after the exponential growth phase (after 4 h) and reached maxima value of 9.17 ±0.11 U min^{-1} at 48 h, when the cell population also at its maximum (9.31±0.04 log cfu ml^{-1}). Hence amylase production by the strain *E. durans* Afm50 was tightly linked to cell growth. The relationship between pattern of cell growth and amylase production was found in *L.fermentum* (Goyal *et al.*, 2005; Liu and Xu, 2008; Fossi *et al.*, 2011). The decline of cell growth and amylase activity occurred after 48 h of incubation and could be attributed to the raise of acidity due to lactic acid production in fermented broth (Singh *et al.*, 2006). Giraud *et al.* (1994) investigated the degradation of raw starch by a wild amylolytic strain of *L. plantarum*. The authors found maximum amylase activity of 60 U ml^{-1} with a controlled pH 6.0 compared with amylase activity of 2 U ml^{-1} under non-controlled pH conditions. The effect of pH on amylase activity of *E.durans* Afm50 on soluble starch is shown in Fig.1. The amylase activity by *E.durans* Afm50 was significantly influenced by pH. The amylase enzyme activity was observed in pH range of 5 to 6.5. The highest amylase activity was recorded (9.17±0.01 U min^{-1}) at pH 5.5.which was the optimal pH. The activity of extracellular enzyme (cell free supernatant as enzyme source) stabilized at the pH ranged from 5 to 6 at 60º C. At 60º C, the stability of extracellular amylase was poor beyond 4.5 and alkaline pH. The extracellular amylase from LAB had good stability at pH range from 5 to 6 as reported for *L. manihotivorans* (Aguilar *et al.*, 2000) and *L. plantarum* (Giraud *et al.*, 1994). In the present study, the stability of the amylase of *E.durans* Afm50 at the pH of 5.0 may

Table 1 : Time course of viable cell population and amylase activity of *E. durans* Afm50

Time (h)	*E. durans* Afm50	
	Viable cell population (log cfu ml^{-1})	Amylase activity (*U min^{-1})
0	3.20±0.04	0.09±0.00
2	3.40±0.29	0.69±0.01
4	4.13±0.06	1.30±0.02
6	5.16±0.07	2.89±0.03
8	7.04±0.08	4.52±0.05
12	8.06±0.09	5.67±0.07
24	8.93±0.10	7.24±0.08
36	9.21±0.08	8.47±0.10
48	9.31±0.04	9.17±0.11
60	9.27±0.01	9.00±0.10
72	9.12±0.02	8.89±0.10

Data are Mean ± Standard error
*U = one enzyme unit is defined as amount of enzyme required to liberate 1 µmol of glucose at 37° C

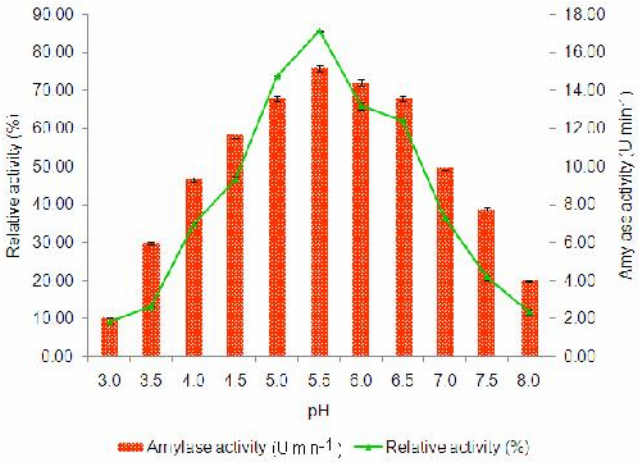

Fig. 1 : Effect of pH on amylase activity and stability of *E. durans* Afm50 in soluble starch; Error bars represent standard error of the mean values

be related to the accumulation of the active enzyme in the broth during the period of cell growth.

The amylase activity of *E.durans* Afm50 displayed in starch and rice flour at concentration of 1 to 10 % is presented in Table 2. The enzyme exhibited the maximum activity at 6 % of pure starch and in 6 % rice flour concentration. The activity exhibited beyond 6 % concentration was higher than the activity recorded in the concentration range of 8 to 10 % The maximum activity of 15.15 U min^{-1} was recorded at 6 % soluble starch concentration. The result shown in Table 2 revealed that the activity was very low upto 4 % rice flour concentration. The enzyme activity recorded at 6 % substrate concentration (6.97±0.03 U min^{-1}) was two fold high compared to 4 % substrate level (3.00±0.09 U min^{-1}). Significant reduction in activity was recorded when the substrate was increased to 8 % (6.72±0.05 U min^{-1}) and 10 % (6.55±0.06 U min^{-1}) levels from 6 % concentration. Nevertheless, the activity in rice flour was less than in soluble starch. Kinetic parameters of extracellular amylase enzyme produced by *E.durans* Afm50 was represented in Table 3. The Km value was low in soluble starch (0.77 mg ml^{-1}) than in the rice flour. The Vmax value of soluble starch is 15.63 U min^{-1} mg^{-1}. This result suggested that amylase activity is high in soluble starch rather than rice flour. These observations suggested the high affinity of the enzyme to pure starch rather than to rice flour. Very few amylolytic LAB are capable to convert starch at high substrate concentration (Petrova and Petrov, 2012). Shibata et al. (2007) carried out sago starch fermentation using *E.faecium* No.78 with 2 % substrate. It may be explained by the likelihood that synthesis of carbohydrates degrading enzymes in some microbial species leads to catabolic repression by substrate such as glucose and fructose (Teodoro and Martins, 2000) considering the hammer milled rice flour used here contained low molecular weight compounds. According to Teodoro and Martins (2000), glucose present in rice flour prevented amylase gene expression and not only secretion of preformed enzyme.

Table 2 : Effect of concentration of soluble starch and rice flour on amylase activity of *E. durans* Afm50

Concentration (%)	Amylase activity (*U min^{-1})	
	Starch	Rice flour
1	9.17±0.01	1.37±0.02
2	10.22±0.08	1.80±0.06
4	12.35±0.04	3.00±0.09
6	15.15±0.02	6.97±0.03
8	14.78±0.01	6.72±0.05
10	14.71±0.01	6.55±0.06
S.E.±	0.054	0.081
C.D. (P=0.05)	0.117	0.176

Data are Mean ± Standard error
*U = one enzyme unit is defined as amount of enzyme required to liberate 1 µmol of glucose at 37° C

Table 3 : Amylase enzyme kinetic parameters of *E. durans* Afm50

Kinetic parameters	Line weaver-Burk plot	
	Soluble starch	Rice flour
V_{max} (U min^{-1} mg^{-1})	15.63	10.10
K_m (mg ml^{-1})	0.77	6.92

Conclusion :

In this study, probiotic lactic acid bacteria *Enterococcus durans* Amf50 exhibited extracellular amylase production in MRS starch medium and maximum activity reached at pH 5.5. The activity of extracellular amylase stabilized at the pH range of 5 to 6. The amylase activity of *E.durans* Afm50 displayed in starch and rice flour at concentration of 1 to 10 % and exhibited the maximum activity at 6 % of pure starch and in 6 % rice flour concentration. The presence of amylase activity in the probiotic lactic acid bacteria can be used in food fermentations which lead to develop functional foods.

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