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Partial purification, characterization of *Lactobacillus* sp. G5 lipase and their probiotic potential

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Abstract

Lipase from *Lactobacillus* sp G5 isolate was partially purified and characterized. Out of 22 isolates, only seven (G1-G7) were phenotypically and biochemically characterized as *Lactobacillus*. However, only G5 isolate showed highest enzyme activity of 31.27 U/ml and chosen for partial purification and characterization. After ammonium sulphate precipitation and dialysis, specific activity of the enzyme was found to be 0.770 IU. During optimization, the enzyme showed maximum activity at pH 8/40°C. Stability was found at pH range 7-9, with temperature range 30°C-60°C. The enzyme showed maximum activity at a pNPA concentration of 0.150 mg/1.5 ml/1 ml enzyme solution. V_{max} and K_m values were 27.32 U/ml and 0.04 mg/min. Among several substrates tested (tween-20, olive oil, castor oil, palm oil, glycerol tributyrate and ghee), maximum affinity was observed towards glycerol tributyrate. Further, the G5 isolate showed good number of probiotic qualities viz., tolerated 1.5% bile salt for 4 h and at 2% the viability decreased, while luxuriant growth was observed at pH 3 and survived at pH 1 and 2 for 4 h. In addition, G5 exhibited resistance to lincomycin and was sensitive to chlorotetracyclin. The results obtained in the present study would indicate the stability of *Lactobacillus* G5 isolate in hydrolyzing lipids and probably a promising probiotic candidate.

Introduction

In recent years, probiotics has drawn much attention for improving human health in natural way. WHO/FAO defined probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host. During last decade, research works showed that combination of probiotic therapy and conventional therapies helped to fight existing diseases and protect from future infections since probiotics have the potential to fight infection, and to modulate the immune system. The ability to activate and enhance the immunity of the host is one of the beneficial aspects of probiotics on human health (Gill, 1998). The mechanism of probiotic action includes competition with harmful gut flora for adhesion to the epithelium of the gastrointestinal tract, for nutrients, enhancing the host immunity to pathogens, producing antibacterial substances. Hydrogen peroxide, bacteriocins, lactic and acetic acids are primarily responsible for non specific inhibition of pathogens (Bielecka *et al*., 1998; Asha and Gayathri, 2012a). These antagonistic properties are useful in probiotic products. Lactic acid bacteria show anti-carcinogenic action, anti inflammatory effects and also take part in alleviating symptoms of lactose intolerance (Fooks *et al.*, 1999; Asha and Gayathri, 2012b). Probiotic bacteria can reduce the risk of occurrence of bacterial intestinal disorders and prevent diarrhea. Moreover, they are also characterized by anticholesterol activities (Fooks *et al.*, 1999; McNaught and MacFie, 2001). They reduce the cholesterol by producing lipase and also by assimilating fat in the body. Apart from this, successful probiotic bacteria should be able to survive gastric conditions and colonize the intestine, at least temporarily, by adhering to the intestinal epithelium (Lee and Salminen, 1995). Such microorganisms appear to be promising candidates for probiotics. However, these beneficial characters are strain specific and more research is required to find out which strain works best for what conditions.

Lipases are unique enzymes with broad biotechnological applications as they hydrolyze fats into fatty acids and glycerols. Several studies showed that probiotic isolate producing lipase can be used to reduce cholesterol levels, to overcome malabsorption, aids in the proper digestion of fats in the diet (Gayathri *et al*., 2011; Bae *et al*., 2002) and also in food industry, extensively in dairy industries for the hydrolysis of milk fat, lipolysis of butter fat and cream, improving the cheese flavor and enhancing the rate of cheese ripening. The short chain (C4 and C6) fatty acids released develop a sharp, tangy flavour, while the medium chain (C12 and C14) fatty acids impart a soapy taste to the product (Ghosh *et al*., 1996).

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In the present study, the lipase was produced from a *Lactobacillus* spp. isolated from goat's curd collected from migrating shepherd population and the probiotic properties of the isolate were characterized. Because this population showed a high degree of disease resistance and endurance towards climatic changes and would maintain the goat's curd since many years for their routine consumption. The results obtained in the present study would indicate the stability of the isolate in hydrolyzing lipids and probably a promising probiotic isolate.

Materials and Methods

Isolation and identification of Lactobacillus spp.

The goat's curd was collected from different regions of mid Karnataka state, India. The deMan Rogosa Sharpe (MRS) agar medium was used as a selective medium for *Lactobacillus* spp. The curd samples were diluted serially 10-fold using 0.85% physiological saline and pour plated. The plates were incubated at 37°C for 24 to 48 h anaerobically. Morphologically distinct colonies on the MRS agar medium were selected and stroke on to the fresh MRS agar plates to obtain pure culture of *Lactobacillus*. Then the colonies were observed for their colony morphology viz., color, texture, size, appearance of margin etc., and stained using Gram's staining method. Gram-positive isolates were further tested for motility test, catalase test, indole test and gelatin liquefaction test. Fermentation tests using various fermentation broths such as glucose, fructose, lactose, arabinose, xylose, sucrose and maltose were carried out (Asha *et al*., 2012) and identified based on Bergy's manual of systematic bacteriology. Further, the isolates were maintained on MRS slants.

Screening for lipase producers

To screen potent lipase producers from the *Lactobacillus* spp., the isolates were growth on the lipase screening tributyrin agar and incubated under microaerophilic condition for 72 h at 37°C and observed for opaque lipolytic zone (Sirisha *et al*., 2010).

Lipase production by submerged fermentation

The isolates showing opaque zone were inoculated to MRS broth and incubated for 24 h. 2% of the broth culture (0.1 OD at 660 nm) was inoculated into the lipase production medium and incubated for 6 days at 37°C with periodical mechanical shaking at 150 rpm using rotary shaker. Then the fermentation broth was centrifuged at 5000 g for 30 min. The supernatant was treated with 0.4M $CaCl_2$ to precipitate the fatty acids followed by centrifugation at 12800 g for 20 min. To this supernatant, 70% (v/v) chilled acetone was added slowly with continuous stirring and kept at 20°C for 4 h to facilitate protein precipitation. Further, the precipitate was obtained by centrifuging again at 12800 g for 20 min. The pellets were resuspended in Tris-HCl buffer (pH 8) to allow the solubilization of proteins and unsolubilized proteins were removed by centrifugation (Padmapriya *et al*., 2011). The supernatant was taken as a source of crude enzyme.

Lipase assay

Activity of lipase was determined using p-Nitrophenyl acetate as a substrate. When enzyme acted on the p-Nitrophenyl acetate (pNPA), it hydrolyzed ester linkage and released the free p-nitro phenol (pNP). The amount of pNP released during enzymatic hydrolysis was quantified spectrophotometrically at A_{410} after 10 min incubation of the reaction mixture. One unit (1U) was defined as the amount of enzyme that liberated, i.e., 1µmol of pNP/min under the standard assay conditions. The reaction mixture consisted of 2.5 ml substrate, 2.5 ml 50 mM Tris-HCl buffer (pH 8) and 1ml crude enzyme solution. The pNPA solution was prepared by adding the solution A (0.001 g pNPA in 1ml isopropanol) into solution B (0.01 g gum arabic, 0.02 g Sodium deoxycholate, 50 µl Triton X-100 and 9 ml of 50 mM Tris-HCl buffer, pH 8) with stirring. The isolate showing maximum lipolytic activity was microphotographed using Scanning Electron Microscopy.

Partial purification of lipase enzyme

Partial purification of lipase was achieved by ammonium sulfate precipitation followed by dialysis. Ammonium sulfate was added to the known volume of crude enzyme solution to give a concentration of 70% (w/v) saturation at 4°C. Precipitation was allowed for 10 h, and followed by centrifugation at 5000 g in a cooling centrifuge for 20 min. The precipitate was dissolved in a minimal amount of 50 mM Tris–HCl buffer (pH 8), and dialyzed for 24 h with three changes in the same buffer (Shu *et al*., 2006).

Total protein estimation

The total protein content after each step of purification was estimated by Lowry's method (Lowry *et al.*, 1951).

Determination of optimal pH, temperature and substrate concentration for lipase assay

The optimum pH for the lipase assay was determined by incubating the enzyme–substrate at various pH from 3 to 10 in the following buffers:

50 mM sodium acetate buffer (pH 3–5), 50 mM potassium phosphate buffer (pH 5–7), 50 mM Tris–HCl buffer (pH 7–9), and 50 mM glycine–NaOH buffer (pH 9–10). Lipase activity in each buffer was measured using the standard assay as described previously. The optimal temperature for the lipase was determined by incubating the reaction mixture at various temperatures from 10°C to 70°C for 10 min and at A_{410}. Effect of pNPA concentration on lipase activity was determined by using pNPA substrate solution at different volumes ranging from 0 to 3 ml with 1 ml enzyme solution and each volume was made up to 4 ml using Tris-HCl buffer (pH 8). After 10 min of incubation at room temperature, A_{410} was recorded.

Substrate affinity of lipase

Affinity of lipase towards various substrates such as tween 20, olive oil, glycerol tributyrate, castor oil and ghee was tested titrimetrically using 0.2N NaOH as titrant in presence of phenolphthalein indicator. One unit of lipase activity was defined as the amount of enzyme releasing one mole of free fatty acids in 1 min under standard assay conditions (Sadasivam and Manickam, 1996).

Probiotic characterizations of lipase producing Lactobacillus isolate (G5)

It was aimed at characterizing the capability of the isolate to resist antibiotics, acidifying activity, resistance to bile salts and survivability in the environment with different pH.

Acid resistance test

The survivability of bacteria in the acid environment was characterized at different pH, which was determined by regulating and stabilizing the MRS broth to pH 1, 2 and 3. 2% (v/v) 24 h old inoculum (1.0 OD at A_{610}) was inoculated to each test tube, serially diluted and incubated at 37ºC. 100 µl of appropriate dilution were then pour plated at 0, 1, 2, 3, 4 and 5 h. After 48 h of incubation at 37ºC, the number of CFU/ml was determined by plate count method and the test was replicated thrice (Goderska and Czarnecki, 2007; Both *et al*., 2010).

Bile salt resistance

The ability of isolated species to grow in presence of bile salts was determined in MRS broth (Goderska and Czarnecki, 2007). Briefly, MRS broth tubes were enriched with 0.0, 0.5, 1.0, 1.5 and 2.0 % (w/v) of bile salt (Lobo chemie, India) and were inoculated with 1 ml of inoculum (1.0 OD at A_{610}) and each tube was serially diluted and incubated at 37ºC. 100 µl of appropriate dilution were then pour plated at 0, 2, 4 and 6 h. After 48 h of incubation at 37ºC, the number of CFU/ml was determined by plate count method and the test was replicated thrice.

Acidifying activity

Acidification can be measured by the change in pH (ΔpH) at the time of incubation. Briefly, 50 ml of MRS broth was inoculated with 2% of G5 culture and incubated at 37°C. The pH was measured at 0, 2, 4 and 6 h (Hanna instruments, Woonsocket, R1 02895). The acidification values are then expressed as pH decrease, calculated as the difference between the value immediately after inoculation and values at 0, 2, 4 and 6h of incubation (ΔpH = initial pH - final pH). The test was replicated thrice. Acidifying activity was considered as fast, fast-medium and slow when pH value decreased by 0.4 pH after 3, 3-5 and >5 respectively (Ayad *et al.*, 2004).

Antibiotic resistance test

Antibiotic resistance of the isolate to various antibiotics was determined by Kirby-Bauer's method. Isolate was tested with regard to its sensitivity to novobiocin, tobromycin, colistin, cycloserine, Lincomycin, cefuroxime, carbenicillin, erythromycin,nitrofurantoin,kanamycin,gentamycin, chlorotetracycline (Himedia, India). Plates were incubated at 37ºC for 48 h and zones of inhibition were measured in millimeters including the diameter of the discs of 6 mm. The test was replicated thrice.

Results

A total of 22 isolates were obtained on MRS selective medium, among which only 07 (G1 to G7) isolates were identified as Lactobacilli by phenotypic and biochemical characterization. The isolates were Gram positive bacilli, single or in chain of few. The isolates did not show positive reaction to catalase, gelatin liquefaction, motility and indole production tests. All the isolates were negative for Vogues-Proskauer (VP) and while Methyl Red (MR) was positive. In carbohydrate fermentation tests, majority of the isolates reduced glucose, fructose, lactose, arabinose, xylose, maltose and sucrose. But G3 and G6 were failed to utilize mannitol. Biochemically, all isolates were relatively homogenous and produced acid only and no gas production was observed. In qualitative assay for lipase production by *Lactobacillus* isolates using plate assay method, out of seven isolates, all the tested isolates showed opaque lipolytic zone on tributyrin agar medium when flooded with 1% $HgCl_2$. However, the isolates were showed difference in the diameter of the opaque zones, larger diameter of 27 mm was observed for G5

Table 1. Acid resistance test of *Lactobacillus* isolate G5

Time (h)	pH 1	pH 2
	mean(X 10^9 CFU/ml) ± SD	mean (X 10^9 CFU/ml) ± SD
00	3.45 ± 0.1798	3.8 ± 0.0081
01	2.53 ± 0.0336	2.8 ± 0.0258
02	1.65 ± 0.0244	1.84± 0.0294
03	0.84 ± 0.0322	1.34 ± 0.0374
04	0.14 ± 0.0197	0.44 ± 0.0223
05	No growth	No growth

Table 2. Resistance to bile salt by *Lactobacillus* isolate G5

Conc. of bile salt (%)	mean (X 10^9 CFU/ml) ± SD			
	0 h	2 h	4 h	6 h
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
0.0	2.2 ± 0.1291	3.2 ± 0.2160	4.3 ± 0.1633	6.2 ± 0.1414
0.5	2 ± 0.0816	1.8 ± 0.1291	1.6 ± 0.1291	1.1 ± 0.1825
1.0	2 ± 0.0812	1.9 ± 0.0812	1.9 ± 0.0812	1.7 ± 0.1414
1.5	1.3 ± 0.0408	1.03 ± 0.1494	1.05 ± 0.0408	0.95 ± 0.0408
2.0	0.97 ± 0.0208	0.88 ± 0.0173	0.7 ± 0.0081	0.6 ± 0.0081

isolate and minimum for G2 with 8 mm.

In quantitative assay using pNPA, G5 showed the highest activity of 31.27 U/ml compared to other isolates. In both qualitative and quantitative assay, G5 has shown comparatively high lipolytic activity. Hence it was considered as *Lactobacillus* isolate with high lipolytic activity and Scanning Electron Microscopy (SEM) of G5 isolate was carried out (Figure 1) and it was selected for further study. After lipase production by submerged fermentation, it was partially purified by ammonium salt precipitation and subjected to dialysis. The total protein content after salt precipitation and dialysis was found to be 310 µg/ml and 35 µg/ml with a specific activity of 0.066 IU and 0.770 IU, respectively. Optimization of the enzyme assay was done at different temperature, pH and substrate concentration. The enzyme has shown maximum activity at pH 8 and temperature 40°C. Stability of the enzyme was found at a pH range between 7 and 9 and temperature 30°C and 70°C. The enzyme showed maximum activity at a pNPA concentration of 0.114 mg/1.5 ml with 1 ml enzyme solution. V_{max} and K_m values were 27.32 U/min and 0.024 mg/ml respectively (Figure 2). Among different substrates used the enzyme has shown maximum activity of 0.18 U with glycerol tributyrate compared to other substrates and one unit of lipase activity was defined as the amount of enzyme releasing one mole of free fatty acids in 1 min under standard assay conditions.

The G5 isolate was also characterized for probiotic properties such as resistance to antibiotics, bile salt resistance, resistance to acidity and acidifying activity. G5 isolate showed distinct pattern of zone of inhibition to the wide range of antibiotics tested and in which, for novobiocin 23.33 (±0.4666), tobramycin 24.3 (±0.4666), colistin 8.83 (±0.8498), cycloserine 7.33 (±0.9380), lincomycin 6.2 (±0.1633), cefuroxime 8.2 (±0.216), carbenicillin 8.7 (±0.2449), erythromycin 32.33 (±1.2472), nitrofurantoin 8.3 (±0.4714), kanamycin 38 (±1.6329), gentamycin 48.6 (±0.476) and chlorotetracyclin 51 (±0.8124) (mean in mm ±

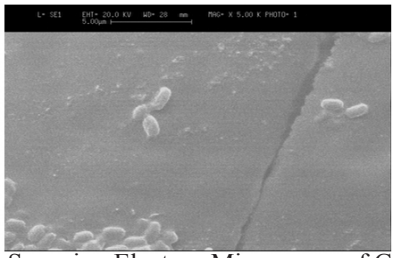

Figure 1. Scanning Electron Microscopy of G5 isolate

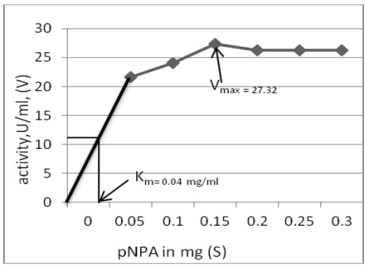

Figure 2. Effect of pNPA concentration on lipase activity

SD) zone of inhibition was observed. The isolate G5 showed maximum resistance to lincomycin with a zone of inhibition (6.2 mm) (±0.1633) and relatively lesser to chlorotetracyclin (51 mm) (±0.8124). Resistance of the isolate to various pH range was determined by plate count method. During the incubation for 1 to 5 h at pH 1, 2 and 3, the G5 isolate was showed optimum growth at pH 3 even after 5h of incubation. While at pH 1 and 2, the survivability was decreased gradually. After 5 h of incubation no growth was observed in pH 1 and 2 (Table 1). In bile resistance test, G5 isolate showed up to 1.5 % survivability and was stable for 4 h and at 2% bile concentration and viability decreased with time of incubation (Table 2). The G5 isolate was also tested for its acidifying activity by measuring the change in pH (Δ pH) after 0, 2, 4 and 6h of incubation and after 2 h of incubation the Δ pH was 0.2 and after 4 h, the Δ pH was 0.5.

Discussion

Outcome of the present study reveals that the G5 isolate would be a promising probiotic candidate with lipolytic activity. The isolate showed maximum lipolytic activity at pH 8 and stabilized over a pH range between 7 and 9. On storage at temperature above 40°C, pancreatic lipases lose their activity, but microbial lipases are more resistant to heat inactivation and thermal stability is one of their desirable characterisitics (Janssen *et al*., 1994), which was corroborated in the present study. The maximum lipolytic activity was found to be at 40°C and was thermally stable at a temperature range between 10°C and 60°C but stability decreased beyond 70°C. Similar results have been reported for *Lactobacillus*

lipase (Padmapriya *et al*., 2011). In many cases, lipases appear to obey Michaelis–Menten kinetics. Michaelis–Menten kinetics is characterized by two parameters, K_m and V_{max}. The latter is the maximum rate of reaction and K_m is a measure of the affinity of an enzyme for a particular substrate. A low K_m value represents high affinity. For an enzyme, K_m values range widely (Guit *et al.*, 1991). The lipase from G5 isolate showed maximum activity at a pNPA concentration of 0.150 mg/1.5 ml with 1 ml enzyme solution. V_{max} and K_m values were 27.32 U/min and 0.04 mg/ml, respectively. However, K_m and V_{max} of a purified lipase of *Pseudomonas fragi* CRDA 323 were 0.7 mg/ml and 0.97 x 10^{-3} U/min, respectively (Pabai *et al.*, 1995). For a *Pseudomonas cepacia* lipase, K_m and V_{max} values were 12 mM and 30 mM/min, respectively when pNPP was used as a substrate (Pencreac'h and Baratti, 1996). The production of lipase is mostly inducer dependent, and in many cases, oils act as good inducers of the enzyme. The lipolytic activity of lipase from the G5 isolate was greatly varied with different substrates tested. The enzyme showed highest activity in presence of glycerol tributyrate; moderate to the castor and olive oils; and least to ghee, tween 20 and palm oil. However, Gao and Breuil (1995) obtained high levels of lipase activity when vegetable oils (olive, soyabean, sunflower, sesame, cotton seed, corn and peanut oil) were used as the carbon source and maximum lipase production observed with olive oil, indicating the variation in affinity of the enzyme towards different substrates.

According to the guidelines of probiotic organisms as reported by FAO/WHO, widely used *in vitro* tests are resistance to gastric acidity and bile compounds based on both survival and growth studies. In addition, evaluation of antibiotic resistance, acidifying activity and other tests are also in use. Before reaching the human intestine to colonise and to establish metabolic activity, the probiotic isolate must travel through stomach and survive in the acidic pH 1.5 to 2 (Dunne *et al*., 2001). Survivability of the isolate in low pH and high levels of bile allows the probiotics to survive through gastric transient in the stomach and helps to reach intestine and thus balancing gut flora. As the rate of establishment increases, the microbes enhance their active metabolic pathways resulting in the release of some organic molecules which are beneficial to host. Therefore, pH 3 is set as standard for acid tolerance while screening for probiotics (Shah and Dave, 1998; Sahadeva *et al.*, 2011). In the present study, the G5 isolate showed luxuriant growth at pH 3 even after 5 h incubation. But the number of viable cells began to decrease at pH 1 and 2 after 1h incubation. Survivability of cells decreased drastically after 3 h incubation at pH 1 and 2. In one of the studies, acid tolerance of *L. acidophilus* and *Bifidobacterium* strains was at pH 2 (Goderska and Czarnecki, 2007). However, in an another study, that there were no viable cells at pH 2 after 30 min, but, at pH 3, the number of viable *Lactobacillus acidophilus* La-5 cells were decreased in time, whereas in case of *Lactobacillus casei* 01, the number of viable cells found to be constant (Both *et al*., 2010). Although acid tolerance of *Lactobacillus* sp. was characterized by other workers, the present G5 isolate showed luxuriant growth at pH 3 and survived at even lower pH for 3 h. Thereby the G5 isolate fulfilled one of the essential characteristic required to consider as probiotics.

High levels of bile salt in the body aids in lipid metabolism, but at the same time it denatures the membranes of probiotics and thereby diminishes its benefits to host. However, survivability to high levels of bile allows lactic acid bacteria to survive in the small intestine. During first hour of digestion the level of bile is almost equal to 2% oxgall. Afterwards 0.3% is the nomal level of bile. Hence, 0.3% was considered as critical concentration for screening resistant strains to be used as probiotics (Gilliland *et al*., 1984). In bile resistance test, G5 isolate showed resistance to 0.5 and 1.0% of bile and the survivability of the cells was almost stable up to 4 h incubation. At 2%, the survivability was decreased with increased incubation, but after 6 h of incubation 1/5th of the cells compared to 1h of control (without bile salt) were survived. In another study, similar results were obtained for *Lactobacillus* spp. isolated from goat's milk (Tambekar and Bhutada, 2010). The results of acidifying test showed that the G5 is a fast to medium acidifying isolate since it decreased the pH by 0.4 units in 3-5 h incubation.

The Resistance of probiotics to antibiotics helps in replenishing normal microflora in an individual after an antibiotic therapy. Probiotics have been used to prevent antibiotic associated diarrhoea, which results in imbalance in the gut microflora caused by antibiotic therapy (Adagbada *et al*., 2012). In the present study G5 isolate was tested with a series of antibiotics and zone of inhibition was recorded after 48 h incubation. The isolate showed less resistance to chlorotetracycline, gentamycin, kanamycin, erythromycin with 51 ± 0.8124, 48.6 ± 0.4760, 38 ± 1.6329 and 32.33 ± 1.2472 zone of growth inhibition (mean in mm ± SD) respectively. Whereas it was resistant to colistin, carbenicillin, nitrofurantoin, lincomycin, cycloserine, and cefuroxime with less

zone of inhibition. However, *L. acidophilus* was also found to be resistant to colistin, gentamicin, kanamycin, nitrofurantoin and streptomycin (Goderska and Czarnecki, 2007). While the present isolate showed resistance to different spectrum of antibiotics would indicate the usage of G5 isolate even during antibiotic therapy. Further study is required to exploit these characteristics in appropriate formulation of probiotic bacteria in terms of species, number of CFU/ml, phase of growth and kind of adjunct to be used for delivery for the betterment of gastrointestinal health.

Conclusions

Present study was aimed to partially purify and characterize the lipase from *Lactobacillus* sp isolated from goat's curd of shepherd population. Probiotic characterization of the highest lipase producing isolate (G5) was also carried out. Lipase is one of the extensively used enzyme in the field of food and medicine. In this context, probiotics with lipolytic activity gains much significance. It can be used as an enzyme supplement in diet which confers health benefits.

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