RESEARCH ARTICLE

Effect of yeast extract on cell growth of three *Lactococcus lactis* ssp. *cremoris* strains in cheese bulk starter

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Submitted: August 8, 2019; Revised: December 1, 2019; Accepted: December 10, 2019
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ABSTRACT

Bulk starter systems are used in large scale cheese factories to produce large volumes of starter culture in order to inoculate cheese vats. High number of cells and high starter activity in cheese vat is critical to produce high quality natural cheese. This study investigated the effect of two commercial yeast extracts (YE-A and YE-F) on increasing cell number and starter activity in a cheese bulk starter system. Cheese bulk starter (BS) culture unit was simulated using laboratory scale fermentation system. Bulk starter fermentation was carried out at controlled temperature (27 ºC) and agitation (180 rpm) using three Lactococci strains (Lactococcus. lactis ssp. cremoris strains 49, 831, 1926 [ASCRC]). Growth of starter culture organisms during BS fermentation was analysed by determining lactic acid production, pH reduction, viable plate counts and OD measurements. Yeast extract supplementation to the bulk starter media significantly increased the lactic acid production of the test Lactococci strains (Lactococcus lactis ssp. cremoris strains 49, 831, 1926 [ASCRC]) in the bulk starter culture compared to the traditional bulk starter media. Cell growth in the bulk starter was increased up to two fold due to YE supplementation. Selected levels of YE-A gave higher growth to the mixed culture compared to YE-F. Chemical analysis and RP-HPLC analysis showed that both yeast extracts contained higher free amino acids and peptides contents compared to skim milk powder (SMP) media. YE-A (0.5%) had the highest free amino acids and peptide contents followed by YE-F (0.2%). The free amino acids and peptides present in yeast extracts apparently enhanced the growth of the mixed culture in the bulk starter media.

Keywords: *Lactic acid production, fermentation, optical density measurement, RP-HPLC, free amino acids, peptides*

INTRODUCTION

Starter culture performance and activity represents one of the most important aspects of cheese manufacturing process. Reliable and consistent acid production by starter cultures in the cheese vat is critical to achieve the desired final quality of cheese, especially for certain cheese varieties like cheddar. Starter culture added to the cheese vat should be active enough to develop lactic acid at required rate in order to attain the desired acidity of cheese milk within a predetermined period (generally pH of 5.4 within 5 h) to achieve the required

final quality of the product (Powell *et al.*, 2003; Fox *et al.*, 2000; Fox, 2003; Parente and Cogan, 2004).

At present, commercial cheese manufacturers obtain their starter cultures from specialist starter manufacturers in concentrated form (frozen or freeze dried) which are delivered to the cheese vat either by direct inoculation or after growing in a bulk starter vessel. However, large cheese factories require a large volume of suitable cultures on a regular basis to inoculate cheese vats. Consequently, the maintenance of bulk starter units on site at factories to propagate starter culture is becoming a common practice in many large cheese factories (Limsowtin *et al.*, 1997; Broome *et al.*, 2003).

The intermittent occurrence of starter culture inhibition is a serious problem in cheese factories. In incidences of starter inhibition cheese manufacturers usually add additional levels of bulk starter to the cheese vats in order to compensate for the inhibition. However, during extended periods of inhibition this may lead to exhaustion of the bulk starter supply in the factory limiting the day's cheese production (Roginski *et al.*, 1984; Packham, 2002). Alternatively, manufacturers can increase the bulk starter capacity (i.e. increase cell numbers and activity of starter organisms in the bulk starter) in order to meet the increased starter culture demand during periods of starter inhibition.

A number of studies has shown that addition of yeast extract to the culture growth medium significantly increased the growth and activity of LAB by providing essential nutrients to the medium (Ibrahim and Bezkorovainy, 1994; Benthin and Villadsen, 1996; Champagne *et al.*, 1999; Champagne *et al.*, 2003; Gaudreau *et al.,* 1999; Altaf *et al.*, 2007; Berrette *et al.*, 2001; Whitehead *et al.*, 1993, Mudannayke *et al.*, 2018).

The aim of this research was to investigate the effect of yeast extract as a growth media supplement in optimizing the bulk starter culture growth and, to identify the active components in yeast extract which give the growth enhancement effect.

MATERIALS AND METHODS

Media

Reconstituted skim milk

Reconstituted skim milk (RSM) was prepared by blending skim milk powder with deionized water to 10% (w/v) which was pasteurized at 85 °C for 15 min or autoclaved at 121 °C for 15 min depending on the requirement.

1/10 M17 broth

1/10 M17 broth (Oxoid, UK) was prepared by dissolving 3.72 g M17 powder and 0.5 g lactose in 1 L de-ionized water (pH to 7.1). The broth was then dispensed

into test tubes (9 mL) and autoclaved at 121 °C for 15 min. The medium was stored at 4 °C for maximum of 2 months prior to use.

M17 Agar

M 17 agar (Oxoid, UK) was prepared by dissolving 24.1 g of M17 agar powder in 480 mL of deionized water (pH 7.1) and autoclaving at 121 °C for 15 min. Following autoclaving 5 mL of 1 M $CaCl_2$ and 15 mL of lactose were added to the medium which was then poured into plates in 20 mL volumes. The agar plates were stored at 4 °C for a maximum of 2 months prior to use.

Starter culture strains

The three starter culture strains used in the study *Lactococcus lactis* ssp. *cremoris* strains 49, 831, 1929 [ASCRC] were obtained from Dairy Innovation Australia- Culture Division, Werribee, Victoria, Australia.

Yeast extracts

Two commercial yeast extracts were used in the study; 60% Autolysate® (Yeast extract-A(YE-A)) and Flavex powder type 0506-14® (Yeast extract-F (YE-F)) obtained from Mauri Yeast Australia, Queensland, Australia and Halcyon Proteins, Victoria, Australia, respectively.

Bulk starter simulation

A factory bulk starter unit was simulated using a laboratory scale fermentation system, as described in Mudannayake *et al.* (2018). One litre glass fermenter vessels containing the appropriate media were kept in a temperature controlled water bath (Polystat CC1, CE hurber, Offenburg). Bulk starter growth was carried out at controlled temperature of 27 °C, under continuous agitation at a speed of 180 rpm. Three fermenter vessels were run simultaneously. The pH probes (L3015 Model GT-DJ-225-2m- double junction, PH Scientific (Pvt.) Ltd., NSW, Australia) were inserted into each fermenter vessel to monitor the pH throughout the fermentation period. pH data logging was carried out via the sensoray logging software (Total Turnkey Solutions, Vic, Australia) while the pH of the media was maintained at 6.00 by automatic addition of 46% (v/v) NaOH connected to the fermenters via valves to control flow rate. The Glass alkali reservoirs were placed on load cells (Celtron LPS- 1.0 kg, Scale components, Queensland, Australia) to determine the mass of alkali delivered over time (Mudannayake *et al.*, 2018). The fermenter vessels containing RSM media were inoculated with the given mix starter strains (*Lactococcus lactis* ssp. *cremoris* strains 49, 831, 1929[ASCRC]) at a percentage of 0.006% and fermentation was run over night.

Bulk starter media

The bulk starter media were prepared from 10% (w/v) reconstituted skim milk (RSM) with added yeast extracts levels of either YE-A or YE-F

followed by pasteurisation. A series of bulk starter fermentation trials were conducted using the laboratory scale fermentation system, using different levels of YE-A or YE-F to find the optimum YE addition level from each yeast extract type (Mudannayake *et al.,* 2018). Accordingly, 10% RSM supplemented with 0.5% (w/v) YE-A or 0.2% (w/v) YE-F were used in this study, while 10% RSM with no added yeast extract were used as the control. Four fermentation experiments were carried out separately, following the same experimental conditions, in four different days.

Sampling

Samples (3x10 mL) were taken through the sampling port, when the volume of 46% (w/v) NaOH added to each fermenter vessel equalled to 0.4% of the fermenter volume, which is the industry bulk starter harvesting point. The samples were immediately chilled to 4°C and tested within 48 h.

Analysis of bulk starter growth

Measurement of pH and lactic acid production

The pH change during bulk starter growth was monitored using double junction glass pH electrodes. The rates of lactic acid production during bulk starter growth were determined by calculating the amount of 46% (w/v) NaOH added to bulk starter vessels over the fermentation period.

Measurement of cell growth

Cell counts

Bulk starter samples were serially diluted with 1/10 M17 agar and 0.1 mL of the appropriate dilutions were plated on M17 agar spread plates. The white round colonies were counted after incubation at 30 °C for 48 h. Cell counts were performed in triplicate for each bulk starter sample.

Optical density

Cell growth in the bulk starters were monitored by determining optical density at one hour intervals. Prior to the optical density measurement, the caseins in the bulk starter samples were solubilised using the method described by Kanasaki *et al.* (1975) and Broome *et al.* (1982). The culture medium (0.25 mL) was added to 1.6 mL ice cold 0.2% (w/v) EDTA tetra-sodium salt (pH 12.5) (Sigma,St. Louis, MO) in a test tube and mixed vigorously using a vortex mixer. The solution was then mixed with 0.9 mL of ice cold 0.2% (w/v) EDTA di-sodium salt (pH 5.5) (Sigma,St. Louis, MO) and the optical density was measured at 480 nm using a UV- visible spectrophotometer (UV-2550, Shimadzu, Japan).

Microscopic observation

After harvesting bulk starter samples were observed under the light microscope at 1000x magnification.

Chemical analysis

Determination of total free amino acids

The total free amino acid content of the yeast extracts and skim milk were determined by the Cd-ninhydrin method as described by Folkertsma and Fox (1992) and Baer *et al*. (1996). YE-A, YE-F and skim milk solutions were prepared at concentrations of 0.25, 0.20 and 10% (w/v), respectively, using deionized water. Absolute ethanol (700 µL) was added to 300 µL sample aliquots and allowed to stand for 30 min before centrifugation at 11,000 g for 10 min.

Standards were prepared using leucine at concentrations of 0, 0.25, 0.5, 0.75, and 1.0 mM. Samples, standard solutions and blanks (100 µL) were mixed with 200 µL water in microcentrifuge tubes and 600 µL of Cd-ninhydrin solution added to each microcentrifuge tube, before mixing gently and heating at 84 °C for 5 min. The samples were then centrifuged at 11,000 g for 10 min and 200 µL of supernatant carefully added to a microplate well and read at 510 nm using microplate reader. Analyses were performed in triplicate.

Determination of trichloroacetic acid (tca) soluble amino nitrogen

Trichloroacetic acid soluble amino nitrogen content was determined using Trinitrobenzenesulfonic (TNBS) assay as described by Bubnis and Ofner (1992). YE-A, YE-F and skim milk samples were prepared at concentrations of 0.25, 0.20 and 10% (w/v), respectively, using deionised water. Standard solutions were prepared using glycine at concentrations of 0, 5, 10, 15 and 20 mM.

Trichloroacetic acid (TCA) extraction

Aliquots of samples, standards and blanks (0.5 mL) were mixed with 0.5 mL of 24% Trichloro-acetic acid and allowed to stand for 10 min. The samples were then centrifuged at 11,000 g for 10 min and 0.5 mL of the supernatant was removed and added to clean test tubes for the TNBS assay.

Trinitrobenzenesulfonic (TNBS) assay

Aliquots (0.5 mL) of the extracted samples were mixed with 0.5 mL of 5% sodium citrate to prevent formation of precipitate, 1.65 mL of sodium borate solution (0.12 M sodium borate in 0.12 M NaOH) and 0.25 ml of 0.2% Trinitrobenzenesulfonic acid (TNBS) were added to each sample, before incubation in the dark at 37 °C for 45 min, following incubation 0.5 mL of 2

M hydrochloric acid and 0.5 mL of sodium sulphite were added and the absorbance was measured at 420 nm using UV-visible spectrophotometer. Analyses were performed in triplicate.

HPLC analysis

Determination of free amino acids by RP-HPLC

Sample preparation

YE-A (1.0%), YE-F (0.4%) and skim milk powder (10%) solutions were prepared using deionised water. Aliquots (300 µL) of the prepared samples and blank were mixed with 700 µL absolute ethanol in microcentrifuge tubes and allowed to stand for 30 min. The samples were then centrifuged at 11,000 g for 5 min and 0.5 µL of the supernatant was carefully removed and diluted 1:1 with HPLC grade water.

Derivatisation

A sample (50 µL) of the above diluted extractions was mixed with 50 µL 0.4 M borate buffer in microcentrifuge tubes. Aliquots (100 µL) of 16 mM Fmoc-Cl solution (9-fluorenylmethyl chloroformate) were then added to each sample and allowed to stand for 90 s to precede derivatisation. Then, 60 µL portions of cleavage reagent (0.85 M NaOH, 0.5 M hydroxylamine hydrochloride and 2-methyl-thio-ethanol) were added and allowed to stand for another 3.5 min. The reaction was then stopped by adding 100 µL of quenching reagent (acetonitrile-water-acetic acid 20:3:2), and the solution was centrifuged at 11,000 g for 5 min. A portion of the supernatant (120 µL) was added to 150 µL vials for HPLC analysis.

Separation protocol and operating conditions

The HPLC system was equipped with a solvent degasser, quaternary gradient solvent pump, multiple autosampler and a detector (Waters Coporation, Milford, USA). Analysis was carried out at 25 °C using a Phenomenex Luna 5 µ C18 (2) (250 x 4.6 mm) column and guard column. Peak detection was at 263 nm and data analysis was performed using Millenium[32] software (Waters Coporation, Milford, USA).

Samples (30 µL) were injected in to the column and eluted at a flow rate of 0.8 mL/min using gradient of 100% solvent A (50 mM sodium acetate, 7 mM triethylamine, 10% acetonitrile, adjusted to pH 6.5 with acetic acid) for 5 min followed by a gradient from 12 to 100% solvent B (acetonitrile- water 90: 10 v/v) over a 25 min and 100% solvent B for 15 min. Run time was 40 min per sample (Munoz *et al.,* 2003, Broome *et al.*, 2006)

Determination of peptides by RP- HPLC

Sample preparation

YE-A (0.5%), YE-F (0.2%) YE-F and skim milk powder (10% (w/v)) solutions were prepared using deionised water. Sample aliquots of 600 µL were mixed with 1.4 mL of absolute ethanol and centrifuged at 11,000 g for 5 min. A portion of the supernatants (1.0 mL) was removed, diluted 1:1 with 0.2% Trifluoroacetic acid and held overnight at 4 °C. The extractions were then filtered through 0.45 µm cellulose acetate disposable filter and 600 µL of sample was added to 1.0 ml vial for the HPLC analysis.

Operating conditions

Analysis was carried out at ambient temperature using a Bio-Rad Hi-Pore RP 318 (5 µm, 300 Å, 250 x 4.6 mm) column and guard column. Peak detection was done at 210 nm and data analysis was done by Millenium[32] software. Samples (30 µL) were injected in to the column and eluted at a flow rate of 1.00 mL/min using a gradient of 100% solvent A (0.1% Trifluoroacetic acid) for 5 min followed by a gradient from 0 to 100 % solvent B (0.1% Trifluoroacetic acid in acetonitrile: water 50: 50 v/v) over 30 min and 100% solvent B for 15 min. Run time was 50 min per sample (Poveda *et al.*, 2003; Broome *et al*, 2006).

Statistical analysis

Statistical analysis was performed using Minitab 16 statistical software. One way Analysis of Variance at 95% confidence level was used to check for any significant difference, and Tukey's test was performed for multiple group comparisons and separation of the means.

RESULTS

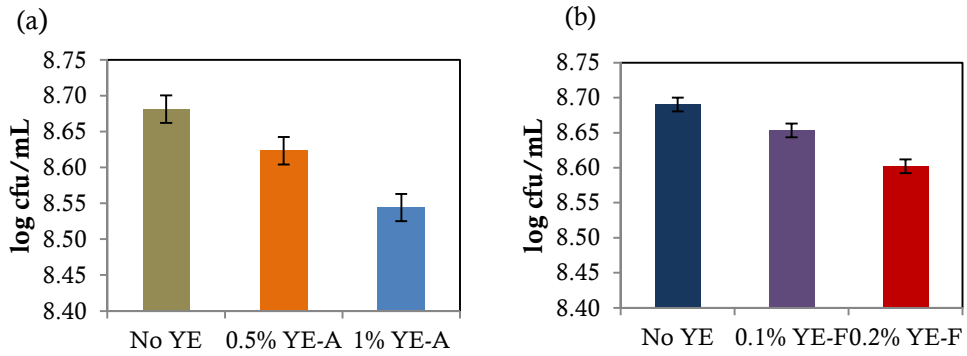
Effect of yeast extract on cell growth in bulk starter

The yeast extracts supplementation to the bulk starter media (0.5% YE-A and 0.2% YE -F) significantly (P<0.05) increased the lactic acid production of the test *Lactococci* strains (*Lc. lactis* ssp. *cremoris* strains 49, 831 and 1929 [ASCRC]) in the bulk starter compared to the no yeast extract added bulk starter media (Table 1).

Table 1: Total lactic acid production by mixed culture in the different media

Level of YE in 10% RSM medium	Lactic acid concentration of the media at 15.5 h fermentation period (g L^{-1})
no YE	4.13 ± 0.50^{a}
0.2% YE-F	13.15 ± 0.52^{b}
0.5% YE-A	19.00 ± 1.23^{b}

Bulk starter samples were plated on M17 agar and after incubating 48 h at 30 °C, the colonies were counted. Interestingly, both YE added bulk starter samples showed lower colony counts than the no YE added bulk starter culture samples (Figure 1). It was observed that the bacterial colonies on the agar plates with YE added bulk starter samples were larger than the no YE added bulk starter samples.



(a) Control and YE-A (b) Control and YE-F.

Figure 1: Cell numbers (cfu mL^{-1}) after growth and harvesting of bulk starter samples. Cell count results presented are the means of two independent triplicated trials. (a) Control and YE-A, (b) Control and YE-F.

Microscopic examination (x1000 magnification) of the bulk starter culture samples showed that a distinctly larger proportion of cells in the yeast extract added bulk starter samples formed longer cell chains (5-6 cells/chains) compared to the no yeast extract added bulk starter samples where a larger proportion of the cells existed as single cells or as pairs (Figure 2). This observation would account for the lower plate counts in the yeast extract added bulk starter culture samples even though the cell numbers were greater.

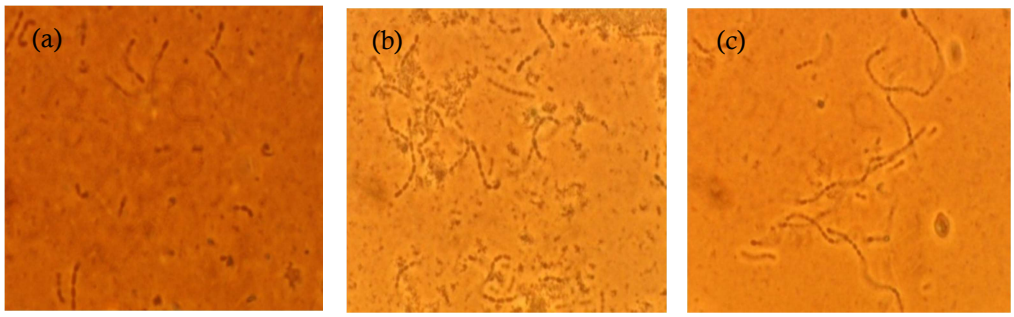
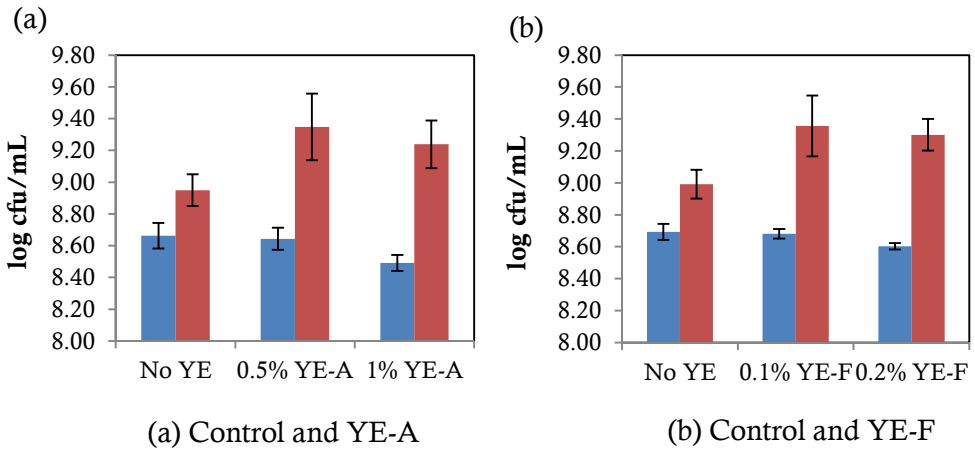


Figure 2: Light microscopic views (x1000) of the starter organisms in the bulk starter culture after harvesting. (a) Bulk starter culture with no YE supplementation; (b) Bulk starter culture supplemented with 0.2% YE-F; (c) Bulk starter culture supplemented with 0.5% YE-A

In order to estimate the total cell numbers in the bulk starter cultures, the plate count values were corrected for the chain length (by multiplying cfu/mL values by the average number of the cells per chain) as suggested by Gaudreau *et al.* (2002). The corrected cell counts gave a more accurate value for the total cell numbers in the bulk starter culture samples (Figure 3).



(a) Control and YE-A (b) Control and YE-F

Figure 3: Cell numbers (cfu mL^{-1}) after growth and harvesting of bulk starter samples corrected for the average chain length (■): Uncorrected cell counts; (■): Cell counts corrected for the average chain length. Average chain length for no YE added samples were taken as 2 while average chain length for YE added samples were taken as 5 based on the microscopic examinations. (a) Control and YE-A and (b) Control and YE-F.

The optical density measurements (Figure 4) showed that growth of the mixed culture in two YE added bulk starters were significantly higher compared to no YE added bulk starters, confirming that YE has increased the cell growth in the bulk starter.

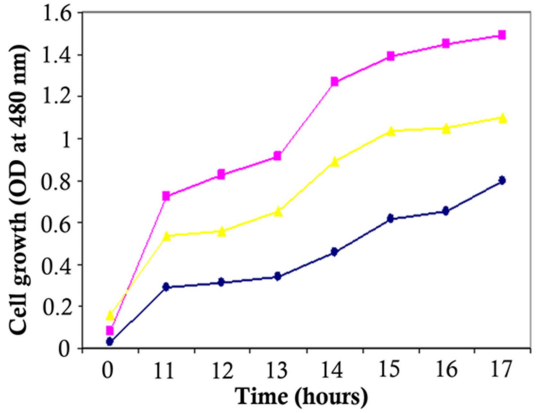


Figure 4: Changes in optical density of mixed *Lactococci* culture in the presence of two yeast extracts, during simulated bulk starter fermentation. ●: control (minus YE); ▲: 0.2% YE-F; ■: 0.5% YE-A.

3.2 Chemical composition of yeast extract

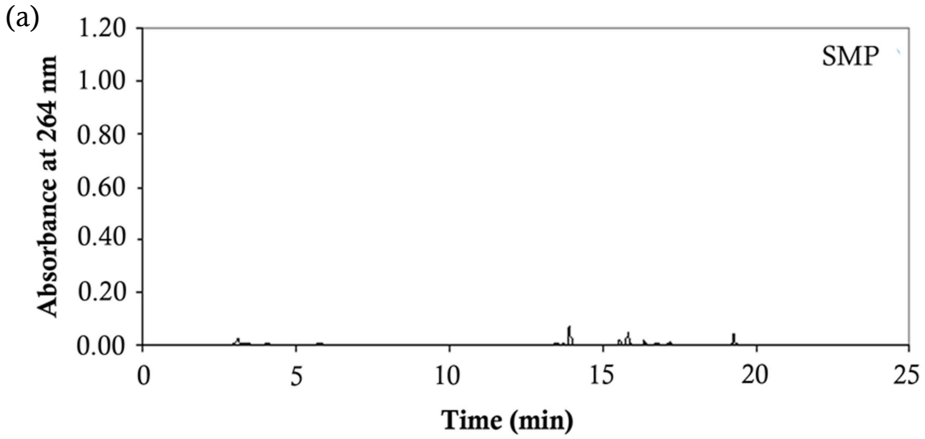
In this study the levels of two yeast extracts used for the chemical analysis were different and presented for the experimentally determined optimum levels. As shown in Table 2, the two yeast extracts contained significantly higher total free amino acid contents compared to the skim milk powder medium. YE-A (0.5%) contained 4 times higher total free amino acid content compared to YE-F (0.2%) (It should be noted that levels of two yeast extracts used for the chemical analysis were different and presented for the experimentally determined optimum levels).

Table 2: Total free amino acid content and TCA soluble amino nitrogen content of yeast extracts and skim milk powder.

	Total free amino acid content (mM)	TCA soluble amino nitrogen content (mM)
10% SMP	0.24 ± 0.02 [a]	1.56 ± 0.20 [a]
0.2% YE-F	1.07 0.20 [b]	2.99± 0.52 [a]
0.5% YE-A	4.44 ± 0.50 [b]	18.89 ±1.52[b]

All values are means of three independent duplicated determinations±standard deviation (SD).

The TCA soluble amino nitrogen content is an indicator of the amount of free amino acids and small and large peptides present in the media. As shown in Table 2, the two yeast extracts contained higher contents of amino acids and small and large peptides compared to skim milk powder, 0.5% YE-A having the highest amount (18.89 mM) followed by 0.2% YE-F (2.99 mM). The qualitative RP-HPLC analyses were carried out to compare the free amino acids and peptide profile in YE-A, YE-F and RSM. RP-HPLC chromatograms of free amino acids (Figure 5) showed that both yeast extracts contained higher levels of free amino acids compared to SMP.



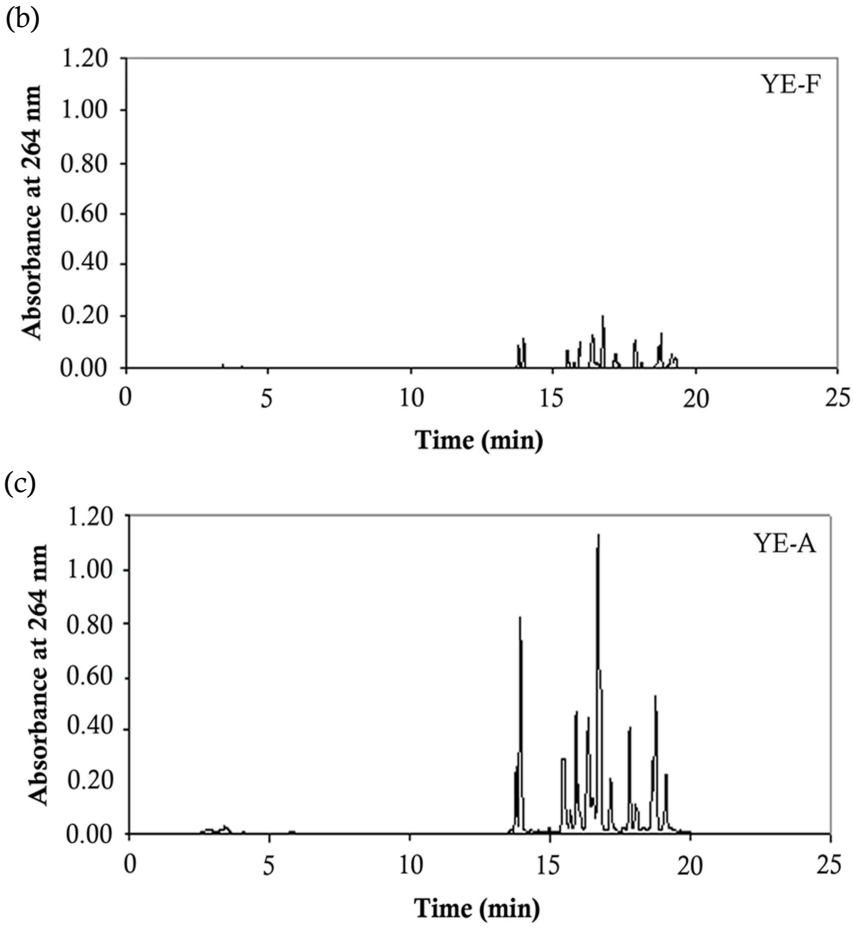


Figure 5: Comparison of the RP-HPLC chromatograms of free amino acids (a) Reconstituted skim milk powder (10%); (b) YE-F (0.2%); (c) YE-A (0.5%).

The YE-A has comparatively more free amino acids compared to YE-F. In this study, the individual amino acids were not identified. RP-HPLC chromatograms of peptide profile (Figure 6) also showed that YE-A has higher amount of peptides followed by skim milk powder and YE-F.

DISCUSSION

Lower cell counts were observed in YE added bulk starter cultures compared to the no YE added bulk starter cultures. However, the increased cell growth in YE supplemented starter cultures were confirmed in optical density (OD) measurements. According to the OD measurement results, 0.5% YE-A supplementation resulted in two-fold increment while 0.2% YE-F supplementation resulted in one-fold increment in final cell growth compared to the no YE supplemented bulk starter cultures.

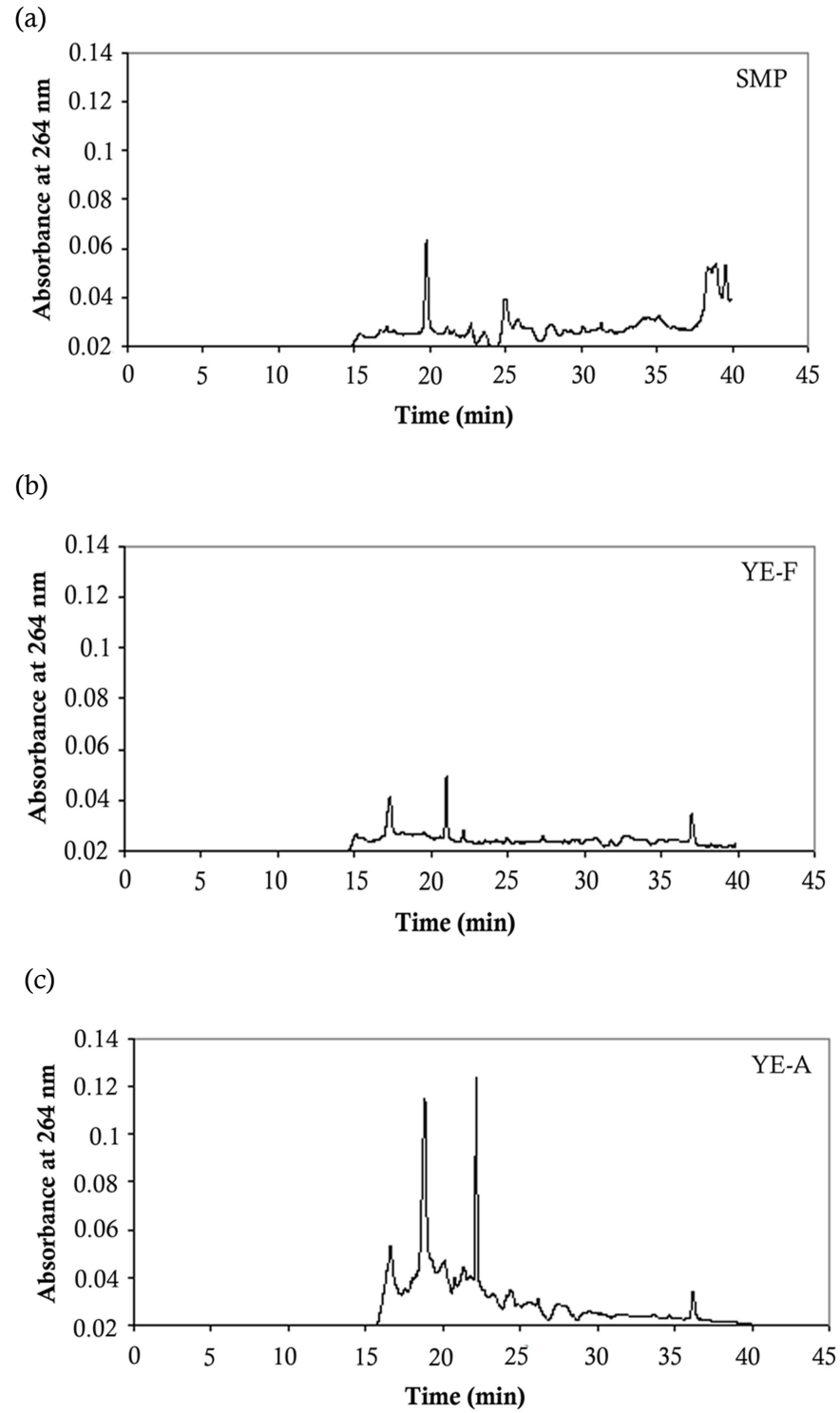


Figure 6: Comparison of the RP-HPLC chromatograms of peptide profiles (a) Reconstituted skim milk powder (10%); (b) YE-F (0.2%); (c) YE-A (0.5%).

The reason for lower cell counts in YE added cultures were found as the variation in bacterial chain lengths. Microscopic examination of the bulk starter culture samples showed that distinctly larger proportion of cells in YE supplemented medium existed in long cell chains (6-7 cells per chain) whereas majority of cells existed as single cells or as pairs in no YE added medium.

This can be explained that, although rapid cell growth and cell division takes place in YE supplemented media, *Lactococci* cells are not capable of rapidly producing cell wall hydrolysing enzymes (peptidoglycan hydrolases) which are required for the cell wall division/separation (Huard *et al.*, 2004). Therefore, incomplete degradation of cell walls causes daughter cells to stay together forming longer cell chains in the medium.

This result was in good agreement with Gaudreau *et al.* (2002). They showed that the lesser correlation between OD results and viable cell count results with respect to growth of lactobacillus cultures in YE supplemented media was due to longer bacterial chain lengths in medium supplemented with YE.

Yeast extract supplemented bulk starter cultures showed higher activity when inoculated to simulated cheese vats and reduced the pH of milk faster than the normal bulk starter cultures. This indicates that YE supplemented bulk starter cultures have higher cell numbers per unit volume compared to the no YE supplemented bulk starter cultures.

Chemical analyses of the media showed that both yeast extracts had higher total free amino acid and small and large peptide levels compared to SMP. YE-A had the highest amount of total free amino acids and peptides. YE-F had the next highest amount while SMP had the lowest amount of free amino acids and peptides. RP-HPLC chromatograms of amino acids and peptide profiles also showed that YE-A have the highest levels of peptides and amino acids which is the most likely reason for improving the growth of the test *Lactococci* strains in the bulk starter system. According to RP-HPLC chromatograms YE-F has high amount of free amino acids compared to SMP; however, peptide profiles showed it has slightly lesser peptides than SMP. Therefore, it would appear that high amount of free amino acids present in yeast extracts were predominantly responsible for the growth stimulation of test *Lactococci* strains while peptides also play an important role in growth stimulation. These results were consistent with results of Smith *et al.* (1975) and Benthin and Villadsen (1996).

CONCLUSION

Lactococci cell growth in laboratory scale bulk starter vessels were increased by two-folds due to YE supplementation to the skim milk medium. The study showed that YE-A gave higher cell growth enhancement to the mixed *Lactococci* strains compared to YE-F. The free amino acids and peptides present in yeast extracts apparently stimulated the cell growth of mixed *Lactococci* strains in bulk starter media. This result also reflects the fact that *Lactococci* strains can

assimilate free amino acids and peptides present in the media and do not necessarily require complete hydrolysis of milk proteins (caseins) to obtain amino acids and peptides for their growth.

ACKNOWLEDGEMENT

Authors would like to acknowledge The University of Melbourne, Australia and Culture Division, Dairy Innovation Australia for facilitating and funding the research. The authors also kindly acknowledge Ms. Carmel Gray, Ms. Lucy Sheehy and Mr. Hanny Ong at Culture Division, Dairy Innovation, Werribee, Vic 3030, Australia for their assistance and support.

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