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Biochemical characterisation of dairy yeasts and their application in cheese as anaerobic adjunct cultures

A thesis presented in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Food Technology at Massey University, Palmerston North, New Zealand

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Abstract

Yeasts are traditionally used as part of the surface microflora in surface-ripened cheeses, where they contribute positively to the flavour of the cheese. The primary objective of this study was to investigate the potential of three dairy yeasts to provide attributes as adjuncts in anaerobically ripened cheeses. *Geotrichum candidum* (B9001), *Yarrowia lipolytica* (B9014) and *Candida kefyr* (B9006), obtained from the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, were studied. They showed diverse metabolic activities in laboratory media, which were influenced by the growth conditions. The metabolic activities of special interest were the lipase and proteinase activities and the production of volatile compounds, as these are important for cheese ripening and flavour development.

Lipase activity (*p*-nitrophenyl butyrate assay) and proteinase activity (fluorescein isothiocyanate β -casein assay) were determined in three fractions prepared from yeast cultures and designated as extracellular fraction, washed-cell fraction and intracellular fraction. Lipase activity of *G. candidum* was detected only in the extracellular fraction and increased five fold when induced by safflower oil in a shake culture (0.16 $\mu\text{M}/\text{min}/\text{mL}$ supernatant at 24 h). Lipase expression was delayed in static cultures. *Y. lipolytica* showed lipase activity in extracellular, washed-cell and intracellular fractions under all conditions. Static cultures in both glucose and safflower oil media showed higher lipase activity than shake cultures. The lipase activity of *Y. lipolytica* was higher in the late stationary phase than in the log phase under all conditions tested. The highest lipase activity was detected in a 192 h static culture grown in safflower oil medium (0.13 $\mu\text{M}/\text{min}/\text{mg}$ dry cell weight, 0.3 $\mu\text{M}/\text{min}/\text{mg}$ dry cell weight and 4.29 $\mu\text{M}/\text{min}/\text{mL}$ supernatant in the intracellular, washed-cell and extracellular fractions respectively). *C. kefyr* did not show any lipase activity ($< 0.03 \mu\text{M}/\text{min}/\text{mL}$ culture) under any of the growth conditions tested.

Proteinase activity was detected in the intracellular fraction of 72 h shake cultures of *G. candidum* grown in both glucose medium and safflower oil medium (154 and 122 RFU/min/mg dry cell weight respectively) but was not detected in static cultures. Proteinase activity was absent in the *Y. lipolytica* cultures under all conditions tested

(< 10 RFU/min/mL culture). *C. kefir* showed low proteinase activity (12–74 RFU/min/mL supernatant) in the extracellular fraction only in shake cultures grown in glucose medium.

Volatile compounds of the headspace were sampled and analysed using solid phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS). The concentrations of volatile compounds were highest in shake cultures grown in glucose medium for all three yeasts. All yeasts produced several alcohols. Several esters were also detected in the *G. candidum* and *C. kefir* cultures whereas aldehydes were detected only in the *G. candidum* cultures.

G. candidum and *Y. lipolytica* were selected for cheese production trials because of their active cheese ripening enzymes. These yeasts, grown under different conditions, were added to Cheddar cheese (10 L vat). The yeast adjuncts influenced the cheese ripening by lipolysis [in terms of the production of free fatty acids (FFAs) analysed by gas chromatography–flame ionisation detector (GC–FID)] and the production of volatile compounds (SPME–GC–MS), whereas proteolysis (analysed by size-exclusion high performance liquid chromatography) by yeast enzymes was not obvious.

The influence of *Y. lipolytica* as an anaerobic adjunct to cheese ripening was dependent on the growth conditions used during its propagation in laboratory media. The concentration of total FFAs was very high (37.1 mg/g cheese at 6 months) when a 192 h *Y. lipolytica* culture grown in safflower oil medium was added to a cheese make, whereas the cultures grown in glucose medium did not have any detectable effect. Addition of *G. candidum* culture to the cheese curd was more effective than its addition to the cheese milk.

Both *G. candidum* and *Y. lipolytica* lipase(s) selectively hydrolysed the long-chain unsaturated fatty acids from the milk triglyceride in the cheese environment. Also, *Y. lipolytica* lipase exhibited some selectivity towards hydrolysis of butyric acid from the milk fat in the cheese.

2-Heptanone, 3-methyl-2-butanone and 2-nonanone were detected ($1\text{--}10 \times 10^6$ relative peak area) only in the cheeses with yeast adjuncts but not in the control cheese.

Enhancement of the production of both conjugated linoleic acid (CLA) and ethyl esters in a washed-curd, dry-salted cheese (375 L vat), made with *G. candidum*, *Y. lipolytica*, *Propionibacterium freudenreichii* ssp. *shermanii*, *Lactobacillus fermentum* and *Lb. rhamnosus*, was only partially successful. Higher concentrations of ethyl esters (> five fold; analysed by SPME–GC–MS) were produced in the cheeses made with yeast adjuncts. However, the concentration of total CLA (free plus esterified; analysed by GC–FID) did not increase although a higher concentration of free linoleic acid (> 10 fold), the substrate for CLA synthesis, was produced in the cheeses made with yeast adjuncts.

A study of the formation of aromatic volatile compounds by *C. kefir* in a medium containing L-phenylalanine (L-phe) showed that the yeast's ability to produce phenyl ethanol, phenyl ethyl acetate and benzaldehyde (analysed by SPME–GC–MS) was enhanced with an increase in the initial L-phe concentration (in the experimental range; analysed by enzymatic assay using phenylalanine ammonia lyase), but the yield was very low (20–27%). The initial concentration of glucose (in the experimental range; analysed by enzymatic assay using Peridochrom glucose reagent) did not affect the production of these aromatic volatile compounds.

This study successfully showed that the yeasts *G. candidum* and *Y. lipolytica*, when used as anaerobic adjuncts, can influence the ripening and flavour development in Cheddar and washed-curd, dry-salted cheeses. The study also showed the capability of *C. kefir* to produce aromatic volatile compounds from amino acid fermentation but the yields need to be increased by further manipulation of the medium components and the culture conditions before this capability can be used commercially.

To my wife Jinita and daughter Nilotri
For your love and support

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