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Partial purification of an antifungal protein produced by *Enterococcus faecalis* CHD 28.3

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Abstract - The antifungal protein produced by *Enterococcus faecalis* CHD 28.3 isolated from the Cheddar cheese was partially purified from the culture supernatant using ultrafiltration, anion exchange and gel filtration chromatography. Employing a 10 kDa cut off membrane, ultrafiltration of the culture supernatant resulted in a recovery of 44.6% of the antifungal protein with 1.7 fold increase in the specific activity. Anion-exchange chromatography using DEAE-Sepharose matrix followed by purification of the samples using high resolution gel filtration chromatography employing Superose-12 column/FPLC system led to a very low (~0.5%) recovery of antifungal activity with an increase in specific activity by 11.9 fold relative to initial value in crude supernatant. The molecular mass of the antifungal protein from the high resolution gel filtration was estimated to be around 11 KDa. The partially purified protein obtained after DEAE-Sepharose chromatography step was completely inactivated by heat treatment at 72 °C for 15 seconds.

Key words: antifungal activity; antifungal substances; *Enterococcus faecalis*; lactic acid bacteria (LAB); protein purification.

INTRODUCTION

Fungal contamination constitutes a serious and frequently disturbing problem in the dairy industry as it accounts for huge losses attributed to spoilage of dairy foods, particularly cheese and other fermented dairy products. The considerable drop in pH of the milk caused by the growth of lactic acid bacteria (LAB) in fermented dairy products makes such foods a breeding ground for highly opportunistic fungi to proliferate and thrive therein. Food-borne fungi, both yeasts and moulds, invariably result not only into food spoilage but also can pose a serious threat to the health of the consumers as some mould species can be pathogenic or toxigenic for humans (Suzuki *et al*., 1991; Batish *et al*., 1997; Guinane *et al*., 2005; Schnürer and Magnusson, 2005).

Contamination of fermented food products like indigenous curd (*dahi*), srikhand, yoghurt, salami sausage, and cheeses of different types with undesirable mycoflora, viz., *Aspergillus, Penicillium, Fusarium, Rhizopus* and *Mucor*, particularly in the prevailing humid tropical conditions of the Indian subcontinent, poses a consistent risk to the consumer's health. Many of these commonly occurring moulds are capable of producing highly health-damaging toxic metabolites designated as mycotoxins such as aflatoxins, penicillium toxins, trichothecenes, fumonisin, ochratoxin A and patulin (Batish *et al*., 1997; Schnürer and Magnusson, 2005).

The role of organic acids like lactic, acetic and propionic acids as well as those of proteinaceous factors (Thimon *et al*., 1992; Roy *et al*., 1996; Sorensen *et al*., 1996; De Lucca and Walsh, 1999) has been envisaged in suppressing fungal growth. Recently, a number of antifungal metabolites, *e.g.* cyclic dipeptides, phenyl-lactic acid, proteinaceous compounds, and 3-hydroxylated fatty acids have been isolated from *Lactobacillus plantarum* (Cabo *et al*., 2002; Lavermicocca *et al*., 2003; Schnürer and Magnusson, 2005).

In earlier series of studies, Magak'yan and Chuprina (1978) demonstrated the inhibition of *Aspergillus niger*, *Aspergillus glaucus* and *Cladosporium herbarium* by *Lactobacillus plantarum*. Similarly, *Lactococcus lactis* subsp. *diacetylactis* exhibited weak inhibition against commonly occurring moulds. El-Gendy and Marth (1980) reported the arrested growth of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus ochraceus* in the presence of some lactic cultures. The antifungal activity of several lactic cultures against *Aspergillus fumigatus, Aspergillus parasiticus* and *Rhizopus stolonifer* were observed by Batish *et al*. (1989, 1990, 1991) and Suzuki *et al*. (1991).

Various strains of *Bacillus subtilis* were also reported to produce the iturin peptide. These are small cyclic peptidolipids characterized by a lipid-soluble β-amino acid linked to a peptide containing D and L-amino acids (De Lucca and Walsh, 1999). Another antifungal peptide, bacillomycin F, inhibited the

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growth of fungi including *Aspergillus niger*, *Candida albicans*, and *Fusarium oxysporum* (Mhammedi *et al*., 1982). A novel glycopeptide known as cepacidine, produced by *Pseudomonas cepacia* AF 2001A, has been shown to be a potent antifungal agent (Lim *et al*., 1994). Sorensen *et al*. (1996) studied the fungicidal properties of several potential compounds produced by *Pseudomonas syringae*, including syringotoxin B, and syringostatin A. These compounds were fungicidal for *Candida*, *Cryptococcus*, and *Aspergillus* isolates. An antifungal hydrophilic peptide A12-C of 770 Da has been purified to homogeneity from supernatants of sporulated cultures of *Bacillus licheniformis* A12. The peptide A12-C has been shown to be active against several fungi like *Microsporum canis* CECT 2797, *Mucor mucedo* CECT 2653, *Mucor plumbeus* CCM F 443, *Sporothrix schenckii* CECT 2799 and *Trichophyton mentagrophytes* CECT 2793 (Galvez *et al*., 1993).

Screening and identification of novel antimicrobial proteins and peptides with improved fungicidal properties will have tremendous impact on the food preservation and safety as well as on biotechnological processes and products. Lactic acid bacteria (LAB) are widely used as starter cultures for dairy and vegetable fermentation. Some LAB strains are known to produce wide variety of bacteriocins active against food-borne pathogens (Klaenhammer, 1988; Nes *et al*., 1996; Stiles, 1996; Cotter *et al*., 2005). Such kind of beneficial attributes have led to utilizations of bacteriocins as food additives (Stiles, 1996; Montville and Chen, 1998; Ennahar *et al*., 1999). In addition, given the preference of consumers toward more natural food preservatives, identification of biological agents with bacteriostatic and fungistatic properties produced by LAB could significantly improve the acceptability of food items lacking chemical preservatives (Schnürer and Magnusson, 2005). More recently The widespread contaminants of bakery products *Aspergillus niger*, *Penicillium roqueforti* and *Endomyces fibuliger* have been reported to be inhibited by strong antifungal activity of *Leuconostoc citreum*, *Lactobacillus rossiae* and *Weissella cibaria* (1 strain) which inhibited all fungal strains to the same or a higher extent compared with 0.3 % calcium propionate and the fermentation products of these three strains were characterized by low pH values, and a high content of lactic and acetic acids (Valerio *et al*., 2009).

In this context, we previously reported the antifungal potential of *Enterococcus faecalis* CHD 28.3 isolated from a four months old Cheddar cheese. The culture supernatant inhibited several commonly occurring toxigenic moulds; the antifungal substance was shown to be proteinaceous in nature (Roy *et al*., 1996). By extending the same study, we showed that the antifungal principle produced by the culture CHD 28.3 exerted a lethal fungicidal effect on the spores and early development of conidiophores, especially before vesicle differentiation, causing stagnation of the entire asexual reproduction process (Roy *et al*., 2001). In the present study, we report results pertaining to purification of the antifungal protein (AFP) from the CHD 28.3 culture by using different separation techniques.

MATERIALS AND METHODS

Bacterial strain identification. 16S rRNA sequencing of the Cheddar cheese isolate CHD 28.3, originally identified as *Lactococcus lactis* (Roy *et al*., 1996), was carried out to confirm previous identification. The strain CHD 28.3 was maintained as frozen glycerol stocks at –80 °C in Elliker's broth (Hi-Media, India; Elliker *et al*., 1956) with 15% (v/v) glycerol (Qualigenes, India). Cultures were re-activated by inoculation (1% v/v) in M17 broth containing sodium β-glycerophosphate (15 g/l, Hi-media) or Elliker's broth at pH 7.2 and incubation at 32 °C.

Antifungal assay. The strain *Aspergillus flavus* IARI (Culture Collection Div. of Microbiology, Indian Agricultural Research Institute, New Delhi, India) was used as indicator strain to assay the antifungal activity of the protein produced by the CHD 28.3 culture. The mould cultures were propagated on potato dextrose agar acidified with tartaric acid (0.1%), and activated by periodic sub-culturing at monthly intervals.

The CHD 28.3 culture grown for 48 h in Elliker's broth (pH 7.2) was centrifuged at 12000 rpm for 10 min at 4 °C. The antifungal activity produced by CHD 28.3 in the cell-free culture supernatant was assayed by cut-well agar assay and the activity expressed as arbitrary units per ml (AU/ml) (Roy *et al*., 1996). The crude cell-free culture supernatant fluid was stored at –20 °C when not immediately used.

The antifungal activity of the selected strain CHD 28.3 was determined by following the well agar diffusion method (Batish *et al*., 1990). Approximately, 30 ml of molten potato dextrose agar (pH 3.4 $\pm$ 0.2) was transferred to the sterile Petri dish and allowed to solidify. An aliquot of 100 µl of mould spore suspension of *A. flavus* IARI or *A. flavus* NCIM (National Collection of Industrial Microorganisms, India) 555 was spread uniformly over the dried agar surface with a sterile bent glass rod. Plates were dried at 37 °C for 1 h and wells of 6 mm diameter were cut with a sterile borer followed by addition of the 100 µl of CFS or concentrated retentates after the ultrafiltration into the wells. The plates were incubated at 25 °C for 12 to 16 h to facilitate the diffusion. The plates were inverted and further incubated at 25 °C for 3 to 5 days for the growth of the indicator mould and were observed for clear zones around the wells. The zone of inhibition was measured in terms of millimetre.

Purification of the antifungal protein (AFP). The antifungal protein in the culture supernatant was purified by the following protocol.

Ultrafiltration. The crude cell free supernatant (100 ml) was concentrated to 25 ml by ultrafiltration using stirred cell ultrafiltration device fitted with a 10 KDa cut off membrane (YM 10 Diaflo-membrane, Millipore, USA). The four times concentrated retentate was washed twice with 20 mM sodium phosphate buffer (pH 8.0). An aliquot of 100 µl of each of the retentate and the filtrate was examined for the presence of antifungal activity by agar well diffusion assay against *A flavus* IARI and *A. flavus* NCIM 555 as previously described (Roy *et al.*, 1996).

Ion Exchange Chromatography. The DEAE-Sepharose 2B (Sigma Chem.) in 20% alcoholic suspension was washed (equilibrated) with four bed volumes of sterile glass distilled water to remove the alcoholic traces. The DEAE-Sepharose-2B matrix was packed into a glass column (1.5 x 20 cm) and equilibrated with four bed volumes of 20 mM sodium phosphate buffer, pH 8.0 under cold conditions (5 °C). An aliquot of 15 ml concentrated sample obtained from ultrafiltration step was layered onto the column and the protein was allowed to bind with the matrix. The column was washed with 55 ml of 20 mM sodium phosphate buffer (pH 8.0) and the bound proteins were eluted with a linear sodium chloride gradient (0 to 500 mM), pH 8.0, at a flow rate of 30 ml h^{-1}. A total of 45 fractions (3 ml each) were collected and a small aliquot from each fraction was

used to assay the antifungal activity and determination of absorbance at 280 nm. Fractions containing antifungal activity were pooled and concentrated using a 10 KDa ultrafiltration membrane. The three times concentrated retentate was washed with 50 mM sodium phosphate buffer (pH 7.0) and an aliquot of the same was used to assay the antifungal activity against *A. flavus* IARI.

High resolution gel filtration of the AFP using FPLC. Further purification of pooled fractions containing distinct antifungal activity from the previous steps was carried on gel filtration Superose-12 column (1 x 30 cm) connected with an FPLC system (Amersham-Pharmacia Biosciences, UK). The column was equilibrated with at least two volumes of elution buffer containing 50 mM sodium phosphate and 150 mM NaCl at pH 7.0. The elution buffer was filtered through a 0.2 μm membrane and degassed by constant stirring under vacuum. The protein sample was centrifuged at 14000 *x g* for 5 min to sediment any particulate material. An aliquot of 500 μl of the concentrated protein sample was injected into the column. The flow rate and fraction collection time were set to 0.4 ml/min and 3 min, respectively. The collected fractions were subjected to cut-well agar assay for the antifungal activity against *A. flavus* IARI. The fractions showing antifungal activity were pooled and concentrated using the 10 KDa cut off small scale ultrafiltration device, Centricon-10 (Millipore, USA).

Estimation of protein concentration. The dye binding method of Bradford (1976) was used for estimation of protein concentration. The standard curve was prepared by using BSA (Sigma Chem).

Molecular weight determination. Molecular weight of the purified AFP was determined by gel filtration chromatography using Superose-12 column. The column void volume (V_o) was calculated with the help of blue dextran. The standard curve (Log Mw versus V_e/V_o, where Mw is the molecular weight and V_e is the elution volume of a given protein) was obtained using chromatography protein markers (BioRad Lab, USA), such as β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa) as molecular weight markers. The protein markers were dissolved in 50 mM Tris-HCl, pH 7.5 containing 40% glycerol and 0.1% (w/v) sodium azide and diluted 100 times with 50 mM phosphate buffer (pH 6.5) before loading on Superose-12 column. Elution volume of the AFP was calculated by measuring antifungal activity in the eluted fractions.

Heat stability of the antifungal substance
The AFP samples at various stages of purification were subjected to heat treatment at 72 °C for 15 s to determine the heat stability of AFP. The residual activity of AFP was determined by cut-well assay against *A. flavus* NCIM 555.

RESULTS AND DISCUSSION

Identification of the bacterial strain CHD 28.3
Strain CHD 28.3 was previously isolated from a four months old Cheddar cheese and after biochemical and morphological tests it resulted as *Lactococcus lactis* ssp. *lactis* (Roy *et al*., 1996). Members of the genus *Lactococcus* and *Enterococcus* show similar biochemical and morphological characteristics and cause ambiguity in their identification by biochemical and microbiological methods. This prompted us to confirm previous identification by 16S rRNA sequencing, which indicated that CHD 28.3 belongs to *Enterococcus faecalis*.

AFP purification and characterization
The AFP produced by *E. faecalis* CHD 28.3 was purified using a combination of ultrafiltration and chromatographic methods. The recovery of protein at different steps of purification is reported in Table 1. The concentrated retentate obtained after the ultrafiltration of the cell free supernatant of CHD 28.3 exhibited antifungal activity against *A. flavus* IARI (inhibition zone of 26 mm diameter, ~36 AU) and *A. flavus* NCIM 555 (28 mm; 40 AU). The ultrafiltration step resulted in a recovery of only ~ 44.6% with about 1.7 folds increase in the specific activity of AFP. Ultrafiltration is an efficient method to concentrate proteins; however it may lead to lower recovery when the molecular size cut off of the membrane used is close to the molecular weight of the protein. The low recovery of AFP indicates that the protein could be of low molecular weight.

The concentrated retentate from ultrafiltration step after washing with 20 mM phosphate buffer (pH 8.0) was further purified by anion-exchange chromatography. The elution profile obtained from DEAE-Sepharose using a linear gradient of 0-0.5 M NaCl at pH 8.0 in the presence of 20 mM phosphate buffer is shown in Fig. 1. The activity profile indicates that AFP eluted at the beginning of the gradient. The antifungal activity was detectable in the fractions numbers 21-24, which correspond to the NaCl concentrations of 70-150 mM. More strongly bound proteins were subsequently eluted with 1 M NaCl. The

Table 1 - Summary of the yield and specific activity of antifungal protein produced by *Enterococcus faecalis* CHD 28.3 at various stages of purification

Steps	Volume (ml)	Protein (mg)	Activity (AU/ml)	Total activity (AU)	Specific activity (AU/mg protein)	Recovery (%)	Purification (fold)
Crude culture supernatant	100.00	36.00	23.00	2300	63.9	100	1.00
Membrane Ultrafiltration	25.00	9.51	41.00	1025	107.8	44.57	1.69
DEAE-Sepharose	15.00	4.032	39.80	597	148.1	26.00	2.32
Gel Filtration (FPLC) (Superose-12)	1.00	0.016	12.20	12.2	762.5	0.530	11.93

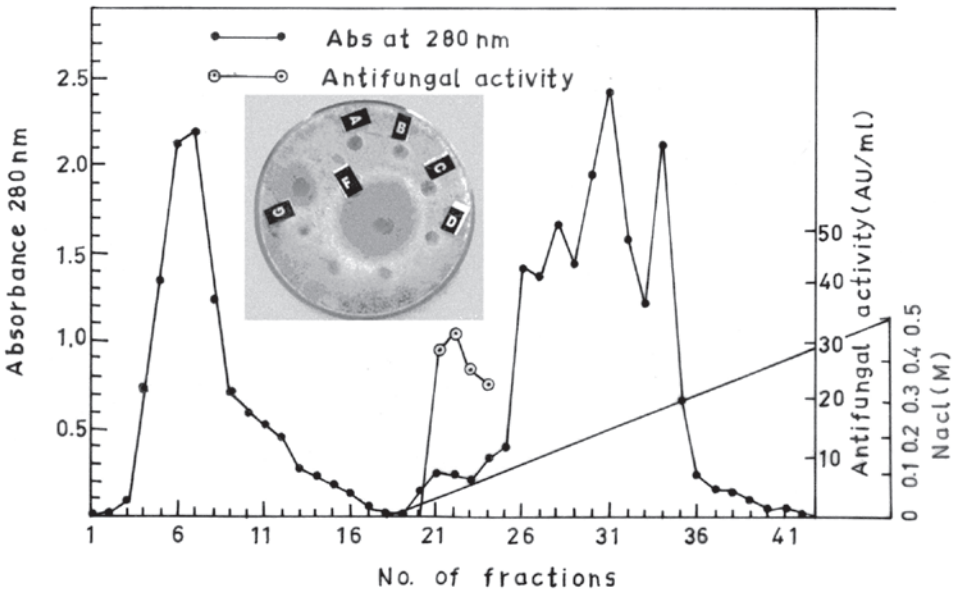

FIG. 1 - DEAE-Sepharose anion exchange profile of proteins obtained from concentrated culture supernatant of *Enterococcus faecalis* CHD 28.3. The inset plate shows the antifungal activity produced by CHD 28.3 against *Aspergillus flavus*. Fraction numbers 21-23 from DEAE-Sepharose chromatography were pooled, concentrated and washed with phosphate buffer before loading in to the wells at various dilutions: 20 mM phosphate buffer (A), 20 mM phosphate buffer with 0.5M NaCl (B), 1:3 dilution (C), 1:2 dilution (D), 1:1 dilution (G), and pooled and concentrated sample (F).

purified protein exhibited strong biological activity as revealed by a large zone of inhibition (28.5 mm; 40.0 AU) (Fig. 1). This step resulted in AFP recovery of 26% with an increase in the specific activity by ~2.3 folds of the initial activity in the crude supernatant.

DEAE-Sepharose purified proteins in fractions 21-24 showing antifungal activity were subsequently pooled and concentrated by high resolution FPLC purification. The elution profile of proteins as shown in the Fig. 2 was obtained by using Superose-12 gel filtration column. Fractions were pooled and concentrated before setting up the assay. Sample from pool I (fractions 13-15) showed major activity against *A. flavus* IARI. Table 1 shows that gel filtration resulted in very high specific activity, however the total recovery was only 0.5% of the initial activity indicating large scale loss of antifungal protein at the gel filtration step. The extremely low recovery of AFP after this step could be attributed to loss of protein moiety during excessive concentration through a 10 KDa cut off membrane needed to reduce the volume of sample before loading on Superose-12 column and after elution to determine the AFP activity. No AFP activity was detected in any of the fractions obtained after the gel filtration without concentration (data not shown). The loss of protein could also be caused by binding of protein to the column. Several workers have reported an unusually low recovery (~0.04-15%) of bacteriocins in the final step of purification by reverse phase chromatography or by gel filtration (Mortvedt *et al*., 1991; Muriana *et al*., 1991).

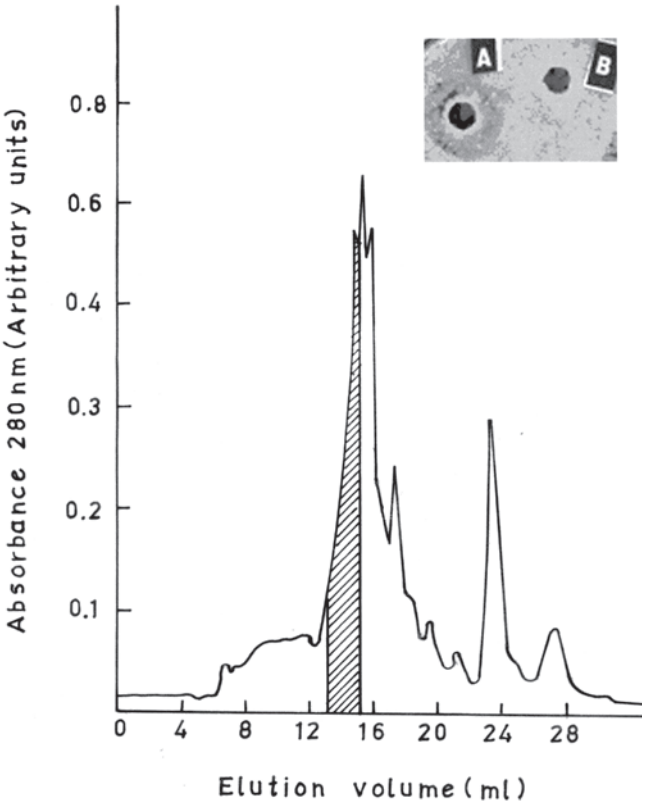

FIG. 2 - Chromatographic profile of pooled and concentrated fractions (21-23) collected from DEAE-Sepharose anion exchange chromatography on FPLC using Superose-12 gel filtration column. Fractions number 13-15 from gel filtration chromatography were pooled, concentrated before loading in to the wells. The inset plate shows activity profile of pooled samples (fraction No. 13-15), concentrated (well A) and without concentration (well B).

Molecular mass and thermal stability of AFP

The molecular mass of AFP in pool I from gel filtration, which were the fractions showing the highest biological activity, was found to be ~11 kDa. In general, even though specific activity of AFP increased, the recovery of protein at every step of purification decreased sharply, which suggests that

AFP was either getting deactivated or lost at each step of the purification process. The AFP molecular mass of 10-11 KDa suggests that there may be significant loss of protein during concentration carried out using an ultrafiltration membrane of a comparable pore size of 10 KDa cut off. The loss of activity due to deactivation of protein might be significant also, however to a lesser extent than the direct loss of protein in ultrafiltration as indicated by a protein loss of ~55% in the first step of 4 fold concentration by ultrafiltration. Moreover, the specific activity was increased at each subsequent step of purification, suggesting the physical loss because of filtration and adsorption rather than chemical deactivation.

The AFP preparation obtained after DEAE-Sepharose ion-exchange chromatography completely lost the antifungal activity when subjected to heat treatment at 72 °C for 15 s. The heat stability of partially purified AFP was observed to be lower than AFP in crude Elliker's broth. The lowering of heat-sensitivity of partially purified protein could be attributed to loss of certain compounds that may protect the protein in the crude preparation. Ellison and Kaulter (1970) reported a substantial decrease in the heat stability of bacteriocin from *Clostridium botulinum* type E with increasing level of purification. It has been proposed that the loss of activity of purified antimicrobial proteins upon heating may be because of denaturation of the protein in dilute solutions or the loss of co-factors (Davey and Richardson, 1981). The antimicrobial proteins by virtue of associating with other bacterial proteins also attained increased biochemical stability as was observed by Lyon and Glatz (1991) for the bacteriocin PLG-1 from *Propionibacterium thoenii.* In a separate investigation the production of the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) by lactic acid bacteria has been reported by Ström *et al*. (2002). In another study Magnusson *et al*. (2003) working on antifungal proteins from the LAB, were able to identify antifungal cyclic dipeptides after HPLC and several other active fractions suggesting a rather complex nature of the antifungal substances. We have not observed binding of some other factors with the AFP, but the loss of activity with increased degree of purification indicates that AFP might be stabilized by some chemical factors present in the crude extract.

CONCLUSION

Consumer demands for minimally processed foods and reduced use of chemical preservatives have stimulated research on antifungal lactic acid bacteria as biopreservatives. The antifungal protein studied has the potential to serve as a potent inhibitor of mould growth. The present finding suggests applications of antifungal lactic acid bacteria for biopreservation in dairy and food industry.

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