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Hydrolysis of Proteins in Cheddar Cheese Whey and Utilization as a Growth Medium for Lactic Acid Bacteria

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Cheddar cheese whey is the major by-product obtained during the manufacturing of cheese. Cheese whey was hydrolysed with four different proteolytic enzymes, pepsin, Trypsin, Papain and rennet. Amongst this four enzymes used, papain gave highest proteolytic activity. Further, papain concentration and time for hydrolysis were optimized. Whey was hydrolysed with a range of papain concentration (50-90 mg/10 ml). Growth parameters of four lactic acid bacteria (*L. helveticus* MTCC 5463, *S. thermophilus* MTCC 5461, *L. mesenteroides* and *L. lactis)* were checked in hydrolysed whey. Mean viable counts were found to be highest in whey hydrolysed with 50mg/10ml concentration for 4 h.

Keywords: Cheddar cheese whey, papain, lactic acid bacteria.

Whey is transparent watery liquid which remains after removal of fat and casein from milk. Technically whey is termed as milk serum, however, practically it is greenish-yellow residual fluid obtained on coagulation of milk. (Agustriyanto and Fatmawati 2009). Whey is a byproduct of the cheese- and casein-manufacturing industry, with an annual worldwide production of about 115 million. Utilization of this valuable by-product leads to the financial advantage in dairying, as well as, it reduces the organic load and treatment costs on the effluent treatment plant by reducing the consumption of electrical energy (Mallik and Kulkarni, 2010).

Fermentation of whey by Lactic Acid Bacteria (LAB) usually focuses on the production of lactic acid. Alternatively, whey or whey permeate has the potential as a culture medium for the propagation of dairy cultures. Whey or UF whey permeate are cheap and readily available sources for use as fermentation media. (Parente and Zottola, 1991)

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Bhuvaneshwari and Sivasubramanian (2011) investigated the treatment of the organic wastes using microbiological process for effective usage of waste and to develop value added products from it. The organic wastes used in this processes were domestic wastes, vegetable wastes, fruit wastes, bakery wastes and whey. They used *Lactococcus lactis subsp lactis for* synthesis of lactic acid. The optimal production of lactic acid and bacterial growth were 35.45 g/l and 1.34 g/l respectively from whey by *Lactobacillus rhamnosus*.

Lavari *et al.* (2014) explored double use of cheese whey (culture medium and thermoprotectant for spray drying of lactobacilli) for their capacity to produce biomass of *Lb. paracasei* JP1, *Lb. rhamnosus* 64 and *Lb. gasseri* 37. All the cultures were found to ferment the media and at highest biomass production, the viability of the cultures ranged between 8-9 log cfu/g of biomass. Therefore, above literature suggested that whey can be used as a fermentation medium.

Lactic acid bacteria (LAB) biomass is one such product that has numerous applications in the pharmaceutical and food industries. Lactic acid bacteria have complex growth factor requirements including B vitamins, several amino acids, and

purine and pyrimidine bases. Whey protein hydrolysate (WPH) is a potential nutrient supplement which is readily useable by microbes. It can be readily produced by dairy processors on-site, either by direct hydrolysis of whey, or by the hydrolysis of WPC. (Fitzpatrick *et al*., 2001)

Therefore, present study was contemplated to investigate effect of hydrolysis of proteins in Cheddar cheese whey on growth of lactic acid bacteria.

MATERIALS AND METHODS

Uunsalted cheddar cheese whey was collected from Amul Dairy, Anand.

Papain (30,000 USP units/mg), pepsin (1:10,000), trypsin (Bovine pancreas) and rennet (from Mucor miehei, Type-II) were purchased from Himedia (Mumbai, India).

The pure strains of *Lactobacillus helveticus* MTCC 5463, *Streptococcus thermophilus* MTCC 5461, *Leuconostoc mesenteroides* and *Lactococcus lactis* were acquired from the culture collection of Dairy Microbiology Department, Anand Agricultural University, Anand, Gujarat. The strains were activated from its frozen form (stored in 10% glycerol at -80 °C) by giving one transfer in respective broths. This was followed by 2 successive transfers into sterile respective broths under incubation conditions of 37ºC for 12 h.

High performance liquid chromatography

Whey samples were centrifuged at 14,000 rpm (Eppendorf Centrifuge, US) for 10 min at 4°C. Then, supernatants were collected and ultrafiltered through 3 kDa cut off membranes (Merck Millipore) at 2000 rpm for 15 min at 10°C. Permeates were collected, filtered through a 0.45 ¼m disposable hydrophilic filter and 20 µl water soluble extract (WSE) was injected in the HPLC (Shimadzu, Japan) through microinjector (Hamilton, Switzerland). In the present study RP-HPLC was used for the separation purpose of different peaks. RP-HPLC (Shimadzu LC-20, Japan) was performed as described by Rodríguez-Figueroa *et al*. (2012); Papadimitriou *et al.* (2007). A binary gradient HPLC system was used fitted with C18 column (ZicHilic) white pore analytic column (5¼, 250x4.6 mm). Sample was applied using microinjector with 20 ¼l loop. Eluent-A was 0.1 % v/v TFA in deionised water and Eluent-B was 0.1% v/v TFA in mixture of 60:40 acetonitrile and deionised water. Separation was conducted at room temperature at flow rate 0.8 ml/min with eluent-A for 5 min and linear gradient, from 0% to 80% of eluent-B, for 5 min. The column finally eluted with 100% eluent-B for 10 min. Absorbance of elute was monitored at 214 nm using variable wavelength spectrophotometric detector.

Enzymatic hydrolysis

To 40 ml of 75 °C heated cheddar cheese whey, 2.1 ml of 0.1% of each enzyme solution was added. (Kananen *et al.,* 2000) Optimum pH for Pepsin was 2, for Trypsin 8 and for rennet and Papain 6.5 was adjusted. In order to facilitate peptic, tryptic and rennet hydrolysis temperature was kept 37 °C constantly for a period of 5 h, whereas for papain hydrolysis 50°C (Wroblewska *et al.,* 2004) for a period of 5 h. After hydrolysis hydrolysed whey was heated at 95ºC for 10 min in order to inactivate the enzyme. (Kim *et al.,* 2007). Among these four enzymes, papain was able to hydrolyse proteins in whey therefore further optimization of papain treatment was done. In order to optimize papain concentration, 10– 100 mg/10ml papain concentration were added to whey and measure of extent of hydrolysis was done hourly. Measure of hydrolysis was estimated by OPA (o-Phthalaldehyde) method. (Church *et al.,* 1983).

RESULTS AND DISCUSSION

Hydrolysis of Whey Protein by proteolytic enzymes

Four proteolytic enzymes were used. i.e., papain, trypsin, pepsin and rennet. Measure of proteolysis was done spectrophotometrically and data of optical density are shown in Table 1.

The table showes that compared to control absorbance increased significantly in all enzymes used to hydrolyse whey. Among all enzymes highest increase in absorbance was found in the whey hydrolysed with papain. As incubation period increased extent of hydrolysis increased. Maximum hydrolysis of whey was obtained after 6 h of incubation when hydrolysed with papain.

Lieske and Konrad (1996) selected the cysteine proteinase papain for hydrolysis because it has a broad specificity and the ability to digest whey protein at a natural pH and in presence of

known interactions of whey constituents. The best performance given by papain enzyme may be attributed to its broad specificity and ability to digest whey protein at a natural pH.

Due to its broad specificity and high rate of hydrolysis, papain was selected to hydrolyze proteins in whey.

Data in Table 2 shows optimization of papain concentration. As incubation time increased change in optical density was statistically significant. Compared to control within treatments the value of absorbance differed significantly from each other. Increase in papain concentration resulted in increase in absorbance up to 4 h. After that there was decrement in absorbance. Absorbance of 1.196 was obtained after 4 h of hydrolysis in 90 mg/10ml added whey. Highest hydrolysis was obtained after 4 h of hydrolysis.

Table 1. Proteolysis of whey using different enzymes

Time	Absorbance obtained using different enzyme				
(Hours)	Control	Pepsin	Trypsin	Papain	Rennet
0	0.226	0.274	0.260	0.323	0.278
1	0.257	0.259	0.246	0.464	0.315
2	0.269	0.289	0.293	0.567	0.246
3	0.258	0.276	0.360	0.614	0.232
4	0.275	0.272	0.296	0.603	0.282
5	0.246	0.342	0.340	0.626	0.274
6	0.244	0.329	0.319	0.663	0.289

Source of variation	Treatment (T)	Incubation period(P)	Interaction(Tx P)
SEm	0.01	0.01	0.03
Test (P < 0.05)	*	*	*
CD	0.03	0.04	0.08
CV%		14.82	

Table 2. Proteolysis of whey using different concentration of papain

Papain conc.	Time (Hours)					
(mg/10ml)	0	1	2	3	4	5
0	0.261	0.319	0.261	0.294	0.312	0.232
10	0.434	0.713	0.744	0.744	0.740	0.729
20	0.517	0.767	0.747	0.780	0.866	0.819
30	0.604	0.790	0.860	0.861	0.957	0.886
40	0.637	0.790	0.826	0.908	0.979	0.945
50	0.621	0.805	0.880	0.946	1.033	0.952
60	0.626	0.836	0.895	1.020	1.104	1.131
70	0.641	0.838	0.911	1.028	1.091	1.100
80	0.641	0.864	0.969	1.046	1.188	1.125
90	0.663	0.886	0.947	1.033	1.196	1.095
100	0.665	0.884	1.000	1.058	1.185	1.099

Source of variation	Treatment (T)	Incubation period(P)	Interaction(T x P)
SEm	0.02	0.01	0.05
Test (P < 0.05)	*	*	*
CD	0.03	0.04	0.08
CV%		9.97	

keeping this hypothesis in mind growth characteristics of lactic acid bacteria were checked in papain hydrolysed whey. 50 – 90 mg/10ml papain concentration for 4 h of hydrolysis at 50ºC was checked for growth of lactic acid bacteria. Whey was hydrolysed with different concentration of papain. Time allowed for hydrolysis was 4 h. Range of papain concentration chosen was on the basis of obtained optical density after hydrolysis. After hydrolysis enzyme was inactivated by heating at 95ºC for 10 min. Whey was cooled to 37°C and lactic cultures (*Lb. helveticus* MTCC 5463, *Streptococcus thermophilus* MTCC 5461, *Leuconostoc mesenteroides* and *Lactococcus lactis*) were inoculated at the rate of 2% (v/v), incubated at 37°C and samples were withdrawn after 0, 6, 12 and 24 h of incubation. All the samples were analysed for changes in pH and viable counts were measured only at 12 h of incubation. Whey without papain hydrolysis was kept as control.

It appears from the results (Table 3) that treatment (hydrolysis of whey) has significant effect on changes in pH during storage. After 24 h of growth of *Lb. helveticus* MTCC 5463, *Streptococcus thermophilus* MTCC 5461 and *Leuconostoc mesenteroides* highest drop was found in whey hydrolysed with 50mg/10ml of papain. Among all papain treatment given, 50 ml/ 10 ml papain hydrolysed whey had pH of 2.74, 2.88 and 3.30 for *Lb. helveticus* MTCC 5463, *Streptococcus thermophilus* MTCC 5461 and *Leuconostoc mesenteroides* respectively after 24 h of incubation. It can be seen from the data that drop in pH was significant as the incubation period increased. Between treatment and period there was statistically significant interaction. 24 h growth of *Lactococcus lactis* resulted in highest drop of pH in whey hydrolysed with 90mg/10 ml of papain. However, this drop in pH was at par with that of whey hydrolysed with 50mg/10 ml of papain. It can be seen from the data that drop in pH was significant as the incubation period increased. However, interaction between treatment and period was again statistically non-significant.

Data in Table 4 shows that hydrolysis has impact on total viable count. Among all treatments after 12 h of incubation highest count of all four lactic cultures were obtained in 50 mg/10ml papain hydrolysed whey. Mean viable counts were 8.86 log cfu/ml, 8.68 log cfu/ml, 8.63 log cfu/ml and 8.93 log cfu/ml for *Lb. helveticus* MTCC 5463, *Streptococcus thermophilus* MTCC 5461, *Leuconostoc mesenteroides* and *Lactococcus lactis* respectively.

Lactic acid bacteria have complex growth factor requirements including B vitamins, several amino acids, and purine and pyrimidine bases. Whey protein hydrolysate (WPH) is a potential nutrient supplement which is readily useable by microbes. It can be readily produced by dairy processors on-site, either by direct hydrolysis of whey, or by the hydrolysis of WPC (Fitzpatrick *et al*., 2001).

Probiotic bacteria grow slowly in milk because of a lack of proteolytic activity, and the usual practice is to add yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) to reduce the fermentation time. *Lactobacillus delbrueckii* ssp. *bulgaricus* produces essential amino acids owing to its proteolytic nature, and the symbiotic relationship of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* is well established; the former organism produces amino nitrogen for the latter organism. Such starter cultures may necessitate the incorporation of micronutrients (peptides and amino acids) through whey powder (WP), whey protein concentrate (WPC), acid casein hydrolysate (ACH), or tryptone for reducing the fermentation time and for improving the viability of probiotic bacteria. (Dave *et al.,* 1998)

Vasala *et al.,* (2005) studied pretreatment of whey-protein-containing media by the proteolytic microbe *B. megaterium. Lactobacillus salivarius* ssp. *salicinius*, a lactic acid bacterium species that can grow at high salt concentration, was used to ferment lactic acid in cheese whey (with 3 g l^{-1} whey protein content) and lactose mother liquor (90 g l^{-1} lactose, 9 g l^{-1} proteins, 30 g l^{-1} minerals). The contribution of protease enzymes or proteolytic microbes to acid production by lactobacilli was examined. Efficient conversion of lactose to lactic acid was obtained in the presence of additional proteolytic activity.

In 1998, Gomes and co-workers found that the growth and acid production of *B. lactis* in milk were affected by the addition of proteinase-mediated hydrolyzate and, to a lesser extent, by neutrase mediated hydrolyzate; a higher degree of hydrolysis of either hydrolyzate resulted in greater

Table 3. Changes in pH during growth of lactic cultures

Incubation period (h)	*Lactobacillus helveticus* MTCC 5463 (mg/10ml)Papain Concentration						*Streptococcus thermophilus* MTCC 5461 (mg/10ml)Papain Concentration						*Leuconostoc mesenteroides* (mg/10ml)Papain Concentration						*Lactococcus lactis* (mg/10ml)Papain Concentration					
	C	50	60	70	80	90	C	50	60	70	80	90	C	50	60	70	80	90	C	50	60	70	80	90
0	6.19	5.64	5.63	5.62	5.63	5.62	6.50	5.93	5.91	5.91	5.92	5.91	6.40	5.84	5.83	5.82	5.85	5.83	6.29	5.69	5.71	5.57	5.70	5.70
6	3.98	3.88	3.96	4.02	4.05	4.07	4.27	4.05	4.15	4.16	4.18	4.14	5.23	4.51	4.85	4.80	4.83	4.81	5.23	4.71	5.01	4.55	5.04	4.85
12	3.15	3.09	3.18	3.27	3.33	3.43	3.49	3.27	3.31	3.38	3.44	3.46	4.09	3.84	3.78	3.81	3.79	3.67	4.91	4.09	4.23	4.16	4.19	4.01
24	2.78	2.74	2.82	2.91	2.97	3.12	3.07	2.88	2.93	2.98	3.03	3.11	3.40	3.30	3.32	3.35	3.32	3.31	3.84	3.68	3.73	3.83	3.72	3.60

Source of Variation	Treatment (T)	Incubation period(P)	Interaction (T X P)	Treatment (T)	Incubation period(P)	Interaction (T X P)	Treatment (T)	Incubation period(P)	Interaction (T X P)	Treatment (T)	Incubation period(P)	Interaction (T X P)
SEm	0.04	0.03	0.07	0.04	0.03	0.07	0.11	0.09	0.23	0.02	0.02	0.04
Test (P < 0.05)	*	*	*	*	*	*	NS	*	NS	*	*	NS
CD	0.10	0.08	0.21	0.10	0.08	0.20	NS	0.26	NS	0.06	0.05	NS
CV%	3.18	2.95	8.72	19.76								

Table 4. Changes in total viable count during growth of lactic cultures

Incubation period (h)	*Lactobacillus helveticus* MTCC 5463 (mg/10ml)Papain Concentration						*Streptococcus thermophilus* MTCC 5461 (mg/10ml)Papain Concentration						*Leuconostoc mesenteroides* (mg/10ml)Papain Concentration						*Lactococcus lactis* (mg/10ml)Papain Concentration					
	C	50	60	70	80	90	C	50	60	70	80	90	C	50	60	70	80	90	C	50	60	70	80	90
12	8.73	8.86	8.64	8.66	8.62	8.64	8.57	8.68	8.60	8.68	8.66	8.67	8.43	8.63	8.57	8.48	8.53	8.59	8.70	8.93	8.71	8.62	8.61	8.49

Source of Variation	Treatment (T)	Treatment (T)	Treatment (T)	Treatment (T)
SEm	0.05	0.08	0.15	0.06
Test (P < 0.05)	*	NS	NS	*
CD	0.14	0.24	0.46	0.20
CV%	0.93	1.58	3.06	1.28

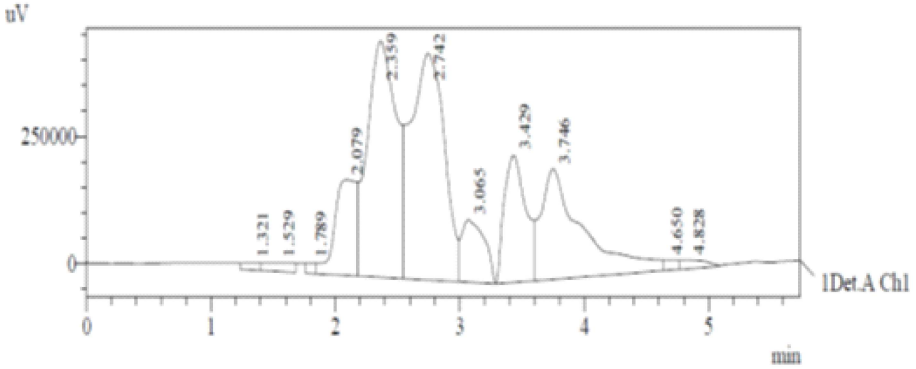


Fig. 1. HPLC chromatogram of raw whey

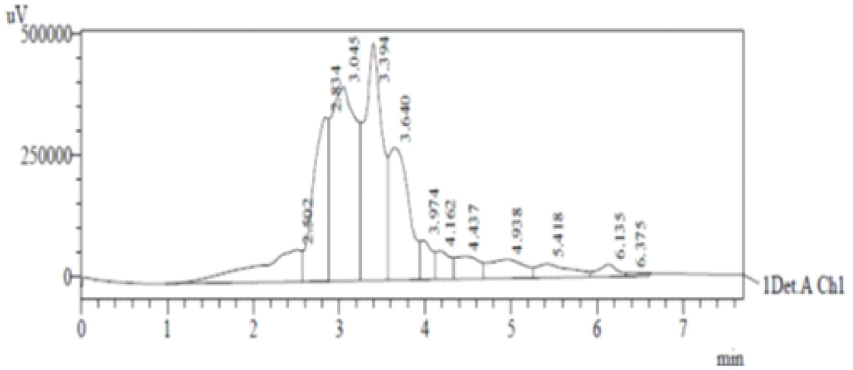


Fig. 2. HPLC chromatogram of papain hydrolysed whey

Peptides production through papain hydrolysis analysed by RP-HPLC

In order to confirm papain hydrolysis in whey. HPLC of raw whey and hydrolysed whey was compared. Results are given in Fig 1 and Fig 2

The ability of papain to hydrolyse the whey proteins was also confirmed by comparing HPLC chromatogram (Shimadzu LC-20, Japan) of raw whey and that of the papain treated whey. The chromatogram produced for raw whey and papain hydrolysed whey is presented in figure 1 and 2 respectively. A comparison of papain hydrolysed whey protein was shown by peptides fraction recording percentage area under peak from the HPLC chromatographic profiles. Peptides produced by papain hydrolysed whey was maximum compared to raw whey (control).

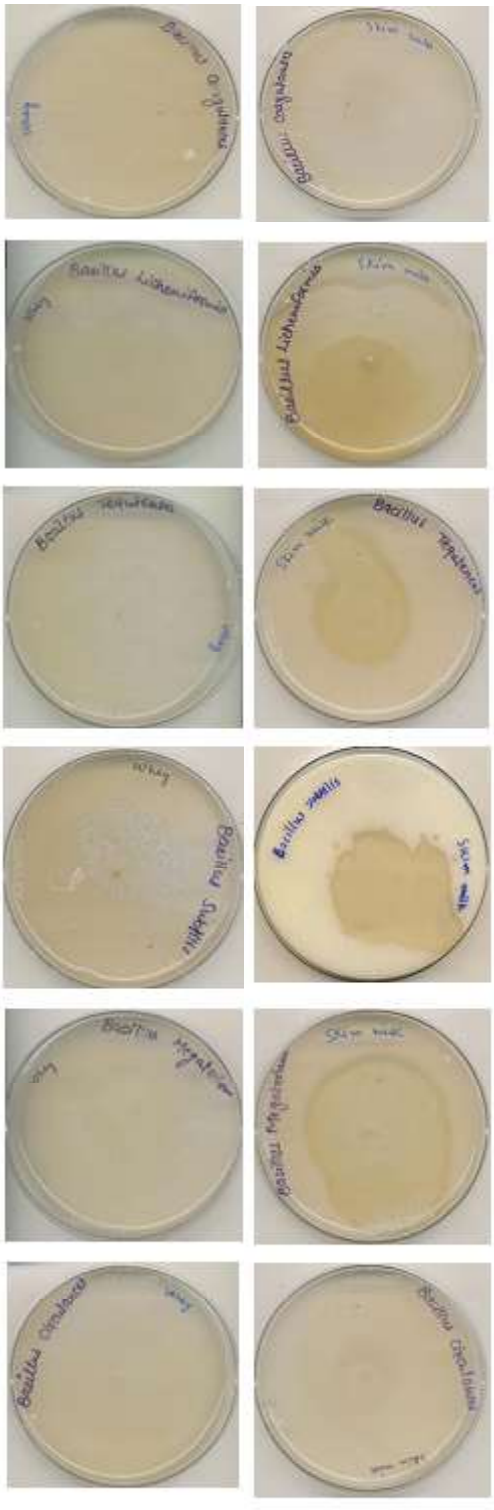
Hydrolysis of whey protein with proteolytic cultures or their hydrolysate

For hydrolysis of whey proteins with proteolytic cultures *Bacillus subtilis, Bacillus megaterium, Bacillus circulans, Bacillus coagulans, Bacillus licheniformis* and *Bacillus tequilensis* were inoculated in whey at the rate of 2% incubated at 37ºC up to 24 h and samples were analyzed for extent of whey protein hydrolysis. Change in optical density through proteolysis by cultures was measured by OPA method. Samples were withdrawn after 4, 8, 12 and 24 h. Results shows that even after 24 h there was no change in optical density. This might be due to inability to break down whey protein.

In order to test the inability of the procured cultures to hydrolyse whey protein agar plate method was done. All the cultures were spotted on both skim milk agar plate and whey agar plate. After 12 h of incubation all the cultures produced a clear hydrolysed zone on skim milk agar plate. Whereas no zone was obtained on whey agar plate (Fig: 3)

Growth of lactic acid bacteria in papain hydrolysed whey

Hydrolysis of whey with papain produced higher amount of small peptides and amino acids. These smaller fragments can easily be utilized as a source of nitrogen by lactic acid bacteria. So



Whey agar Skim milk agar

Fig. 3. Proteolytic zone formation by *Bacillus* sp. on whey agar and skim milk agar plate

biomass increase and greater acid production. The growth of *B. lactis* on unsupplemented milk was poor. When supplemented with milk hydrolyzate, growth was improved; the exponential phase of growth occurred during the first 8 to 10 h following inoculation. (Gomes *et al.,* 1998)

The efficiency of addition of milk hydrolyzate on *B. lactis* in coculture with *L. acidophilus* could be related to the directly accessible pools of free amino acids or bioactive peptides milk hydrolyzate obtained from treatment of milk with proteinase (MHP) as a potential source of nitrogen for cell growth and concomitant proteolytic activity of *L. acidophilus*. (Gomes *et al.,* 1998)

Whole whey was hydrolysed for 12 h with Protease 2A and Trypsin using two concentrations of enzyme (20 and 40 g/kg protein).Samples were assayed for total viable counts of adventitious microflora that survived thermization, total acidity, total concentration of free amino acids, peptide profile and overall degree of hydrolysis. The highest total concentration of free amino acids was observed when hydrolysis was effected by Protease 2A, and the major variations in amino acid qualitative composition occurred between 2 and 6 h (Pintado *et al.,* 1999)

Above studies supported that hydrolysis of whey protein or addition of whey protein hydrolysate has positive impact on growth of lactic acid bacteria.

CONCLUSION

It is evident from the above study that, hydrolysis of whey with papain enzyme resulted in significant changes in growth parameters of the lactic cultures. Amongst all treatments, hydrolysis with 50 mg/10 ml for 4 h at 50ºC was found to be the most conducive for the growth of all four lactic cultures. Therefore, simply by hydrolysing with papain this cheap by-product of cheese industry can be very useful as a growth medium for lactic acid bacteria.

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