Results & Discussion
5. RESULTS AND DISCUSSION

The results obtained in the present investigation on the isolation of lactic acid bacteria from food products (dairy products and jams), screening of LAB isolates for bacteriocin activity, optimization of cultural and environmental parameters for bacteriocin production, partial purification and characterization of the bacteriocin produced by selected lactococcal isolate (*Lactococcus lactis* subsp. *lactis* CCSUB202) and effectiveness of the bacteriocin in controlling the growth of lactic and non-lactic acid bacteria are presented in Tables 8 to 37 and Figures 12 to 47.

5.1 Isolation of LAB from food products: - A total number of 162 well isolated colonies (Table 6) on MRS Plates obtained by plating 137 food product samples from 31-buffer milk, 16-cow milk, 16-goat milk, 18-curd, 22-cheese, 10-pasteurized milk (Parag, Kailash, Gopal ji, Amul, Madhusudan, Saras, Param), 5-cream, 8-sweets (mava-burf, mava-ladoo, ghevar), 5-apple jam and 5-mango jam, collected from various cattle yard, dairy and sweet shops in Meerut region were transferred to MRS broth, incubated at 37°C and subsequently examined microscopically for shape and arrangement of cells after Gram's staining and gas production. The gas forming Gram-positive cocci and lacticular shaped cells occurring in pairs and chains were counted as *Leuconostoc* and the non-gas forming Gram-positive rods were considered as *lactobacilli*. The Gram positive cocci that appeared in pairs short chains (Figure 12) without any gas production, catalase negative, therefore, were not considered as the species of *Staphylococcus* or *Micrococcus*, which are catalase positive were taken to be *lactococci*. As per the characteristics described by Collins et al., 1984 the isolated strains were identified.

The distribution of lactic acid bacteria comprising *Lactococcus*, *Lactobacillus* and *Leuconostoc* isolated from different food products are shown in Tables 6 and 7, Figure 13.
An appraisal of the data recorded in Tables 6 and 7 indicate that the lactococci constituted the majority of the lactic acid bacteria examined followed by lactobacilli and Leuconostocs. The respective numbers of Lactococcus, Lactobacillus and Leuconostoc isolates were 91, 48 and 23 and similarly the per cent distribution for the three genera was 58.17%, 29.6% and 14.19%.

The percent distribution of Lactococcus, lactobacilli and Leuconostocs in raw milk (buffalo milk, cow milk and goat milk), curd, cheese pasteurized milk, cream and sweet are presented in Figure 13. The per cent distribution of Lactococcus was 51.2 (raw Milk), 55 (curd), 76 (cheese), 54.5 (pasteurized milk), 83 (cream), 55.5 (sweet) and 40 (Jams). The corresponding values for lactobacili and Leuconostoc in respective products were 32 and 16.2, 30 and 15, 11.5 and 15.3, 27.2 and 18, 16.6 and 0, 34 and 11 and 60 and 0. (Figure 13). It is be observed from the Tables 6 and 7 that out of the total number of 91 Lactococcus isolates, 41 isolates (45%) were from raw milk, 11 isolates (12%) were from curd, 19 isolates (20.8%) were from cheese, 6 isolates (6.5%) were from pasteurized milk, remaining 14 isolates (15.3%) were from cream, sweets and jam samples.

The fact that milk is free from Lactobacillus when it leaves the udder but gets easily contaminated with lactobacilli with feeds, silage, dust, dairy utensils etc. Kandler and Weiss. (1986), Cock and Stouvenel, (2006), may also hold true for Leuconostocs, which also occur along with lactobacilli in plant environments (Garvie, 1986 a, b). The Lactococcus, Lactobacillus and Leuconostocs are generally present in grains, dairy and meat products and vegetables (Dave and Prajapati, 1994; Choi et al., 2000; O’Sullivan et al., 2002; Liu, 2003; Park et al., 2003; Sharma and Garg, 2005; Sharma et al., 2006). In the present study Lactococcus, Lactobacillus and Leuconostocs could be isolated from cheese, similar observations were earlier made by Prentice and Brown (1983) and Litopoulos-Tzontaki et al. (1998).

The isolation of lactic acid bacteria from several other food products has also been described including Franz et al., 1990 isolate Lactobacillus
plantarum BFE905 from “Waldorf” salad. Leisner et al., 1999 isolated 92 strains of lactic acid bacteria from Malaysian food ingredient, chilli Bo. Moreno et al., 1999 isolates 167 strains of Lactococcus lactis from different dairy products.

Many other researchers have isolated lactic acid bacteria from different food products (Hammer, 1993; Salminen and Von Wright, 1983; Roissart, 1994; Boonmee et al., 2003; Ziadi et al., 2005; Do-Won et al., 2006). The majority of the investigations are carried out of ready-to-eat foods including cheese (Loessner et al., 2003) and meat products (Mattila et al., 2003; Lungu and Johnson, 2005). however, fish (Nilsson et al., 1997) and vegetables (Bennik et al., 1998). Lactobacillus spp., Lactococcus spp., and Leuconostoc were isolated from airag (fermented mare’s milk) by Batdorj et al. (2003), Ying et al. (2004) and Batdorj et al. (2006).

6.2. Screening of lactic acid bacteria isolates for their antibacterial activity: - 162 lactic acid bacteria isolates obtained from different food products were screened for their antibacterial activity against Lactococcus lactis subsp. lactis MTCC3038 and various non-lactic cultures by three different assays viz: preliminary antagonism by Waksman and Lechevalier method (1962), agar-overlay method (Yang, Johnson and Ray, 1992) and agar-well assay (Barefoot and Klaenhammer, 1983).

The observations made in these studies are presented in Tables 8 to 10 and Plates 14A, 14B and 15. Out of the total number of 162 lactic acid bacteria isolates, 92 isolates showed antibacterial activity against Lactococcus lactis subsp. lactis MTCC3038 which was used in the preliminary antagonism method (Waksman and Lechevalier, 1962) and agar-well assay (Tables 8 and 9). The inhibition by 25 isolates was very strong against Lactococcus lactis subsp. lactis MTCC3038 as diameter of the zone of inhibition ranged from 10-16 mm. Among them lactococcal isolate CCSUB202 exhibited a stronger inhibitory activity (diameter of zone of inhibition was 16 mm) against Lactococcus lactis subsp. lactis MTCC3038. None of the remaining 70 lactic
acid bacteria isolates showed inhibitory activity against Lactococcus lactis subsp. lactis MTCC3038. The Lactococcus CCSUB202 isolate displayed moderate to very strong antibacterial activity against non-lactic cultures in the agar-well assay.

However, among them the inhibition by the Lactococcus CCSUB202 was relatively much stronger against Listeria monocytogenes MTCC657 and Listeria monocytogenes MTCC1143 followed by Bacillus subtilis MTCC441, Salmonella typhi MTCC734, Shigella sonnei MTCC2957, Escherichia coli MTCC119, Staphylococcus aureus MTCC96, Clostridium perfringens MTCC450, Enterobacter faecalis MTCC439, Enterobacter aerogenes MTCC111, Streptococcus pneumoniae MTCC1935, Pseudomonas aeruginosa MTCC2581, Micrococcus MTCC108, Klebsiella pneumoniae MTCC109 and Proteus vulgaris MTCC744.

In the agar-well assay, the centrifuged and filter sterilized cell free supernatants of Lactococcus CCSUB202 isolate grown in MRS broth (pH 8.5) at 37°C for 24 h were used in checking the antibacterial activity against Lactococcus lactis subsp. lactis MTCC3038 and all non-lactic indicator strains. The pH of the culture supernatant was in the range of 4.0 to 4.5 and the results are given in Tables 9 and 10 respectively.

Lactococcus CCSUB202 strain showed the inhibition of the lactic strain (diameter of inhibitory zone 16 mm) against Lactococcus lactis subsp. lactis MTCC3038 in agar-well assay (Table 9). The Lactococcus CCSUB202 strain displayed a very strong inhibitory activity against Listeria monocytogenes MTCC657, Listeria monocytogenes MTCC1143, Bacillus subtilis MTCC441, Staphylococcus aureus MTCC96, Clostridium perfringens MTCC450, when tested by agar-well assay (Table 10). However, remaining 24 isolates of LAB showed a moderate zone of inhibition against the strain of Listeria monocytogenes MTCC657, Listeria monocytogenes MTCC1143, Bacillus subtilis MTCC441, Staphylococcus aureus MTCC96, Clostridium perfringens MTCC450 in agar-well assay.
The inhibition of *Lactococcus lactis* subsp. *lactis* MTCC3038 and non-lactic indicator strains by the *Lactococcus* CCSUB202 in the three assay systems has also been shown in Figure 16.

Agar overlay (Yang et al., 1992) method is one of the most sensitive and widely used bioassays for the detection of microbial antagonism. The antagonistic activity observed against various indicator strains in this assay is the cumulative effect of all the antimicrobial metabolites secreted by test culture (producer strain) into the medium that get concentrated and diffused around the colony of the test culture much before the indicator is added. Out of 162 isolates, the failure of 70 LAB isolates to inhibit lactic indicator strains (*Lactococcus lactis* subsp. *lactis* MTCC3038) in this method reveals that the concentration of antibacterial substance such as organic acids, bacteriocin secreted by them was not sufficient to antagonize the sensitive culture (*Lactococcus lactis* subsp. *lactis* MTCC3038). The pH of the LAB culture supernatants used in the agar-well assay was in the range of 4.0 to 4.5. In spite of this low pH, the same 70 isolates once again failed to inhibit sensitive culture in agar-well assay, further substantiating the fact that indicator strains was insensitive to the concentration of organic acids produced by the test cultures.

A large number of bacteriocins of lactic acid bacteria have a narrow spectrum of antibacterial activity and are usually active against closely related bacteria. Thus, selection of lactic acid bacteria, which are relatively insensitive to the concentration of organic acids encountered in screening procedures of indicator strains, enhances the probability of the detection of bacteriocin-like activity. Lactic acid bacteria have been widely used as indicator of antagonistic activity while screening isolates belonging to the genera *Lactobacillus* (Garriga et al., 1993; Jimenez-Diaz et al., 1993; Vignolo et al., 1993; ten Brink et al., 1994; Franz et al., 1998; Choi et al., 2000; Faustino Jozala et al., 2005; Chen et al., 2006; Batdorj et al., 2006), *Leuconostoc* (Mathieu et al., 1993; Yang and Ray, 1994a), *Lactococcus* (Kozak et al., 1978; Geis et al., 1983; Morgan et al., 1995; Rodriguez et al., 1985; Moreno et al., 1999; Guerra and Pastrana, 2003).
and *Pediococcus* (Strasser-de-san and Manca-de-Nadra, 1993; Cintas et al., 1995).

Though, all the 92 LAB isolates exhibited antagonistic activities against sensitive strain and both the strains of *Listeria monocytogenes* in the agar overlay assay but examination of the culture supernatants by agar-well assay showed inhibition of sensitive strain and listerial strains by 25 isolates only (the more or less same pH 4.0 to 4.5) of the culture supernatants shows the insignificant role of organic acids in the inhibition of listerial indicator strains by the culture supernatants of 25 isolates. The failure of the 67 isolates to exert antagonistic activity against *Listeria* cultures in the well assay could be due to the insufficient quantities of antibacterial substances present in the culture supernatants used for detecting inhibitory activity or that the antagonistic compounds are secreted only in the agar medium but not in the liquid medium. Schillinger and Holzapfel (1990) observed the inhibition of *Listeria monocytogenes* DSM20600 in the well diffusion assay only by a ten fold concentrated supernatant of *Carnobacterium piscicola* LV61. Although, 65 strains of lactic streptococci exhibited antagonistic effect on the agar but among them only 16 strains showed inhibitory activity in the liquid medium (Geis et al., 1983), similarly, Barefoot and Klaenhammer (1983) reported the detection of inhibitors of some strains of *Lactobacillus acidophilus* only on agar media but not in liquid media. West and Warner (1988) also could not detect the antagonistic activity of *Lactobacillus plantarum* NCDO1193 in a liquid culture, although this strain produced inhibition zone against the other lactic acid bacteria on the agar.

The discrepancy in the observations between agar overlay method and agar-well diffusion assay has been widely reported. Although, 24 strains of *Carnobacteria* showed antagonistic activity in the agar overlay assay, only 18 were found inhibitory to the indicator strains in agar well assay (Schillinger and Holzapfel, 1990). Similarly, Thuault et al (1991) reported that *Cl. tyrobutyricum* was inhibited by 33 strains of *Lactococcus* in the agar overlay method but when the culture supernatants were tested only 4 of them showed inhibition.
zone in the well diffusion assay. Of the 55 strains of *Lactobacillus* exhibiting antibacterial activity in the spot assay, only 6 produced inhibition zones in the well assay (Garriga et al., 1993) while Cintas et al. (1995) reported the inhibition by only 12 isolates of the indicator strains in the well assay even if as many as 55 strains showed antagonistic activities in the agar overlay method.

Although, 25 LAB isolates exhibited a relatively less inhibitory activity against *Bacillus subtilis* MTCC441 and *Clostridium perfringens* MTCC450 De Vuyst 1994 and Stiles (1996) also reported that a bacteriocin nisin is particularly active against *Clostridium*, their spores and against *Listeria monocytogenes*. Yarmus et al. (2000), produced bacteriocin by *Lactococcus lactis* subsp. *lactis* EZ26 (DSM ID-95-131), displays a wide range of inhibition including *Listeria monocytogenes* and *Staphylococcus aureus*. Mauriello et al. (2004) also developed antilisterial bacteriocin from *Lactobacillus curvatus* 32Y. Schobitz et al. (2006), produced bacteriocin like substance, which inhibit *Listeria monocytogenes*.

Most bacteriocin produced by LAB appears to have relatively narrow inhibitory spectra (Klaenhammer, 1988; Kone and Fung 1992; Jack et al., 1995) while some bacteriocins such as nisin and pediocin are active against a wide range of bacterial spectra. Bacteriocin producers have been used as starter cultures in the fermented food for the effective control of spoilage microflora (Mairead et al., 1997). Among the microorganisms inhibited by certain bacteriocins, numerous reports have included the fatal pathogen *Listeria monocytogenes* (Harris et al., 1989; Speelhang and Harlander, 1989; Muriana, 1996; Mauriello et al. 2004; Schobitz et al. 2006). Species of *Bacillus*, *Clostridium*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Mycobacterium* species inhibited by nisin (Daeschel, 1989; Yarmus et al. 2000). Batdorj et al. (2006), produced lactic acid bacteria, supernatant of this bacterium inhibited the growth of several *Lactobacillus* spp. and food-born pathogens including *Escherichia coli*, *Staphylococcus aureus* and *Listeria innocua*. Our results confirmed these findings.
Twenty-five isolates of lactic acid bacteria that produced large inhibition zones against lactic and non-lactic indicator strains in all the three different essays were used for further characterization of the antibacterial substances.

5.3 Identification of lactic acid bacteria isolates: - The detailed taxonomic studies involving various morphological, physiological and biochemical characteristics to identify the LAB isolates to species level are documented in the Tables 11 and 12 the proposed confirming identity of the isolates is given in the Table 13.

It may be observed from Tables 11 and 12 that 17 isolates were Gram-positive cocci with cells being arranged in pairs and short chains and eight isolates were Gram-positive rods with cell being arranged in chains.

Lactococci are homofermentative microaerophilic Gram-positive bacteria, which grow at a temperature of 10°C, but not at 45°C, and produce L (+) lactic acid from glucose. These are characterized by ovoid cells, which appear individually, in pairs, or in chains. It often happens that cells of lactococci themselves extend into a chain, which makes them difficult to differentiate from lactobacilli. The group consisting of Streptococcus, Enterococcus and Leuconostoc also forms cocci that occur as chains or pairs, so it is difficult to distinguish these genera from Lactococcus genera on a morphological basis (Wijtzes et al., 1997). Among the lactococci, Lactococcus lactis subsp. lactis biovar diacetylactis differs from Lactococcus lactis subsp. lactis and cremoris in their ability to utilize citrate with production of diacetyl (Kempler and McKay, 1981). Lactococcus lactis do not possess flagella and do not create endospores (Marshall and Tamime, 1997).

Lactococcus lactis is also characterized by numerous phenotype variations, and it is sometimes difficult to recognize the differences among them. Thus, according to Bergey’s Manual (1994), Lactococcus lactis subsp. lactis biovar. diacetylactis produces ammonia from arginine. Collins (1977), on the other hand, lists strains that do not possess that property, but are the
differentiated from the subspecies cremoris on the basis of the maximum growth temperature and inability to produce ammonium from arginine (Petterson, 1988). Davey and Heap (1993), however, established the existence of Lactococcus lactis subsp. cremoris strains that manifest the arginine metabolism.

In this study out of 25 isolates, sixteen isolates showed growth at 45°C while others were negative. Six isolates—CCSUB143, CCSUB151, CCSUB159, CCSUB171, CCSUB222 and CCSUB253 failed to grow at 10°C but exhibited growth at 45°C. All the isolates were able to grow at pH 8.0 but possessed variable result at pH 4.4 and pH 9.6. Isolates numbers CCSUB114, CCSUB121, CCSUB151, CCSUB155, CCSUB197, CCSUB202, CCSUB213, CCSUB219, CCSUB221, CCSUB222, CCSUB240 and CCSUB253 failed to grow at pH 4.4 and isolates numbers. CCSUB114, CCSUB155, CCSUB165, CCSUB166, CCSUB175, CCSUB197, CCSUB209, CCSUB221, CCSUB222, CCSUB240 and CCSUB253 were failed to grow at pH 9.6. All isolates showed growth in 40% bile except CCSUB143 and CCSUB151. Nine isolates viz. CCSUB144, CCSUB155, CCSUB166, CCSUB175, CCSUB197, CCSUB209, CCSUB213, CCSUB221 and CCSUB227 failed to grow in 4% NaCl while others were able to grow in 4% NaCl. Six isolates viz. CCSUB165, CCSUB171, CCSUB197, CCSUB222, CCSUB240 and CCSUB253 were showed growth in 6.5% NaCl (Table 11 and 12).

Biochemical characterization of the LAB isolates showed that all the isolates were non-gas producers, catalase negative, nitrate reduction negative and gelatin liquefaction negative but possessed variable arginine hydrolysis activity. Two isolates CCSUB159 and CCSUB171 showed relatively less arginine hydrolysis activity. Only nine isolates CCSUB114, CCSUB121, CCSUB128, CCSUB135, CCSUB202, CCSUB210, CCSUB213, CCSUB219 and CCSUB227 showed arginine positive (Table 11 and 12, Figure 17A) while others were arginine negative.
The carbohydrate fermentation pattern revealed the utilization of lactose and salicin by all the isolates, while a variable reaction was observed with mannitol, raffinose and sorbitol. Isolates number CCSUB97, CCSUB165, CCSUB197, CCSUB213 and CCSUB240 were mannitol positive while others were mannitol negative, whereas isolates number CCSUB97, CCSUB143, CCSUB151, CCSUB159, CCSUB165, CCSUB171, CCSUB197, CCSUB222, CCSUB240 and CCSUB253 were positive for raffinose while others were negative. None of the isolates except number CCSUB97 and CCSUB165 showed positive reaction for sorbitol. Fermentation of various carbohydrates by Lactococcus isolate CCSUB202 is depicted in Figure 17B (Table 11 and 12).

Out of the total number of 25 lactic acid bacteria isolates subjected to detailed taxonomic studies, eight were identified as the species of genus Lactobacillus (CCSUB97, CCSUB165, CCSUB143, CCSUB151, CCSUB222, CCSUB253, CCSUB159, CCSUB171) and remaining 17 were identified as genus Lactococcus. Isolates number CCSUB97, CCSUB159 and CCSUB165, CCSUB171 were identified as the strains of Lactobacillus casai and Lactobacillus leichmanii respectively, while other four (CCSUB143, CCSUB151, CCSUB222 and CCSUB253) isolates were identified as Lactobacillus acidophilus. Only two isolates were identified as Lactococcus raffinolactis, nine were identified as Lactococcus lactis subsp. lactis and six were identified as Lactococcus lactis subsp. cremoris. Of these, isolate number CCSUB202 had the broadest spectrum of inhibitory activity against sensitive strain Lactococcus lactis subsp. lactis MTCC3038 in all the three assays. Isolate number CCSUB202, Gram positive coccus, catalase negative, arginine hydrolysis positive and produce no gas from glucose fermentation. Based on these characteristics and analysis of the carbohydrate fermentation, isolate CCSUB202 was presumptive Lactococcus lactis subsp. Lactis (Table 13).

Five species of Lactococcus and three subsp. of Lactococcus lactis have been recognized in the ninth edition of Bergey's Manual of Determinative
Bacteriology (Holt et al., 1994) and Bergey's Manual of Systematic Bacteriology (Garvie, 1986b). They include *Lactococcus garvieae*, *Lactococcus lactis* (subsp. *lactis*, *cremonis* and *hordniae*), *Lactococcus piscium*, *Lactococcus plantarum* and *Lactococcus raffinolactis*. According to Schiefer et al., (1985) and Stiles and Holzapfel (1997) *Lactococcus lactis* species have two subspecies and a biovar: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremonis* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*.

Seven species of the *Lactobacillus* genus have been recognized in the eighth edition of Microbiological Methods (Collins et al., 2004). They include *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus leichmanii*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Lactobacillus salivarius*. The strains included as *Lactobacillus delbrueckii* have been distributed among *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Table 13).

Isolates number CCSUB97, CCSUB143, CCSUB151, CCSUB185, CCSUB171, CCSUB222 and CCSUB253 from dairy products were short rods. This structure and arrangement probably contributes to resistance to lyophilization (Chamba et al., 1994) all the isolates belonged to a group of *Lactobacillus* as defined by Kandler and Weiss (1986). Magnusson and Trautadollir (1982) and Malin and Steentstrom (1984) reported similar observations with *Lactobacillus* isolated from herring (clupea harengus).

The ability to grow at 10°C but failed to grow at 45°C is limited to *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus brevis* (Garvie, 1986b). None of the isolate in the study could be identified as *Lactobacillus plantarum* and *Lactobacillus brevis* as they failed to utilize sorbitol but CCSUB97 and CCSUB185 utilize sorbitol. Thus the isolates number CCSUB97 and CCSUB185 identified as *Lactobacillus casei* (Table 11).

*Lactobacillus leichmanii* the only *lactobacilli* that can hydrolyse arginine but failed to grow at 10°C as our isolates number CCSUB159 and CCSUB171
showed the same characters thus they were identified as *Lactobacillus lechmanhii* according to Collins et al., 2004. Four isolate numbers CCSUB143, CCSUB151, CCSUB222 and CCSUB253 were showed same biochemical and carbohydrate fermentation accepts arginine hydrolysis as *Lactobacillus lechmanhii*. These isolates were showed arginine negative. *Lactobacillus salivarius* also showed arginine negative but, this strain (*Lactobacillus salivarius*) ferment mannitol and sorbitol while isolated strains (CCSUB143, CCSUB151, CCSUB222 and CCSUB253) were not ferment both of the sugars. Thus isolates number CCSUB143, CCSUB151, CCSUB222 and CCSUB253 presumptively identified as *Lactobacillus acidophilus* (Collins et al., 2004) (Table 11). *Lactococcus* occur in pairs and short chains, the ability to hydrolyse arginine is limited to the *Lactococcus garvieae*, *Lactococcus lactis* subsp. *hondiae*, *Lactococcus lactis* subsp. *lactis* (Garvie, 1986b; Collins et al., 2004). None of the coccus isolates in the study could be identified as *Lactococcus lactis* subsp. *hondiae* and *Lactococcus lactis* subsp. *garvieae* as they can ferment mannitol but failed to grow at 4% NaCl. These characters compared with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and found similar to *Lactococcus lactis* subsp. *lactis* thus isolates number CCSUB114, CCSUB121, CCSUB126, CCSUB135, CCSUB202, CCSUB210, CCSUB213, CCSUB219 and CCSUB227 were identified as *Lactococcus lactis* subsp. *lactis*. Other eight isolates number CCSUB144, CCSUB155, CCSUB156, CCSUB175, CCSUB209, CCSUB221, CCSUB197 and CCSUB240 had not ability to hydrolyse arginine but isolate CCSUB187 and CCSUB240 were identified as *Lactococcus raffinolactis*. Six isolates CCSUB144, CCSUB155, CCSUB166, CCSUB175, CCSUB209 and CCSUB221 were failed to arginine hydrolysis and also failed to ferment mannitol and raffinose (Table 12).

Therefore, on the basis of the taxonomic schemes of Garvie (1986b). Holt et al., (1994) and Collins et al (2004). Above six isolates number (CCSUB144, CCSUB155, CCSUB166, CCSUB175, CCSUB209 and
CCSB221), which failed to hydrolyse arginine, were classified as *Lactococcus lactis* subsp. *cremoris.*

In the present study eight strains belonged to different species of *Lactobacillus,* two isolates each of *Lactobacillus casei* and *Lactobacillus leichmanii* number CCSUB97, CCSUB165 and CCSUB159, CCSUB171 respectively. Four isolates CCSUB143, CCSUB151, CCSUB222 and CCSUB253 identified as *Lactobacillus acidophilus* while cocci shaped seventeen strains belonged to *Lactococcus* species (Table 13).

Out of 17, only two strains CCSUB197 and CCSUB240 were identified as *Lactococcus raffinolactis* and six strains (CCSUB144, CCSUB155, CCSUB166, CCSUB175, CCSUB209 and CCSUB221) were classified as *Lactococcus lactis* subsp. *cremoris.* The maximum numbers of 25 isolates (CCSUB114, CCSUB121, CCSUB125, CCSUB130, CCSUB152, CCSUB164, CCSUB180, CCSUB186, CCSUB192, CCSUB201, CCSUB213, CCSUB219 and CCSUB227) obtained in the present study were identified as *Lactococcus lactis* subsp. *lactis* (Table 13 and Figure 18).

It is interesting to note that all the lactococcal isolates i.e. isolate numbers CCSUB114, CCSUB121, CCSUB201 and CCSUB210 (that showed high inhibition activity against *Lactococcus lactis* subsp. *lactis* MTCC3038 in all assays) were found to be different strains of *Lactococcus lactis* subsp. *lactis.*

Similar results were observed by Guessas and Kihal (2004), who isolated and identified lactic acid bacteria (*Lactococcus* species 76.16% *Lactobacillus* species 10.98% and *Leuconostoc* sp. 8.6%), the dominating species was *Lactococcus lactis* subsp. *lactis,* from Algerian raw goat's milk. Hernandez et al. (2005) also isolated one hundred eighty lactic acid bacteria of the genera *Lactococcus, Lactobacillus* and *Leuconostoc* from raw tenerific goat's cheese. Sixty strains of lactic acid bacteria isolated from Vietnamese fermented milk of which, dominated strain *Lactococcus lactis* subsp. *lactis* LTM32 was indentified and partially characterized by Do et al., (2001). Savadogo et al., 2004 isolated and identified lactoococi, lactobacilli,
Leuconostocs, Streptococcus and Enterococcus genus. Twenty representative lactic acid bacteria strains were identified to species level belonging to species Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. lactis biovar. diacetylactis, Lactobacillus confusus, Lactobacillus delbrueckii subsp. lactis, Lactobacillus plantarum, Leuconostoc citreum and Leuconostoc lactis from thirty samples of traditional fermented milk. Eighteen presumptive isolates of lactic acid bacteria from local white cheese made from sheep raw milk were isolated and identified by Haddadin (2005).

Lactic acid bacteria isolated from dairy products included lactococci (Piard et al., 1990; Thuaulet et al., 1991; Gupta and Batish, 1992; Ali et al., 1995; Morgan et al., 1995; and Matinez et al., 1998) lactobacilli (Muriana and Klaenhammer, 1987; Vaughan et al., 1992; Kanatani and Oshima, 1994; Rekhif et al., 1994; Oyetayo, 2004; Todorov et al. 2000; Todorov and Dicks, 2005) and Leuconostocs (Hechard et al., 1992; Mathieu et al., 1993; Malik et al., 1994a and Sudimman et al., 1994).

Several other researchers have also identified the lactic acid bacteria from dairy products (Yarmus, 2000; Sharma, 2002; Savadogo et al., 2004; Hernandez et al., 2005; Sharma and Garg, 2005; Batdarj et al., 2006; Nomura et al., 2006). Weigmann (1905-1908) identified lactic acid bacteria as essential components of the mesophilic microflora in spontaneously fermented cream and milk. Harris et al. (1992) reported that Lactococcus lactis subsp. lactis strains are often assumed to be associated mainly with milk and dairy products. The pediococcal strains from Manchego cheese were identified by Nunez (1976). Tzanetakis and Litopoulou-Tzanatou (1989) were identified 83 pediococcal strains from raw goat milk.

According to Axelsson (1998), due to the phosphoenol phosphotransferase system (PEP-PTS), which ensure their efficient uptake and fermentation of lactose, some of lactic acid bacteria have been adapted well to growth in milk and today the most recognized habitat for lactococci are dairy product.
Although, more reports are available, concerning the isolation of lactococci from dairy sources, the lactococci also have been isolated from various other sources such as vegetable and fruits (Grahn et al., 1994; Kimoto et al., 2000; Choi et al., 2000; Kimoto et al., 2004), beer (Togo et al., 2002), wine (Curran et al., 1995), fermented food (Chen et al., 2005, 2006), Molasses (Todorov and Dicks, 2005).

6.4 Selection of the producer strain and indicator organism:-

Selection of the producer as well as indicator strains was done by estimating the bacteriocin titres of the culture broths of the producers against lactic indicator organisms Lactococcus lactis subsp. lactis MTCC 3041 and Lactococcus lactis subsp. lactis MTCC3038 employed in the screening process and expressing the bacteriocin titre as Arbitrary or Activity Units (AU) per milliter of the culture broth. The results are presented in Table 14 and Figure 19.

The bacteriocin activity of Lactococcus lactis subsp. lactis strains CCSUB114, CCSUB121, CCSUB202 and CCSUB210 was 640 AU/ml, 480 AU/ml, 1280 AU/ml and 640 AU/ml respectively against Lactococcus lactis subsp. lactis MTCC3041. All the lactococcal cultures displayed higher antibacterial activity against Lactococcus lactis subsp. lactis MTCC3038 viz: 960 AU/ml, 640 AU/ml, 1920 AU/ml and 960 AU/ml. Of all the bacteriocin producing lactococcal cultures, Lactococcus lactis subsp. lactis CCSUB202 resulted in the maximum activity of 1920 AU/ml against Lactococcus lactis subsp. lactis MTCC3038. (Figure 19) whereas the bacteriocin activity of the same producer strain against MTCC3041 was 1280 AU/ml (Table 14 and Figure 19).

Bacterial cultures for bacteriocin production and characterization studies have been selected on the basis of their broader antibacterial spectrum of activity (Piard et al., 1990; Jimenez-Diaz et al., 1993; Schved et al., 1993; Cintas et al., 1995 and Rodriguez et al., 1995). Since no differences
in the inhibitory activity among the five producing strains against the lactic and non-lactic indicators employed in the present screening studies were observed, the bacteriocin activity present in respective culture broths was quantified using the two lactic indicator strains. *Lactococcus lactis* subsp. *lactis* CCSUB202, however, exhibited higher activity against all the lactic cultures and was, therefore, retained for further studies. *Lactococcus lactis* subsp. *lactis* MTCC3038 was found to be the most sensitive organism to the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSUB202 and hence used as the indicator strains for the bacteriocin assays.

In some earlier studies, *Lactococcus* species have been used as indicator for the different bacteriocins of different lactococcal strains (Piard et al., 1990; Kojic et al., 1991 and Rodriguez et al., 1995; Sharma, 2002; Sharma and Garg, 2005), *Leuconostoc* species for bacteriocins of *Leuconostoc* (van Laack et al., 1992 and Keppler et al., 1994), *Lactobacillus* spp. for the bacteriocins of *lactobacilli* (Daasheh et al., 1990; Joerger and Kleenhemmer, 1986) and *Lactobacillus* strains have also been used as indicator organisms in the characterization of bacteriocins of pediococci such as pediocin ACH (Bhunia et al., 1988), Pediocin SJ-1 (Schved et al., 1993) some other bacteriocin (Chen et al., 2005, 2006; Badtork et al., 2006; Chen and Yanagida, 2006).

5.5 Optimization of parameters for Bacteriocin production:--

The culture conditions for maximum bacteriocin production by *Lactococcus lactis* subsp. *lactis* CCSUB202 were optimized with respect to culture medium, initial pH of the culture medium, temperature of the growth of the producer culture and period of incubation. In each experiment changes in pH, growth in terms of absorbance at 600nm and bacteriocin activity expressed in terms of AU/ml was recorded at regular intervals. The results obtained during the optimization of bacteriocin production parameters are given in Table 15 to 18 and Figures 20 to 25.
Figure 11: Standard curve for protein estimation (Lowry et al., 1951).
incubation at 37°C and the remaining amount during the next 8 hour of incubation. The bacteriocin titers obtained in MRS, MRS+1.5% Tween 80, MRS II, TGYA, TGEA, Elliker's and Nutrient Agar broths at the end of 8 hour incubation were calculated to be about 50, 50, 33.3, 12.5, 12.5, 12.5 and 0 %, respectively of the highest titres reached in the respective media and they increased to 75, 66.6, 66.6, 50, 50, 50 and 100 % after 16 hour of growth. It is evident from the Figure 20 and 21 that bacteriocin activity remained highest in MRS I broth at all the three time intervals employed in the experiment.

MRS I broth was found to be the most suitable medium for optimal production of the bacteriocin by Lactococcus lactis subsp. lactis CCSUB202 followed by MRS, MRS+1.5% Tween 80, MRS II, TGYA, TGEA, Elliker's and Nutrient Agar broths. The variation in the bacteriocin titres observed could be due to the different cell densities attained in each of the broths at the end of the incubation period. MRS I broth medium in the present study supported good growth of the test culture and hence the maximum bacteriocin production. Although the growth was slightly more in MRS II broth. Lactococcus lactis sp. usually grown in complex medium such as MRS (De men et al., 1960). However, several defined media are published for lactic acid bacteria (Dunn et al., 1947; Kosar and Thomas, 1955; Morishita et al., 1974; Le desma et al., 1977; McFeeters and Chen, 1986; Montel and Labadie, 1986; Grobben et al., 1995; Lauret et al., 1996).

Many studies have demonstrated that culture medium; have a drastic effect on the production of bacteriocin (Villani et al., 1995; Ivanova et al., 1988; El-Shafei et al., 2000; Kabuki et al., 2006).

6.5.2 Effect of initial pH of the growth medium: -

The bacteriocin producing Lactococcus lactis subsp lactis CCSUB202 was grown in MRS I broth adjusted to different initial pH values viz .5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9 changes observed with respect to pH, absorbance and
bacteriocin activity during the course of incubation at 37°C are given in Table 16, Figure 22 and 23.

The growth of the bacteriocin producer in MRS I broth resulted in lowering of the initial pH from 5.5 to 4.02 after 24 h cell growth and 5.5 to 3.84 after 40 hour incubation. The absorbance expressed in terms of O.D. reached up to 0.586 and the bacteriocin activity reached to a maximum extent of 1280 AU/ml during the same time but bacteriocin activity slightly decreased after 40 hour incubation 960 AU/ml and absorbance reached up to 0.734. When the initial pH of the MRS I broth media ranged from 6 to 8.5, the corresponding pH decreased was in the range of 3.73 to 3.97 after 40 h at 37°C, while the absorbance values recorded were in the range of 1.012 to 1.123 Table 16. It was also observed that there was no difference in the bacteriocin activity reached after 8 h in MRSI broth with the an initial pH ranging from 6 to 8.5 and the highest activity of 2560 AU/ml were attained after 24 h at all these initial pH values in the range of 6 to 8.5 except at 7.0 in which case the highest activity was attained after 16 h of growth and remained at the same level even after 24 h of incubation. The Figure 23 also shows that the bacteriocin activity at the end of 8 and 24 h incubation remained same at the initial pH values in the range of 6 to 8.5. The higher activity of 2560 AU/ml was attained after 16 h at pH 7.0, while the same value was reached after 24 h at other initial pH values of MRS I medium. It was also observed that bacteriocin activity was minutely decreased after 40 h.

The bacteriocin production by *Lactococcus lactis* subsp. *Lactis* CCSUB202 was not significantly dependent on the initial pH of the MRS I medium between 6 to 8.5. However, there was a slight decrease in bacteriocin activity when the test culture was grown in MRS I broth with an initial pH 5.5 and 9.0. The decreased activity of bacteriocin could be due to the reduced cell growth (A\textsubscript{600} =0.586 and 0.903) at rather lower initial pH of the medium. Therefore, pH 7.0 of MRS I medium was employed as an initial pH for the production of the bacteriocin by the test strain.
Izildinha Moreno et al. (2000) reported that bacteriocin produced by *Lactococcus lactis* subsp. *lactis* strains were fully or partly active at range of pH 2 to 10 and completely inactivated at pH 12.0. Similar to nisin of ATCC11454, bacteriocin of ITAL383 was stable at neutral and acid pH (2.0 to 6.0), partly active at pH 6.0 to 10.0 and completely inactive at pH 12.0. Thuault et al. (1991) have also reported that the bacteriocin production by *Lactococcus lactis* subsp. *lactis* ADRI 85LO30 is independent of initial pH of the medium in the range of 5 to 7. Coventry et al. (1996) observed no detectable levels of brevicin 286 when the producer strain was grown in MRS broth at an initial pH 4.5, but the production was optimum at a pH 6 to 7.0.

Lower cell densities and consequently lower bacteriocin activity were observed when *Leuconostoc gelidum* was grown in APT broth at an initial pH lower than 6 and 6.5 (Hasting and stiles, 1991). Similar reports concerning the effect of initial pH of the growth medium on the bacteriocin production include a decreased production leuconocin S in APT with an initial pH 7.5 (Lewus et al, 1992) and a 50% decrease in carnosin 44A production by *Leuconostoc Comosum* LA44A when the initial pH of the APT medium was lowered from 6 to 5. The decreased bacteriocin production in most of the cases has been attributed to the reduced cell mass. Kang and Lee (2005), reported that optimal production of bacteriocin from *Enterococcus faecium* GM-1 was obtained when the pH of culture medium between 6.0-6.5. When Messens et al (2002), grew *Lactobacillus amylovorus* DCE471 in MRS medium at pH of 4.0 to 9.0. In the same way, some studies showed that the production of bacteriocins could also be influenced by profile described by the pH values in the culture broth Guessa and Pastrana (2002). It has been observed by several workers (Hurst, 1981; Barefoot and Klaenhammer, 1984; Muriana and Klaenhammer, 1987; Piard et al 1990; ten-Brink et al, 1994; Kanatani et al, 1995; Tahara et al, 1996; Tahara and Kanatani, 1996; Dave and Shah, 1997 and Bogovic–Matijasic and Rogelj, 1998) that maintenance of final pH of the growth medium at a particular level results in increased levels of bactecniocin activity. Nomura et al. (2005) grew LAB in Elliker's broth at pH 9.2 to 9.6.
Todorov and Dicks, (2006) produced bacteriocin in MRS broth with an initial pH 6.0 to 6.5.

5.5.3 Effect of incubation temperature:- It may be seen from the Table 17, Figure 22 and 24 that Lactococcus lactis subsp. lactis incubated at 25, 30, 37 and 40°C in MRS I broth did not show any variation in the pH, growth and bacteriocin activity after 24 h of incubation. The pH values recorded at four different incubation temperatures after 24 h were 3.60, 3.78, 3.75 and 3.82 respectively while the O.D. values were 2.99, 3.06, 3.11 and 3.00 at the four respective temperatures. The highest bacteriocin activity attained after 24 h was 2560 AU/ml at all the four temperature. Although there were no differences in bacteriocin production by the selected strain after 24 h at all the four incubation temperatures, the drop in pH increase in absorbance and the bacteriocin activity differed significantly after 8 h of growth. The pH dropped to 5.70, 4.82, 4.22 and 4.25 at 25, 30, 37 and 40°C, respectively after 8 h while the absorbance (O.D.) values recorded at the corresponding time were 0.82, 1.04, 2.72 and 2.67 at the respective temperatures. The bacteriocin activity after 8 h of incubation at 25, 30, 37 and 40°C was found to be 6.25%, 25%, 62.5% and 37.5% of the total bacteriocin activity observed at the respective temperatures after 24 h incubation. It can also be seen that the highest bacteriocin activity of 2560AU/ml was attained after 24 h of growth at 25°C, 30°C as well as 40°C whereas it was attained after 16 h at 37°C and remained at the highest level without any further decline even at the end of 24 h of incubation. The incubation of the of Lactococcus lactis subsp. lactis CCSUB202 at 45°C and 50°C resulted in a bacteriocin activity of 640 AU/ml and 320 AU/ml respectively after 8 h and remained at the same level without increase nor decrease in the activity up to 16 h incubation but the bacteriocin activity decrease to 320 AU/ml and 160 AU/ml after 24 h. After 16 h growth at 45°C and 50°C in the MRS I broth, the pH value dropped to 4.40 and 4.52, respectively while O.D. increased to 1.10 and 1.14 at the respective
temperature. Though pH value remained constant at 4.40 and 4.52 but the absorbance value decreased to 0.98 and 1.06 after 24 h. it is observed from Table 17, the bacteriocin activity was slightly decreased after 40 h incubation.

The bacteriocin production as affected by temperature of incubation is given Figure 24. It is evident that though there was no difference in the bacteriocin activity after 24 h at all the four temperature of incubation, the activity was maximum at 37°C after 8 h as well as after 16 h of incubation.

There were no significant differences in the growth, drop in pH and bacteriocin production when the test culture was grown in MRS I broth at 25°C, 30°C, 37°C or 40°C. However, relatively faster growth (A600=3.11) at 37°C resulted in the synthesis and secretion of a large proportion (63%) of the bacteriocin into the growth medium in the first 8 h of the incubation period. The bacteriocin production was adversely affected when the test culture was grown at 45°C or higher and this could be attributed to very little growth, rather lysis of the producer strain at the end of incubation.

The bacteriocin production by Lactococcus lactis subsp. lactis ADRI 85LO30 was not significantly dependent on the incubation temperature in the range of 30 to 42°C (Thuault et al, 1991). Do et al, (2001) though reported Lactococcus lactis subsp. lactis LTM32, grew and produced bacteriocin over a range from 15 to 37°C with the maximal growth and bacteriocin production at 30°C. The optical production of bacteriocin from Enterococcus faecium GM-1 was obtained at the temperature between 35 to 40°C. Messen et al (2000) earlier had shown his data that Lactobacillus amylovorus DCE 471 in SSM growth at temperature between 28-44°C and pH between 4.2 to 6.4. According to Kang and Lee (2005) that the optimal production of bacteriocin from Enterococcus faecium GM-1 was obtained at the temperature between 35 to 40°C. Nomura et al. (2005) earlier had shown that optimal LAB growth at temperature 40 to 45°C. Optimal growth temperature usually results in optimal bacteriocin production (Parente and Ricciardi, 1999). However, there are some reports of bacteriocin with maximal production at suboptimal growth
temperatures. In the case of amylovorin L471, show growth at low temperature was suggested to free up more energy for bacteriocin production (De Vuyst et al., 1996) were claimed to be due to different rate-limiting reactions dependent upon temperature, resulting in better utilization of carbon and/or energy at low growth rates and increased availability of essential metabolites for bacteriocin synthesis (Aasen et al., 2000). Another explanation was suggested to be increased degradation or inactivation of the bacteriocin at high temperatures (Leroy and De Vuyst, 1999; Moretro et al., 1991). The production of lacticin RM was found to be temperature dependent (Keren et al., 2004). Biswas et al (1991) though reported an identical pediocin AcH titres at 30 and 37°C but there was a slight decrease in producer’s cell mass and bacteriocin production at 40°C. Similarly, Schved et al. (1993) reported the production of pediocin SJ-1 by acidilactici SJ-1 over the entire temperature range of 30°C to 45°C. On the other hand, no detectable pediocin L50 was observed when the producing strain P. acidilactici L50 was grown at 45°C. Similar reports concerning effect of temperature on the bacteriocin production include, the production of carnosin 44A at a temperature of 4-10°C (van Laack et al. 1992), leucocin 3Talla over a wide temperature range of 0-30°C (Felix et al., 1994) and a number of bacteriocins by several Leuconostoc spp. (yang and Ray, 1994a).

The general observation was that although the bacteriocin production was detected over a wide temperature range, the production was maximum at the optimum temperature of growth of the producer strain and a relatively longer incubation times were needed to achieve the highest bacteriocin titres at low temperatures.

5.5.4 Effect of incubation period: — Lactococcus lactis subsp. lactis CCSUB202 was grown in MRS I broth with an initial pH 7.0 and incubated for upto 36 h at 37°C. The samples were taken at regular intervals and analysed for pH, absorbance and bacteriocin activity. The results are shown in Figure 22 and 25, Table 18.
temperatures. In the case of amylovorin L471, show growth at low temperature was suggested to free up more energy for bacteriocin production (De Vuyst et al., 1996) were claimed to be due to different rate-limiting reactions dependent upon temperature, resulting in better utilization of carbon and/or energy at low growth rates and increased availability of essential metabolites for bacteriocin synthesis (Aasen et al., 2000). Another explanation was suggested to be increased degradation or inactivation of the bacteriocin at high temperatures (Leroy and De Vuyst, 1999; Moretro et al., 1991). The production of lactacin RM was found to be temperature dependent (Keren et al., 2004). Biswas et al (1991) though reported an identical pediocin AcH titre at 30 and 37°C but there was a slight decrease in producer's cell mass and bacteriocin production at 40°C. Similarly, Schved et al. (1993) reported the production of pediocin SJ-1 by acidilactici SJ-1 over the entire temperature range of 30°C to 45°C. On the other hand, no detectable pediocin L50 was observed when the producing strain P. acidilactici L50 was grown at 45°C. Similar reports concerning effect of temperature on the bacteriocin production include, the production of carnosin 44A at a temperature of 4-10°C (van Laack et al., 1992), leucocin 3Talla over a wide temperature range of 0-30°C (Felix et al., 1994) and a number of bacteriocins by several Leuconostoc spp. (Yang and Ray, 1994a).

The general observation was that although the bacteriocin production was detected over a wide temperature range, the production was maximum at the optimum temperature of growth of the producer strain and a relatively longer incubation times were needed to achieve the highest bacteriocin titre at low temperatures.

5.5.4 Effect of incubation period: – Lactococcus lactis subsp. lactis CCSUB202 was grown in MRS I broth with an initial pH 7.0 and incubated for upto 36 h at 37°C. The samples were taken at regular intervals and analysed for pH, absorbance and bacteriocin activity. The results are shown in Figure 22 and 25, Table 18.
Although bacteriocin S50 was secreted throughout the log phase, the highest production occurred in the first 8h of incubation (Kojic et al., 1991). The production of several bacteriocins of *Leuconostocs* such as leucocin A-UAL187 (Hastings and Stiles, 1991), mesenterocin 52 (Mathieu et al., 1993), carnocin LA54A (Keppler et al., 1994), leucocin B-Talla (Felix et al., 1994), helveticin V-1829 (Vaughan et al., 1992), pediocin AcH (Yang and Ray, 1994b) have also been reported to be secreted in the log phase of the growth of the respective producing strains, while other bacteriocin produced during early stationary phase, include lacacin B (Barefoot and Klaenhammer, 1984), helveticin J (Joeger and Klaenhammer, 1986). *Lactobacillus plantarum* LPC010 produced plantaricin S in the log phase while plantaricin T was produced by the same strain in the stationary phase (Jimenez-Diaz et al., 1993). Achemchem et al. (2005), reported that the antimicrobial substance produced by *Enterococcus faecium* strain F58 during the growth phase, with maximum production after 16 to 20 h of incubation. It has also been reported by some workers that the activity declined when the incubation period was extended. Dave and Shah (1997) reported that activity of acidophilicin LA-1 declined when the culture entered death phase. Similar findings were also observed for pediocin L50 (Cintas et al., 1995), sakacin P (Moretro et al., 2000).

Although not conclusively proved, the decline in the bacteriocin activity reported in the above studies during the extended periods of incubation has been believed to be due to the secretion of inhibitors such as proteolytic enzymes that degrade active bacteriocin molecule.

5.6 Optimization of production conditions in Soya nutri nuggets extract media:- In this part of study, production of bacteriocin in soya nutri nuggets extract medium (SNNEM) alone and effect of supplementation of various nutrients individually and in combination in SNNEM on bacteriocin production was studied.
The results indicated that growth of *Lactococcus lactis* subsp. *lactis* CCSUB202 culture in soya nutri nuggets extract did not produce any bacteriocin (Table 19) while the supplementation of various nutrients (carbon sources, salts and Tween 80) in soya nutri nuggets extract (SNNE) resulted in a high level of bacteriocin production (Figure 26 and Table 20, 21). When SNNE were supplemented with combination of nutrients, the culture produced bacteriocin activity of 5120 AU/ml (in SNNEM just double of the bacteriocin activity produced in MRS I medium) Figure 27.

The soya nutri nuggets extract supplemented with carbon sources or salts or Tween 80 individually or supplemented with combination of various nutrients resulted high production of bacteriocin from *Lactococcus lactis* subsp. *lactis* CCSUB202.

The new, SNNEM medium for *Lactococcus lactis* subsp. *lactis* CCSUB202 described here contains only seven components, making it less complex than previously described media as well as supporting better growth. This indicates that the medium will be most suitable for physiological studies of lactococci commonly associated with dairy products, and perhaps many other *Lactococcus* as well.

Wenhva et al. (2004), reported that *Lactococcus lactis* subsp. *lactis* ATCC11454, a nisin producing strain, was grown on complex medium containing soy peptone. Nisin production related to the growth condition of *Lactococcus lactis* subsp. *lactis* ATCC11454, the effect of various media components and concomitant release of nisin into the media (Faustino et al., 2005). Chandrapati and O’Sullivan, (1998) observed 50% increment in nisin expression using sucrose as the carbon source for *Lactococcus lactis* culture. Vessonipenna and Moraes, (2002b), also added sucrose in MRS or M17 in previous work, favors the production of nisin by cells. Carbon source regulation affects cell growth and nisin biosynthesis (De Vuyst and Vandamme, 1992). While an increase in bacteriocin production as a result of adding from 0.75 to 1% Tween 20 or Tween 80 to the medium has been
reported by Garver and Muriana (1994), Huot et al., (1996) and Keren et al. (2004). Nonionic detergents such as Tween 80 may mimic the effect of various food constituents in inducing the production of bacteriocins, and they are known to stimulate protein secretion by affecting membrane fluidity Reese and Maguire, (1969). As described for Lactococcus lactis by Cocaing-Bourquet et al (1995), the removal of ammonium salt from the medium did not affect the growth negatively. Many studies have demonstrated that culture medium; have a drastic effect on the production of bacteriocin (Villani et al., 1995; Ivanova et al., 1998; El- Shafei et al., 2000; Vazquez et al., 2005; Todorov and Dicks, 2005; 2006; Kabuki et al., 2006).

5.7 Optimization of physical parameters for maximum bacteriocin production in SNNEM:- The culture conditions for maximum bacteriocin production by Lactococcus lactis subsp. lactis CCSUB202 in SNNEM optimized with respect to initial pH of the SNNEM, incubation temperature and incubation period. In each experiment, changes in pH, absorbance at 600 nm, bacteriocin activity in terms of AU/ml and zone of inhibition in mm were measured at regular intervals. The results obtained during the optimization study are presented in Table 20 to 24 and Figure 28 to 31.

5.7.1 Effect of initial pH of the SNNEM:- The bacteriocin producing Lactococcus lactis subsp lactis CCSUB202 was grown in SNNEM broth adjusted to different initial pH values viz 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9 changes observed with respect to pH, absorbance and bacteriocin activity during the course of incubation at 37°C are given in Table 22, Figure 28 and 29.

The growth of the bacteriocin producer in SNNEM broth resulted in lowering of the initial pH from 5.5 to 3.83 after 24 h cell growth. The absorbance expressed in terms of O.D. reached upto 1.932 and the bacteriocin activity reached to a maximum extent of 1280 AU/ml during the
same time. When the initial pH of the SNNEM broth media ranged from 6 to 9, the corresponding pH decreased was in the range of 4.02 to 4.64 after 24 h at 37°C, while the absorbance values recorded were in the range of 1.912 b 2.873 Table 22. It was also observed that there was no difference in the bacteriocin activity reached after 8 h in SNNEM broth with the an initial pH ranging from 6.5 to 9.0 and the highest activity of 5120 AU/ml were attained after 24 h at all these initial pH values in the range of 6.5 to 9.0 except at 7.5 in which case the higher activity was attained after 16 h of growth and remained at the same level even after 24 h of incubation. The Figure 29 also shows that the bacteriocin activity at the end of 8 and 24 h incubation remained same at the initial pH values in the range of 6.5 to 9.0. The higher activity of 5120 AU/ml was attained after 16 h at pH 7.5, while the same value was reached after 24 h at other initial pH values of SNNEM. Bacteriocin activity slightly decreased after 40 h at all pH.

The bacteriocin production by *Lactococcus lactis* subsp. *Lactis* CCSUB202 was not significantly dependent on the initial pH of the SNNEM between 6.5 to 9.0. However, there was a slight decrease in bacteriocin activity when the test culture was grown in SNNEM broth with an initial pH 5.5 and 6.0. The decreased activity of bacteriocin could be due to the reduced cell growth (*A*ₐ₉₀ = 1.932 and 1.912) at rather lower initial pH of the medium. Therefore, pH 7.5 of SNNEM was employed as an initial pH for the production of the bacteriocin by the test strain. Chen et al. 2005 and Kabuki et al. (2008) also have done same work.

**5.7.2 Effect of incubation temperature:** - It may be seen from the Table 23 that *Lactococcus lactis* subsp. *lactis* incubated at 25, 30, 37 and 40°C in SNNEM broth did not show any variation in the pH, growth and bacteriocin activity after 24 h of incubation. The pH values recorded at four different incubation temperatures after 24 h were 3.83, 3.72, 3.78 and 3.79 respectively while the O.D. values were 3.02, 3.11, 3.21 and 3.09 at the four respective
temperatures. The highest bacteriocin activity attained after 24 h was 5120 AU/ml at all the four temperature. Although there were no differences in bacteriocin production by the selected strain after 24 h at all the four incubation temperatures, the drop in pH increase in absorbance and the bacteriocin activity differed significantly after 8 h of growth. The pH dropped to 5.71, 5.84, 4.74 and 4.81 at 25, 30, 37 and 40°C, respectively after 8 h while the absorbance (O.D.) values recorded at the corresponding time were 0.921, 1.16, 1.91 and 1.86 at the respective temperatures. The bacteriocin activity after 8 h of incubation at 25, 30, 37 and 40°C was found to be 18.7%, 25%, 62.5% and 37.5% of the total bacteriocin activity observed at the respective temperatures after 24 h incubation. It can also be seen that the highest bacteriocin activity of 5120 AU/ml was attained after 24 h of growth at 25°C, 30°C as well as 40°C whereas it was attained after 16 h at 37°C and remained at the highest level without any further decline even at the end of 24 h of incubation, on further incubation after 40 h, it was observed from Table 23 that bacteriocin activity was minutely decreased. The incubation of the of Lactococcus lactis subsp. lactis CCSUB202 at 45°C and 50°C resulted in a bacteriocin activity of 640 AU/ml and 320 AU/ml respectively after 8 h and remained at the same level with neither increase nor decrease in the activity upto 16 h incubation but the bacteriocin activity decrease to 320 AU/ml and 160 AU/ml after 24 h. After 16 h growth at 45°C and 50°C in the SNNEM broth, the pH value dropped to 4.17 and 4.87, respectively while O.D. increased to 1.24 and 1.18 at the respective temperature. Though pH value remained constant at 4.17 and 4.87 but the absorbance value decreased to 0.95 and 1.02 after 24 h.

The bacteriocin production as affected by temperature of incubation is given Figure 30. It is evident that though there was no difference in the bacteriocin activity after 24 h at all the four temperature of incubation, the activity was maximum at 37°C after 8 h as well as after 16 h of incubation.
There were no significant differences in the growth, drop in pH and bacteriocin production when the test culture was grown in SNNEM broth at either 25°C, 30°C, 37°C or 40°C. However, relatively faster growth ($A_{000}=3.21$) at 37°C resulted in the synthesis and secretion of a large proportion (63%) of the bacteriocin into the growth medium in the first 8 h of the incubation period. The bacteriocin production was adversely affected when the test culture was grown at 45°C or higher and this could be attributed to very little growth, rather lysis of the producer strain at the end of incubation.

5.7.3 Effect of incubation period:- *Lactococcus lactis* subsp. *lactis* CCSUB202 was grown in SNNEM broth with an initial pH 7.5 and incubated for upto 36 h at 37°C. The samples were taken at regular intervals and analysed for pH, absorbance and bacteriocin activity. The results are shown in Figure 28 and 31 and Table 24.

There was sharp decline in the pH value in the first 8 h during which period the pH dropped to 4.78 from an initial pH value of 7.5. The decrease in the pH thereafter was quite marginal, reaching upto 3.89 at the end of 16 h incubation and remained more or less constant at the same value up to 36 h incubation period. After 40 h incubation period, bacteriocin activity was minutely decreased.

It may be observed from the Figure 31 that there was no increase in the absorbance value in the first 2 h. The increase was, however, maximum between 4 and 8 h of incubation during which, the absorbance value increased from 1.618 to 1.718. The increase, thereafter, was marginal reaching a value of 2.313 after 16 h incubation. The highest value of 2.412 was recorded after 24 h with a slight decline in absorbance after 28 and 36 h of incubation.

It can be observed from the Figure 31 and Table 24 that bacteriocin was detected in the growth medium after 4 h of incubation coincided with the
start of log phase of growth of the culture. The bacteriocin production continued throughout the log phase of growth reaching the highest value of 5120 AU/ml after 24 h. The bacteriocin activity remained at the highest level with no further decline in the activity during stationary and death phases of growth of the culture up to 36 h incubation. The bacteriocin production by *Lactococcus lactis* subsp. *lactis* CCSUB202 was observed to be growth associated as it was secreted into the growth medium continuously throughout the log phase reaching the highest titers at the end of this phase, thus, indicating it to be a primary metabolite. It was also observed that there was no detectable lose in the bacteriocin activity in the stationary as well as death phases. The maximum production (63%) of the bacteriocin occurred in the first 8 h of growth while the rest (37%) being produced in the next 8 h of the incubation.

5.8 Mass production of bacteriocin in SNNEM:- Bacteriocin production in SNNEM by batch fermenter (LAB FORS AG CH-4103 Bottmingen/Switzerland) with a capacity of 2 litres was used in present study.

The relationship between absorbance and bacteriocin activity by *Lactococcus lactis* subsp. *lactis* CCSUB202 as influenced by temperature and pH values was assessed. Figure 32 represent fermentation at a controlled temperature 37°C and at constant pH 7.5.

Figure 32 and Table 25 shows that the bacteriocin production by *Lactococcus lactis* subsp. *lactis* CCSUB202 in a batch fermenter was slightly increased as compared to fermentation in shaker culture. In this experiment the highest bacteriocin activity was reached at the end of the exponential growth phase 5280 AU/ml of medium (Figure 32) when absorbance had also reached a maximum value in experiment. Bacteriocin activity was already detectable after 4 h of fermentation when 40% of the absorbance had been produced. Figure 32 shows that maximum activity was obtained at the 16 hour of fermentation (5280 AU/ml). However, the higher absorbance was achieved
in this experiment at 16th hour of fermentation Figure 32. On further incubation after 28 h, the bacteriocin activity was minutely decreased, 5280 AU/ml to 4860 AU/ml.

Traditionally, optimization of bacteriocin fermentation processes has been performed by physiological and metabolic control of their biosynthesis. Bacteriocin are usually produced in complex media (Biswas et al., 1991; De Vuyst and Vandamme, 1992, 1993; Parente and Hill, 1992) under well controlled conditions of temperature and pH (Biswas et al., 1991; De Vuyst and Vandamme, 1992; Parente and Ricciardi, 1994; Parente et al., 1994; Mortvedt- Abildgaard et al., 1995; De Vuyst et al., 1996a) seem to play an important role in bacteriocin production.

Similar behaviour was observed by De Vuyst and Vandamme (1992) using Lactococcus lactis subsp. lactis NIZO22186 and glucose as a carbon source. These experiments achieved 1.27g/l of cell mass in the eighth hour of fermentation and nisin activity of approx. 2000 I.U./ml of medium. Amiali et al. (1998) obtained maximum nisin production at 8 h of fermentation with 41 I.U. of nisin per ml. Several other workers (De Vuyst et al., 1996a; Lejeune et al., 1998; Callewart et al., 1998; Wenhua et al., 2004; Chen et al., 2005; Richard and Murray, 2006) also used fermenter for bacteriocin production.

5.9 Purification of the bacteriocin produced by Lactococcus lactis subsp. lactis CCSUB202: -

The purification of bacteriocin from the culture broth was attempted by different methods viz: precipitation with organic solvents and fractionation with ammonium sulphate, dialysis followed by gel filtration chromatography (HPLC) for further purification of the bacteriocin.

5.9.1 Selection of buffer: - The bacteriocin dissolved in various buffers such as citrate (pH 6.2), acetate (pH 5.8), sodium phosphate (pH 7.0, 7.6) and
potassium phosphate buffer (pH 7.0). The results regarding the effect of different buffers on the activity of bacteriocin of *Lactococcus lactis* subsp. *lactis* CCSUB202 at 4°C and room temperature of storage are presented in Table 26 and Figure 33A, 33B in terms of residual activity (%).

The bacteriocin retained full activity in sodium phosphate buffer (pH 7.0, 7.6) and potassium phosphate buffer (pH 7.0) even after 72 h at 4°C without any loss. While a residual activity of 62.5, 25 and 12.5 per cent was observed after 24, 48 and 72 h, respectively in citrate buffer (pH 6.2) as indicated in Figure 33A. Storage in acetate buffer (5.8) also resulted in loss of about 75, 75 and 87.5 per cent after 24, 48 and 72 h, respectively. These observations indicate that bacteriocin was quite stable in sodium phosphate buffer (pH 7.0, 7.5) and potassium phosphate buffer (pH 7.0) even after 72 h at 4°C, while citrate and acetate buffer exerted deleterious effect on the activity of bacteriocin. The decrease in the activity of bacteriocin may be attributed to the structural modification of the protein caused by constituent of buffer leading to the loss of inhibitory activity. However, there is no report regarding the effect of different buffers on the activity of bacteriocin.

Residual activity of bacteriocin at room temperature in sodium phosphate buffer (pH 7.0, 7.6) and potassium phosphate buffer (pH 7.0) retained full after 24 h but it decreased 37.5, 70 and 37.5, 75 per cent after 48 and 72 h respectively while a residual activity of 30, 12.5 and 12.5 was observed after 24, 48 and 72 h respectively in citrate buffer (pH 6.2) as indicated in Figure 33B and Table 26). Storage at room temperature in acetate buffer (5.8) also resulted in loss of about 75, 87.5 and 87.5 per cent of 24, 48 and 72 h.

Different workers have used different buffers for the purification studies of different bacteriocins. The buffers, which have been used in the purification of bacteriocins include sodium phosphate buffer (pH 7.0) for lactacin F (Muriana and Klaenhammer, 1991), lactacin 481 (Piard et al., 1992), pediocin PAI (Lozano et al., 1992) pediocin L50 (Cintas et al., 1995), sodium phosphate
buffer with 4 M urea for acidocin J 1132 and acidocin J 1229 (Tahara et al., 1996; Tahara and Kanatani, 1996), acetate buffer (pH 5.0) for lactacin B (Barefoot and Klaenhammer, 1984), mesentericin Y105 (Hechard et al., 1992), pediocin SJ-1 (Schved et al., 1993), and citrate buffer for acidophilicin LA-1 (Dave and Shah, 1997).

5.9.2 Solvent fractionation of the bacteriocin:- The bacteriocin from the culture supernatant was recovered by fractionation with various organic solvents viz: isopropanol, ethanol, methanol and acetone (Table 27). The bacteriocin precipitated between 0.0-to 1.0 volume of ethanol and methanol exhibited a recovery of 1.89 and 2.27 %, and 2.65 and 5.89-fold increase in specific activity, respectively. The fractionation of the bacteriocin between 1.0-2.0 and 2.0-3.0 volumes of ethanol and methanol and at all the three volumes i.e.0.0-1.0, 1.0-2.0 and 2.0-3.0 of isopropanol and acetone resulted in a very low recovery and a decrease in the specific activity of bacteriocin.

The fractionation of the bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CCSUB202 using different organic solvents viz. acetone, methanol, ethanol, isopropanol not only afforded very low yields but also resulted in a decrease in the specific activity of bacteriocin preparations, indicating the adverse effect of the organic solvents on the bacteriocin activity of the culture supernatants.

Burianek and Yousef (2000). a solvent extraction method was developed to concentrate lacticin from the culture *Lactobacillus acidophilus* OSU133 also no activity found after potential precipitation of bacteriocin upon addition of methanol, isopropanol and acetonitrile to culture supernatant fluid (1:1,v/v) was explored.

Although recovery of enzymes by fractionation with organic solvents is a common practice. But very few reports are available on the solvent fractionation of bacteriocins of lactic acid bacteria.
5.9.3 Ammonium sulphate precipitation results after dialysis: Bacteriocin from culture supernatant of *Lactococcus lactis* subsp. *lactis* CCSUB202 was concentrated by ammonium sulphate precipitation followed by dialysis. The results presented in Table 28 showed that fractionation of bacteriocin with 0 to 40 per cent and 40 to 60 per cent ammonium sulphate saturation resulted in 20 and 60 per cent recovery of bacteriocin with 6.68 and 15.87 fold increase in specific activity, that is 5044.3 AU/mg and 11976.6 AU/mg protein in comparison to 754.2 AU/mg for crude bacteriocin in culture supernatant. Ammonium sulphate saturation in the range of 60 to 80 per cent and 80 to 100 per cent exhibited very low recovery of bacteriocin with very low specific activity. However, ammonium sulphate fractionation in the range of 0 to 60 per cent saturation resulted in 77.5 per cent of recovery with 12.00-fold purification of bacteriocin (Figure 34).

Ammonium sulphate precipitation is the most common and widely used method to concentrate the antibacterial protein from the culture supernatant of the bacteriocin producing lactic cultures and procedures results in varying degrees of recovery and purity of different bacteriocins.

The other bacteriocins of LAB precipitated with ammonium sulfate include: *lactococcin G*, which was purified to a 35-fold with recovery of 57% (Nissen-Mayer *et al.* 1992), carnosin 44A with 4-fold increase in the activity using 0-60% saturation (van Laack *et al.* 1992) while Piard *et al.* (1992) obtained a 455-fold purified lacticin 481 preparation upon reprecipitation with ammonium sulphate (0-80%) of the pellet recovered from the culture supernatant using 0-60% saturation.

Ammonium sulphate precipitation of lactacin F with 0 to 40 per cent thrice resulted in 64 per cent activity recovery with 320-fold purification and most extraneous proteins were separated out as determined by SDS-PAGE analysis (Muriana and Klaenhammer, 1991). While quite low inocence in specific activity of acidocin 8912 was observed by Tahara *et al.* (1992) but with a high recovery (68.1%). In case of acidocin J 1132 also, ammonium sulphate
precipitation (0 to 65 %) though recovered about 80 per cent activity but no increase in specific activity was observed (Tahara et al., 1996). While, Dave and Shah (1997) reported that on subjecting acidophilic LA-1 preparation from two-stage ammonium sulphate precipitation to SDS-PAGE, the purest form of bacteriocin was obtained. However, the bacteriocin activity was found to be lost to a considerable extent after each purification step.

5.9.4 Gel filtration chromatography:- The bacteriocin preparation obtained after ammonium sulphate precipitation step was further purified by gel filtration (HPLC, AKTA Prime Amersham Biosciences, Sweden) the elution profile of ammonium sulphate precipitated bacteriocin is shown in Figure 35. The bacteriocin was eluted from the Superdex 75 column (HPLC, AKTA Prime Amersham Biosciences, Sweden) in between 60 and 65-fraction number. These fractions were pooled and concentrated. The concentrated fraction exhibited a bacteriocin titre of 5280 AU/ml. It was further observed that purified bacteriocin after gel filtration exhibited a specific activity of 19200 AU/mg and overall poor recovery of 10 per cent but with higher purification fold, that is 25.45-fold increase. Table 29.

A superdex 75 gels have molecular fractionation range of about 3000-70,000 Da. In gel filtration, protein or bacteriocin is separated on the basis of molecular mass. The protein molecules having high molecular weight are eluted first and less molecular weight fractions are eluted later, thus giving an idea about the molecular mass of bacteriocin also. Previously also, gel filtration technique has been successfully used by several workers for the purification of bacteriocin from Lactobacillus sp. (Barefoot and Klaenhammer, 1984; Mortvedt et al., 1991; Muriana and Klaenhammer, 1991; Tahara et al., 1992). Most of the researchers reported that purification resulted in several fold increase in specific activity of bacteriocin but resulted in poor recovery also. These bacteriocins include lactacin B with 2.4 per cent recovery (Barefoot and Klaenhammer, 1984), lactacin F with 41 per cent recovery with
369-fold purification (Muriana and Klaenhammer, 1991) and acidocin 8912 with 13.6 per cent recovery (Tahara et al., 1992). Other methods have also been used for purification of bacteriocin from Lb. acidophilus culture which include organic solvent fractionation and cation exchange and reversed phase HPLC (Tahara et al., 1992; ten-Brink et al., 1994; Tahara et al., 1996; Tahara and Kanatani, 1996).

5.10 Characterization of bacteriocin: - The partially purified bacteriocin of Lactococcus lactis subsp. lactis CCSUB202 was characterized with respect to its inhibitory spectrum, molecular weight, heat stability, pH stability, effect of pH on heat stability, effect of sodium chloride and surfactant on bacteriocin activity. Table 30 to 37 and Figure 36 to 47.

5.10.1 Inhibitory spectrum: - Inhibitory spectrum of partially purified bacteriocin was determined by agar well assay using various lactic acid and non-lactic organisms. It may be seen from the Table 30 and Figure 36. Among the microorganisms inhibited by certain bacteriocin, numerous reports have including the fatal pathogens (Yarmus et al., 2000; Ying et al., 2004; Keren et al., 2004; Kabuki et al., 2006; Batdorj et al., 2006; Todorov and Dicks, 2006).

5.10.2 Determination of molecular weight of bacteriocin: - The partially purified bacteriocin preparation obtained by Gel-Filtration procedure was run on SDS-PAGE. One part of the gel with molecular weight markers was stained with Comassie brilliant blue R-250 and one part of the gel was used to determine bacteriocin activity.

It may be observed from the Figure 37 that the gel stained with the dye had shown bands with molecular weight 3.4 kDa in the bacteriocin sample (Lane B, C and D). The gel overlaid with the indicator strain gave clear zone of inhibition. Super imposing of stained gel over the gel used to detect the
bacteriocin activity revealed that the protein bands detected in the stained gel possess the antibacterial activity. The protein band corresponding to the inhibition zone could have molecular weight approximately 3.5 kDa.

The bacteriocin preparation purified by Gel-filtration method has shown protein bands. Similarly, sakacin A and leuconocin LCM1 purified by the pH dependent adsorption-desorption method showed several protein bands in the SDS-PAGE gels (Yang et al., 1992). Since the direct detection of bacteriocin activity in the stained SDS-PAGE gels and the subsequent comparison with the stained gel allowed the estimation of the apparent molecular weight of the bacteriocin of Lactococcus lactis subsp. lactis 202 to be in the range of 2.5 to 3.4 kDa. Using a similar approach, the molecular weights of mesentericin Y105 and carnosin LA44A were estimated to be 2.5-3.0 kDa (Hechard et al., 1992) and 2.5 – 6.0 kDa (van Laack et al., 1992) respectively. The molecular masses of several other bacteriocins viz. podiocin 5 (Daba et al., 1991), acidocin B (ten Brink et al., 1994) have also been estimated by SDS-PAGE method. Bacteriocins of LAB in general have been characterized as low molecular weight substances. Molecular weights reported for various bacteriocins of LAB nisin (Hurst, 1981; Choi et al., 2000; Izildinha et al., 2000; Sharma, 2002) diplococcin (Davey and Richardson, 1981), Lacticin 481 (Piard et al., 1992), lactococin G (Nissen-Meyer et al., 1992), diacetin B (Ali et al., 1995) from lactococci leuococcin A–VAL187 (Hastings and Stiles, 1991), mesentericin Y105 (Hecahrd et al., 1992), carnosin LA54A (Keppler et al., 1994) from leuconostocs and lactacin B (Barefoot and Klaenhammer, 1984) and acidocin B (ten-Brink et al., 1994), plantaricin S and T (Jimenez-Diaz et al., 1993).

Keren et al. (2004) also detected molecular weight of Lacticin RM by using SDS-PAGE. Bizani et al. (2005) and Chen and Yangida (2006) also used SDS-PAGE for the molecular weight of some other bacteriocin.

Very few bacteriocins of lactobacilli have been reported to have high molecular weights in excess of 30,000 Da (Joerger and Klaenhammer, 1986; Vaughan et
al., 1992). Many other researchers (Hernandez et al., 2005; Batdorj et al., 2006; Kabuki et al., 2006) used tricine-SDS-PAGE for determines the molecular weight of bacteriocin.

5.10.3 Heat stability of bacteriocin: - Lactococcus lactis subsp. lactis CCSUB202 bacteriocin preparation in both crude and partially purified forms was to be stable to different heat treatment viz: 65°C for 30 minutes, 75°C for 30 minutes, 85°C for 10 and 15 minutes, 90°C for 10 and 15 minutes, 100°C for 5, 10, 15, 30 and 60 minutes and 121°C (autoclaving) for 15 minutes. The stability of both the preparations to the last two treatments that is heating (in water bath) at 100°C for 5, 10, 15, 30 and 60 minutes and 121°C for 15 minutes is present in Figure 38 It is also shown in the Figure 39 and Table 31 that the bacteriocin in both the forms completely retained their respective activities at 100°C for 30 minutes, but lost around 20% of the activity after 60 minutes. Autoclaving (121°C for 15 minutes) of the bacteriocin preparation resulted in a loss of about 40% of the initial activity.

The retention of the bacteriocin activity after different heat treatment apply shows that the bacteriocin of Lactococcus lactis subsp. lactis CCSUB202 is extremely heat stable. Bacteriocins of lactic acid bacteria in general are heat stable antibacterial substances. The extreme heat stability is believed to be because of their simple structure. All the bacteriocin described till date have been found to be low molecular weight heat stable peptides (Bhunia et al., 1988; Daba et al., 1991; Schved et al., 1993). The bacteriocin produced by Do et al (2001), was found to be stable at 121°C for 15 min or 100°C for 120 min. The activity of pediocin 5 (Daba et al., 1991), pediocin SJ-1 (Schved et al., 1993) and pediocin L50 (Cintas et al., 1995) was also not affected after heat treatment for 30 min at 100°C. However, bacteriocins of lactobacilli such as helviticin J (Joergen and Klaenhammer, 1986) and helveticin V-1829 (Vaughan et al., 1992) were reported to be heat labile. The heat sensitivity of these bacteriocins is apparent from their size and the
apparent complexity of their protein structure in contrast to other bacteriocins of LAB.

5.10.4 pH stability of bacteriocin:- The result relating to the pH stability of crude bacteriocin are shown in Figure 40 and 41, Table 32. The bacteriocin remained stable at pH 13 for 2 h, 12 for 24 h, 11 for 7 days and at pH 10 for 15 days Figure 40.

The estimation of the residual activities of the pH adjusted culture broths (Figure 41) revealed that the bacteriocin in crude form was extremely stable pH in the range 1-8 retaining 100% throughout the 15 days storage period at 4°C. It lost about 34.0% of the initial activity after 8 hour at pH 9 with no further loss of activity after 15 days of storage. At pH 10, the residual bacteriocin activity was found to be 50% after 8 hour, 12.8% after 7 days and only 4.03% after 15 days of storage. There was no detectable bacteriocin activity after 15 days of storage at pH 11, 7 days at pH 12 and after 8 hours at pH 13. The estimated residual activities of the bacteriocin at pH 11 were 12.8%, 3.12% and 3.12% after 8 hour, 1 and 7 days of storage, respectively, while the residual activity at pH 12 decreased from 3.12% to 0.15% during the storage of 24 hour.

The estimation of residual activities of the partially purified bacteriocin of Lactococcus lactis subsp. lactis CCSUB202 adjusted to different pH values revealed that the bacteriocin retained total activity in the pH range 1-9 even after 15 days of storage at 4°C Figure 42 and 43, Table 33. There was about 66% loss of the bacteriocin activity at pH 10 and 11 after 24 hour and 75% after 15 days. At pH 12 no detectable activity after 7 days and at pH 13 after 8 hours, there was 10% and 3.97% activity after 8 hour and 24 hour at pH 12.

When the pH of the crude bacteriocin adjusted to 13 or above was lowered to the initial value (pH 4) and the pH-reaadjusted samples were assayed for their inhibitory activity against the indicator strain, no inhibitory activity was noticed.
The bacteriocin preparations of *Lactococcus lactis* subsp. *lactis* CCSUB202 thus remained stable and active over a wide range of pH. The bacteriocins of some of the lactococcal strains such as *lactococcin* produced by *Lactococcus lactis* subsp. *lactis* (Thuault et al., 1991) and bacteriocin S50 produced by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (Kojic et al., 1991) also did not lose activity after exposure for 24 hour between pH values 2 and 11. Bacteriocin S50 was also reported to lose the activity at pH 12 within 30 minutes. The activity of lactostreptocins, however, was completely lost when pH was raised to 7 or above (Kozak et al., 1978). Likewise, carnocin LA54A lost about 50% activity between pH 6-7 and 90% at pH 10 (Keppler et al., 1994). Bacteriocin in the present study did not get destabilised during storage for 15 days at 4°C over a pH range 1-8 (crude) and 1-9 (partially purified). The heat-treated crude lactici 481 also did not show decrease in the activity both at pH 4 and 7.5 after 15 days of storage at 5°C. However, unheated lactici 481 lost 50% and 87.5% of the initial activity after 2 and 7 days, respectively at 4°C.

The fact that *Lactococcus lactis* subsp. *lactis* CCSUB202 bacteriocin activity could not be revived when the pH of the samples adjusted to 12 and 13 were brought down to 4 indicated that the loss in its activity was irreversible.

**5.10.5 Effect of pH on the heat stability of the bacteriocin:** The results pertaining to the impact of heat treatment on the crude bacteriocin adjusted to different pH values are delineated in Table 34 and Figure 44 and 45. The crude bacteriocin subjected to heat treatment at 75°C for 30 minutes and 100°C for 10 minutes retained its total activity in pH range 1 to 8 but lost about 41.2% and 96.7% of the activity at pH 9 and 10, respectively, with no detectable activity at pH 11 and above. However, the crude bacteriocin, which was not given any heat treatment, retained total activity in the pH range 1-8 but the loss of activity was found to be about 36.5%, 72.4%, 87.9% and 97% at pH 9, 10, 11 and 12, respectively. The bacteriocin adjusted to pH 12
produced a zone of inhibition in the well Plate assay (Figure 44) while the bacteriocin given the two heat treatments exhibited a zone of inhibition only at pH 10 (Figure 44).

The partially purified bacteriocin remained active retaining total activity in the pH range 1 to 9 and residual activity at pH 10 and 11 was calculated to be about 40% (Figure 46 and Table 35). The same preparation heated at 100°C for 10 minutes retained 100% activity upto pH 8 only, beyond which the residual activities were 80, 20 and 1% at pH 9, 10 and 11, respectively. Almost complete loss of activity of the heated bacteriocin at pH 11 may also be visualized by a very small zone of inhibition Figure 46 and 47.

The bacteriocins of several LAB have, however, been reported to be heat stable under acidic conditions only. An 80% loss of activity of mesenterocin 52 has been reported at pH 7 after a heat treatment of 100°C/15 minutes (Mathieu et al., 1993).

5.10.6 Effect of surfactants:- The ammonium sulphate precipitated, dialysed bacteriocin was treated with a variety of surfactants. It may be noted from the Table 36 that treatment of the bacteriocin with Tween 20, Tween 80 and Triton X-100 registered an increase of 20%, 20% and 60% in the activity, respectively. The bacteriocin activity increased by 20, 200 and 300% upon treatment with 0.1, 0.5 and 1% SDS, respectively. Among the different concentrations of surfactant as controls, only SDS at 0.5 and 1% level exhibited a negligible bacteriocin titre of 200 AU/ml on the indicator strain.

Bacteriocins of LAB have a tendency to form large macro-molecular complexes after aggregating with other bacteriocin molecules or medium components. Non-ionic detergents such as Tween 20, Tween 80 and Triton X-100 at 1% level did not result in significant increase in bacteriocin activity indicating that these agents are not capable of dissociating aggregates of Lactococcus lactis subsp. lactis CCSUB202 bacteriocin. However, anionic detergents, SDS used at 0.5% and 1% level resulted in a 200 and 300%
increase in bacteriocin activity, respectively. The increase in bacteriocin activity could be attributed to the dispersion of bacteriocin complex thereby releasing more units for the activity.

The formation of bacteriocin aggregates has also been reported in bacteriocins such as helveticin J (Joerger and Klaenhammer, 1986), pediocin AcH (Bhunia et al., 1988) and lactacin F (Muriana and Klaenhammer, 1991). Whereas, SDS had a favorable effect on the dissociation and consequent increase in the activity (40%) of lactacin F (Muriana and Klaenhammer, 1991), it had a detrimental effect on the activity of helveticin V-1829 (Vaughan et al., 1992).

5.10.7 Effect of sodium chloride: It may be seen from the Table 37 that the sodium chloride up to a concentration of 0.4 M did not have any deleterious effect on the bacteriocin activity. Treatment of the bacteriocin with 0.5 M and 1 M NaCl, however, resulted in 30% loss in the activity, which increased to 70% with an increase in the concentration of NaCl to 2 or 3 M. The salt solutions as control failed to exhibit any inhibitory activity on the indicator organisms. It seems that NaCl at relatively higher concentration exerts a toxic effect on the bacteriocin molecule and thus, leads to loss of the antagonistic activity.

Studies on NaCl tolerant bacteriocin have been carried out using various lactic acid bacteria such as (Nodo et al., 1980; Coi et al., 1997; Onda et al., 1999, 2002, 2003; Managota et al., 2003; Tanasupavat et al., 2003; Sabia et al., 2003; Hamasaki et al., 2003 Kabayashi et al., 2003, 2004; Chen et al., 2005).