Studies on the Decarboxylation of Acetolactate in Milk Products

Thesis presented by

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Declaration

I hereby certify that the material, which I now submit for the assessment on the programme of study leading to the award of Master of Science is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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ABBREVIATIONS

ALA	α-acetolactic acid
ALD	acetolactate decarboxylase
ALS	acetolactate synthase
ATP	adenosine triphosphate
BCAA	branched chain amino acids
βPgal	β-D-phosphogalactoside galactohydrolase
Cit	citrate
EMP	Embden Meyerhof Pathway
HPr	heat-stable protein
Lb.	*lactobacillus*
Lc.	*lactococcus*
LDH	lactate dehydrogenase
Ln.	*leuconostoc*
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NMR	nuclear magnetic resonance
PDH	pyruvate dehydrogenase
PEP-PTS	Phosphoenol-pyruvate phosphotransferase system
PK	phosphoketolase
PMF	proton motive force
Str.	*Streptococcus*

ABSTRACT

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The effect of different parameters on the decarboxylation of acetolactate (ALA) to diacetyl and acetoin were studied. The distillation volume and the milk solids concentration had no significant effect on decarboxylation of ALA, whereas breakdown of ALA increased with decreasing pH and increasing temperature. Oxygenation increased diacetyl production from ALA, but diacetyl was lost from the model system. Oxygenation did not have an effect on acetoin production from ALA. Metal ions (Cu^{2+}, Fe^{2+}) and haemin caused high breakdown of ALA to diacetyl during steam distillation, with Cu^{2+} being the most effective. The decarboxylation of ALA was a first order reaction.

A new method was developed for the determination of ALA based on steam distillation at pH 3.5 in the presence of Cu^{2+}, which caused complete decarboxylation of ALA to diacetyl. ALA concentrations were calculated from the difference between diacetyl levels in a sample in which ALA was completely converted to diacetyl, and diacetyl levels in a sample with minimal decarboxylation of ALA to diacetyl, which was achieved by distillation at pH 0.8. The method compared well to the Jordan and Cogan [1995] method.

Trials were carried out at laboratory and industrial scale to improve the manufacturing process for lactic butter. An increase in temperature during manufacture increased diacetyl concentrations and converted most of the ALA to diacetyl; it had no significant effect on acetoin concentrations.

Cit^+ strains of *Lactococcus lactis* subsp. *diacetylactis*, 999 and 1166, were grown in the presence and absence of oxygen, leucine, valine, $CuSO_4$, $FeSO_4$ and haemin. Except for oxygen, which increased diacetyl production and decreased growth, there was no significant effect of these compounds on metabolite production.

LITERATURE REVIEW

Starter Cultures

The cultures used in milk fermentations are called starters because they initiate or start the production of lactic acid in the milk. The bacteria commonly found in starter cultures are usually present in raw milk as part of the natural milk microflora, and, therefore, can sour the raw milk if it is left undisturbed for a day or so. This ability was used for many years in the production of starter cultures for cheese and other fermented dairy products without knowing what exactly was happening during the souring of the milk or that bacteria were involved. At the beginning of this century, as knowledge of bacteriology grew, the bacteria involved were identified and the intentional inoculation of milk and cream with these organisms to carry out the fermentation began [Sandine, 1975; Cogan and Hill, 1987].

There are two types of starter cultures, mesophilic and thermophilic. Mesophilic starter cultures have a optimum growth temperature of ~26°C and are used in the production of Cheddar, Gouda, Edam, Blue, Camembert and cottage cheese, cultured butter and buttermilk and sour cream. Thermophilic cultures have higher optimum temperatures (45-50°C) than mesophilic ones, which makes them useful in the production of the so called "cooked" cheeses like Swiss and Italian. Thermophilic cultures are also used in the preparation of yoghurt [Accolas and Auclair, 1983; Cogan and Hill, 1987].

Mesophilic cultures are composed mainly of *Lactococcus lactis* subsp. *cremoris* and the closely related *Lc. lactis* subsp. *lactis*. Both of these organisms are generally considered not to be able to utilise citrate (Cit^-), although many cultures, especially those used as butter and quark starter cultures, also contain lactococci that are able to metabolize citrate (Cit^+) and produce diacetyl. This organism was formerly called *Streptococcus diacetylactis.* It was renamed *Lc. lactis* subsp. *lactis*, due to the fact that the difference between it and *Lc. lactis* subsp. *lactis* is a plasmid that is responsible for citrate uptake. It is now called Cit^+ *Lc. lactis* subsp. *lactis* [Kempler and McKay, 1979; Schleifer et al., 1985].
Besides Cit^- and Cit^+ lactococci, which are involved in acid and flavour formation respectively, Cit^+ *Leuconostoc* sp. are also found in mesophilic cultures, which contribute to the formation of flavour compounds like diacetyl from citrate [Cogan

and Hill, 1987]. The citrate utilizers are also often called generically aroma producers.
The types of aroma bacteria present in mesophilic starter cultures are used to further differentiate them. Cultures containing only *Leuconostoc* sp. are called L type, those containing only Cit^+ *Lc. lactis* subsp. *lactis* are known as D type, and DL cultures consist of both Cit^+ *Lc. lactis* subsp. *lactis* and *Leuconostoc* sp. Cultures without any flavour producers are called O type [Cogan and Hill, 1987].

Thermophilic cultures contain *Str. thermophilus* and one or several lactobacilli, i.e. *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. helveticus* [Accolas and Auclair, 1983].

Mesophilic and thermophilic starter cultures can be further divided into defined and undefined or mixed cultures. Defined cultures consist of one to several phage-unrelated pure cultures whereas mixed cultures are not selected but have evolved from 'good' acid producing starters, which were first used in the late 19th and early 20th centuries and, since then have been transferred numerous times. The number of strains in such cultures is unknown [Sandine, 1975; Cogan and Hill ,1987].
Mixed strain starters have a major advantage over defined cultures in that they are quite phage resistant, due to the fact that they contain many phage resistant or phage unrelated strains. When a phage attack occurs these strains grow, resulting generally in only a small decrease in acid producing ability. But mixed strain starters also have many disadvantages, the most notable being their ability to produce variable amounts of lactic acid. Their use has led to the development of defined strain cultures, which are easier to control [Sandine, 1975; Stadhouders and Leenders, 1982; Cogan and Hill, 1987].
Defined strain starter cultures can be divided into single and multiple strain starters. Single strain starters consist of one strain of *Lc. lactis* subsp. *cremoris* or less commonly *Lc. lactis* subsp. *lactis*. Multiple strain cultures consist of two to six (sometimes more) phage unrelated strains of *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* [Lawrence et al., 1976].

Lactose Metabolism

Lactose is a disaccharide composed of galactose and glucose and is the main energy source for the lactic acid bacteria (LAB) when they are grown in milk.

LAB have two different systems for transporting lactose. *Leuconostoc* sp. and thermophilic cultures use a proton motive force (PMF) system, while *Lactococcus* sp. use group translocation, the so-called phosphoenol-pyruvate phosphotransferase system (PEP-PTS) [Cogan and Hill, 1987]. Both systems require energy. The exact energy compound used in the PMF-system is unknown, although it is likely to be ATP as in galactose transport in *Lc. lactis* subsp. *lactis* [Thompson, 1980]. Lactose is transported against a concentration gradient and often another molecule is transported either into (symport) or out of (antiport) the cell during the transport of lactose [Cogan and Hill, 1987].
In the case of the PTS system, the energy source is PEP [McKay et al., 1969]. In this system lactose is transformed into lactose-P as it is transported into the cell. This is a very complex process involving four different proteins, enzyme I, HPr (a heat-stable protein), factor III (a lactose-specific factor) and enzyme II. The first three are soluble proteins, whereas enzyme II is a lactose-specific membrane-bound component [McKay et al., 1970].

Cultures which transport lactose via the PMF system hydrolyse lactose via ßgal to glucose and galactose. The next steps in metabolism depend very much on the culture itself. In *Lb. helveticus* glucose is metabolised via the EMP system and galactose by the Leloir pathway while all strains of *Lb. delbrueckii* subsp. *bulgaricus*, most strains of *Lb. delbrueckii* subsp. *lactis* and all strains of *Str. thermophilus* excrete galactose in amounts equimolar with the amounts of lactose used. This is thought to be involved, at least in *Str. thermophilus*, with lactose transport. Metabolism of glucose is via the Embden-Meyerhof pathway (EMP). In *Leuconostoc* the fermentation of glucose proceeds via the phosphoketolase (PK) pathway to equimolar concentrations of D-lactate, ethanol and CO_2, while galactose is probably metabolised via the Leloir pathway to glucose-6-P before entering the PK pathway [Cogan and Hill, 1987].

In the lactococci, lactose-P formed during PTS transport is hydrolysed to galactose-6-P and glucose by ß-D-phosphogalactoside galactohydrolase (ßPgal). Galactose-6-P is further metabolized to triose phosphates through the tagatose pathway whereas glucose is fermented via the glycolytic pathway [Lawrence et al., 1976; Cogan and Hill, 1987].

Usually lactate is the only product of sugar metabolism, but when these bacteria are grown on some sugars e.g. galactose other products can be formed, e.g. formate, ethanol and acetate. If the medium also contains citrate, production of acetoin and diacetyl also occurs [Cogan and Hill, 1987]. Based on the discovery that *Lc. lactis*

ATCC 7962, which does not possess ßPgal, grows slowly on lactose converting only 15% of it to L-lactate, it was concluded that the rapid and homolactic fermentation of lactose is dependent on a functional PEP-PTS system and the presence of ßPgal [Thomas, 1976].

Citrate Metabolism

Citric acid is a tricarboxylic acid consisting of six carbon atoms, which is present in milk in only small concentrations (~10mM). Metabolism of citrate to flavour compounds like acetate and diacetyl is important in the production of many fermented dairy products such as butter and quark. In contrast, diacetyl is an undesirable compound in beer. The production of CO_2 during metabolism of citric acid also adds texture to some products, although it is unfavourable in the production of fresh cheeses like quark [Hugenholtz and Starrenburg, 1992].

The ability of cells to utilize citrate is unstable, suggesting that it is plasmid encoded. The existence of such a plasmid was proven by Kempler and McKay [1979] who showed that treatment of cells of Cit^+ *Lc. lactis* subsp. *lactis* with acridine orange resulted in a loss of the ability to utilise citrate and a loss of the plasmid encoding the citrate transport system, citrate permease. This protein is active within the pH range 5.0 to 6.0 in Cit^+ *Lc. lactis* subsp. *lactis* and is considered to be an integral membrane protein [David et al., 1990; Hugenholtz, 1993].

Citrate is not an energy source for Cit^+ *Lc. lactis* subsp. *lactis* [Cogan, 1982; Cogan and Hill, 1987], although an increase in the specific growth rate of Cit^+ *Lc. lactis* subsp. *lactis* was found when citrate was added to a lactose containing medium [Harvey and Collins, 1963a]. Citrate also stimulated the growth rate of heterofermentative lactobacilli in lactose containing media [Drinan et al., 1976]. Hugenholtz et al. [1993] showed that in lactose-limited continuous cultures Cit^+ *Lc. lactis* subsp. *lactis* was able to grow on citrate as the only energy source at low growth rates at pH 5.3, generating a PMF as a result of electrogenic uptake or citrate/product exchange together with proton consumption by the intracellular oxaloacetate decarboxylase [Starrenburg and Hugenholtz, 1991; Hugenholtz, 1993]. A PMF as the driving force for citrate transport was also suggested by David et al. [1990]. A similar mechanism was found for *Ln. mesenteroides*, which generates metabolic energy from citrate metabolism in the form of a proton electrochemical

gradient across the membrane by electrogenic exchange of citrate and D-lactate [Marty-Teysset et al., 1996].
Citrate uptake is inhibited by Cu^{2+} and Fe^{3+}, which at the same time stimulate the production of diacetyl, probably because the citrate uptake activity of the cells is lowered by diacetyl [Kaneko et al., 1990b].

After transport into the cell, citrate is cleaved to oxaloacetate and acetate by citrate lyase (citritase), which is constitutively present in Cit^+ *Lc. lactis* subsp. *lactis*, but induced by citrate in *Leuconostoc* sp. [Speckman and Collins, 1968; Cogan, 1981; Mellerick and Cogan, 1981]. The former is decarboxylated to yield pyruvate [Seitz et al., 1963]. Hence, if cells are grown in the presence of citrate in addition to an energy source such as lactose or glucose, excess pyruvate is produced which cannot be reduced to lactate because of the need to recycle NADH to continue glycolysis. The excess pyruvate not required for the synthesis of cell material has to be removed, which leads to the formation of the typical products of citrate metabolism, acetoin and diacetyl. This effect can be thought of as a detoxification mechanism. Pyruvate therefore is a key intermediate in citrate metabolism [Harvey and Collins, 1963b; Kempler and McKay, 1981; Starrenburg and Hugenholtz, 1991]. It has been found that Cit^+ *Lc. lactis* subsp. *lactis* can produce acetoin and diacetyl even in the absence of citrate as additional source of pyruvate under aerobic growth conditions [Bruhn and Collins, 1970]. In this case NADH oxidase is active and is partly responsible for the reoxidation of NADH to NAD^+ to continue glycolysis. In anaerobically (normal) growing cells this function is carried out by the reduction of pyruvate to lactate and the concomitant production of NAD^+ from NADH by LDH. In aerobically grown cells the pyruvate not required for the latter reaction is used to form diacetyl and acetoin.

Pette [1949] proposed a hypothetical substance which could act as an intermediate for both acetoin and diacetyl production from pyruvate. This hypothetical substance was later shown to be acetolactic acid (ALA), a very unstable compound [De Man and Pette, 1956]. ALA is formed from two pyruvate molecules. Juni [1952] suggested a condensation of pyruvate with "active" acetaldehyde (hydroxy-ethylthiamine pyrophosphate). This mechanism was later confirmed by ^{13}C nuclear magnetic resonance (NMR) [Verhue and Tjan, 1991]. The enzyme that catalyses this reaction, acetolactate synthase (ALS), is expressed constitutively [Cogan, 1981; Snoep et al., 1992], requires thiamine pyrophosphate (TPP) and Mg^{2+} or Mn^{2+} and is inhibited by citrate [Kobayashi and Kalnitsky, 1954; Harvey and Collins, 1961; Brauen and Keenan, 1972; Snoep et al., 1992], although Cogan [1981] found that in some strains of Cit^+ *Lc. lactis* subsp. *lactis* ALS can be partly induced by citrate.

ALS has a pH optimum of about 6.0 [Juni, 1952]. In Cit^+ *Lc. lactis* subsp. *lactis* ALS has a high K_M for pyruvate, 50mM as compared to 10mM in *Leuconostoc* sp. [Snoep et al., 1992; Marugg et al., 1994]; its activity is higher in *Lactococci* sp. than in *Leuconostoc* sp. ALS is allosteric in Cit^+ *Lc. lactis* subsp. *lactis*, whereas it can be allosteric or obey Michaelian kinetics in *Leuconostoc* sp. [Monnet et al., 1994a]. Due to its high K_m for pyruvate in Cit^+ *Lc. lactis* subsp. *lactis*, ALS is only active when the internal pool of pyruvate is high, as it is during co-metabolism of citrate and a fermentable carbohydrate. Therefore toxic excess pyruvate can be removed without competition with other enzymes such as lactate dehydrogenase (LDH) or pyruvate dehydrogenase (PDH). PDH has a K_m of 1mM for pyruvate and therefore has a much higher affinity for pyruvate than ALS, whereas ALS has a higher activity. The two enzymes are also not expressed simultaneously. PDH is only produced aerobically [Snoep et al., 1992; Smith et al., 1993; Monnet et al., 1994a]. *Lc. lactis* subsp. *lactis* produces two different ALS which perform different functions. The gene encoding one of the ALS is part of an operon involved in the biosynthesis of branched chain amino acids (BCAA), the second gene for ALS produces a similar enzyme. The biosynthetic ALS has a pH optimum of 8.0 and contains FAD while the catabolic enzyme has an optimum pH of 6.0 and does not contain FAD. Former is regulated by transcriptional attenuation, and the latter expressed in a constitutive fashion, suggesting that *Lc. lactis* subsp. *lactis* can produce different types of ALS under varying growth conditions [Marugg et al., 1994]. This also explains why Snoep at al. [1992] did not find any feed back control of the pH 6.0 enzyme from BCAA.

Juni [1952] found that in *Enterorobacter aerogenes*, acetoin is produced from pyruvate via ALA by acetolactate decarboxylase (ALD). The enzyme had a pH optimum of about 6.0 and 75% of its activity was conserved within the pH range of 5.4 to 6.9. Phalip et al. [1994] found that ALD of *Lc. lactis* subsp. *lactis* consisted of six identical subunits of 26 500Da and was activated by BCAA, Mn^{2+} and Zn^{2+} but not Mg^{2+}. The enzyme showed allosteric properties in the absence and Michaelian kinetics in the presence of leucine. In Cit^+ *Lc. lactis* subsp. *lactis* ALD has a high K_m for ALA (60mM) compared to *Leuconostoc* sp. (0.3mM) [Monnet et al., 1994a]. ALA and acetoin are optically active compounds. In Cit^+ *Lc. lactis* subsp. *lactis* the acetoin produced is dextrorotatory whereas in *Enterobacter aerogenes* it is levorotatory [Speckman and Collins, 1968; Collins and Speckman, 1974]. It has also been found that only one of the optical isomers of commercial ALA is attacked by the ALD of Cit^+ *Lc. lactis* subsp. *lactis*. This indicates that ALA is enzymatically bound during decarboxylation to acetoin, otherwise the result of decarboxylation would be a racemic mixture [Collins and Speckman, 1974]. Another mechanism for

the production of acetoin was proposed by Kamiya et al. [1993]. They found that under anaerobic conditions a major portion of the ALA is converted to acetoin without involvement of an enzyme. They suggested that ALA is decarboxylated to an unknown intermediate X, which then is converted to acetoin under anaerobic conditions or to diacetyl if enough oxygen is present. Decarboxylation is supposed to be the rate determining step because similar reaction rate constants for the conversion of ALA to acetoin and to diacetyl were found.
Acetoin is either excreted as an end-product or it is further reduced to 2,3-butanediol, a reaction catalysed by butanediol dehydrogenase [Hugenholtz, 1993]. Butanediol dehydrogenase is constitutively present in several strains of Cit^+ *Lc. lactis* subsp. *lactis* and is partly repressed by citrate in some strains [Cogan, 1981].

There are different opinions on how diacetyl is produced. Until the 1960s, it was believed that diacetyl was formed via oxidation of acetoin [Jönsson and Pettersson, 1977]. This theory was then replaced by two main theories. Some workers believe that diacetyl was produced directly in the cell via condensation of acetyl coenzyme A with "active" acetaldehyde involving the enzyme diacetyl synthase [Chuang and Collins, 1968; Speckman and Collins, 1968; 1973; Jönsson and Pettersson, 1977; Kaneko et al., 1990a]. Despite exhaustive studies, no convincing evidence for the existence of this enzyme has ever been found in LAB. The other theory claims that diacetyl was formed outside the cell by oxidative decarboxylation of excreted ALA [De Man and Pette, 1956; Seitz et al., 1963; Verhue and Tjan, 1991]. It cannot be excluded that both mechanisms occur simultaneously. Recent results with *Ln. lactis* indicate a third mechanism, suggesting that the established pathway for acetoin synthesis from pyruvate and ALA catalysed by ALS and ALD is also responsible for the enzymatic formation of diacetyl [Jordan et al., 1996]. The level of diacetyl produced is usually much smaller than that of acetoin [Walsh and Cogan, 1973].

One phenomenon that can occur, which is desirable in beer fermentations but undesirable in dairy fermentations, is the irreversible reduction of diacetyl to acetoin by acetoin dehydrogenase. This enzyme has a pH optimum of 5.5 and is stimulated by Cu^{2+} and haemin [Seitz et al., 1963; Jönsson and Pettersson, 1977; Kaneko et al., 1990a]. It is constitutively present in several strains of Cit^+ *Lc. lactis* subsp. *lactis* and partly repressed by growth on citrate in some strains [Cogan, 1981].

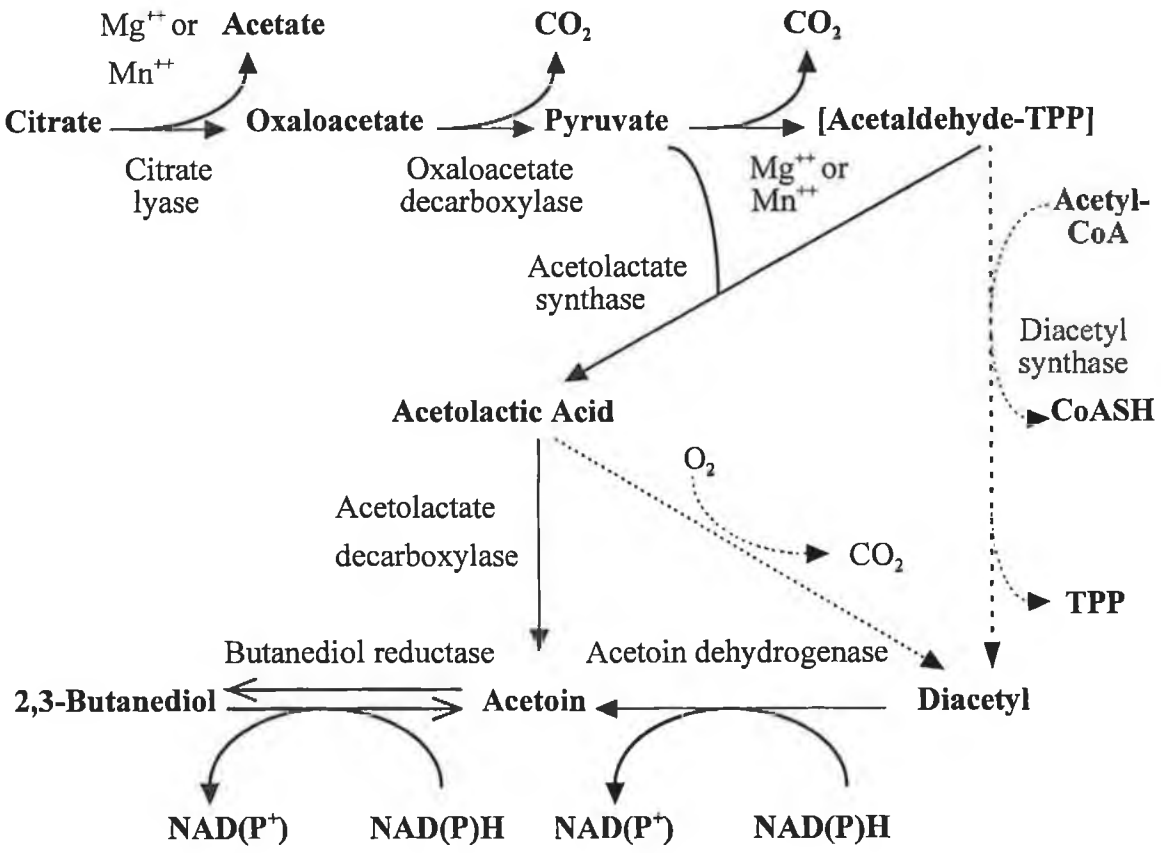

Fig.1: Citrate Metabolism by Cit$^+$ *L. lactis* subsp. *lactis*

Factors influencing Diacetyl and Acetoin Production

One factor affecting the amount of diacetyl and acetoin produced from ALA by Cit$^+$ *Lc. lactis* subsp. *lactis* is pH. In a fermentation controlled at pH 4.5, the maximal specific rate of citrate utilisation, the bioconversion yield and the ratio of diacetyl to acetoin were increased compared to a fermentation controlled at pH 6.5. In contrast, the specific growth rate and specific rate of lactose fermentation were lower at the lower pH value. The pH acts indirectly by increasing the proportion of non-dissociated lactic acid, which is considered inhibitory to growth and lactose fermentation [Cachon and Diviès, 1994].

Temperature also has an effect. The rate of growth of Cit$^+$ *Lc. lactis* subsp. *lactis* and lactic acid production were halved at 18°C compared to 30°C, and more diacetyl was produced at 18°C than at 30°C whereas acetoin production was unchanged at both temperatures. One reason for this could be the effect temperature has on the principal enzymes involved in pyruvate metabolism. While LDH and ALS activities were relatively unaffected by a change in temperature, NADH oxidase activity was

higher at 18°C than at 30°C whereas acetoin dehydrogenase activity was decreased at 18°C compared to 30°C [Bassit et al., 1995].
Experiments investigating the effect of pH and temperature on synthetic ALA showed similar results; the specific rate of ALA decarboxylation increased with decreasing pH and increasing temperature, with diacetyl and acetoin production corresponding to ALA degradation. ALA decarboxylation was a first order reaction [Monnet et al., 1994b].

The amount of diacetyl produced from ALA during fermentation or in a model system with synthetic ALA is also dependent on the redox potential of the surrounding medium. A high redox potential, as found at the beginning of a lactic fermentation, results in production of diacetyl and acetoin. The low redox potential at the end of a fermentation only allows the production of acetoin. Hence, the amount of diacetyl can be increased by keeping the redox potential at a high level, e.g. by bubbling oxygen through the culture or by continuous stirring [Jönsson and Pettersson, 1977; Bassit et al., 1993;, Monnet et al., 1994b].

Another related factor is the initial oxygen concentration in the medium. At 0% O_2 saturation at 30°C, little diacetyl was produced by Cit^+ *Lc. lactis* subsp. *lactis* (0.01mM diacetyl compared to 2.4mM acetoin). Acetoin production increased to 5.4mM when O_2 was increased to 100% saturation while diacetyl production was increased by factors of two, six and eighteen at initial O_2 concentrations of 21, 50 and 100%, respectively. The increase in the ratio of diacetyl to acetoin with increasing O_2 concentrations was linear. The reason for these findings is that the specific activities of ALS and NADH oxidase are lower at lower O_2 concentrations. NADH oxidase replaces LDH, acetoin dehydrogenase and butanediol dehydrogenase in the reoxidation of NADH allowing accumulation of acetoin and diacetyl [Bassit et al., 1993].
When the combined effect of O_2 and temperature was studied, it was evident that the effect of O_2 was more important than the effect of temperature in production of acetoin and diacetyl, whereas the opposite was true for acidification. Maximal diacetyl concentrations and a maximal ratio of diacetyl to acetoin were reached at 18°C and 100% O_2 saturation, maximal acetoin concentrations at 26°C and 100% O_2 saturation and a maximal acidification rate at 30°C and 0% O_2 saturation [Bassit et al., 1994].

The addition of Cu^{2+}, Fe^{2+} or haemin also increases the amount of diacetyl produced by Cit^+ *Lc. lactis* subsp. *lactis*. All three are considered to stimulate the activity of diacetyl synthase. It was observed that Cit^+ *Lc. lactis* subsp. *lactis* produced diacetyl

and acetoin from glucose in the absence of citrate when grown aerobically in the presence of Cu^{2+} and haemin [Kaneko et al., 1990a; 1990b].

Branched Chain Amino Acids and Citrate Metabolism

Dairy strains of *Lc. lactis* subsp. *lactis* are unable to synthesise the three branched chain amino acids (BCAA) valine (val), leucine (leu) and isoleucine (ile) in contrast to their non-dairy counterparts which can grow in the absence of all three BCAA [Godon et al., 1993; Chopin, 1993]. The reasons for this systematic BCAA auxotrophy in dairy strains of *Lc. lactis* subsp. *lactis* are unknown. Chopin [1993] explains the auxotrophy for BCAA in dairy strains as a consequence of their adaptation to growth in milk and dairy products. Milk contains a significant amount of protein and small amounts of free amino acids. Since BCAA are the most frequent amino acids of *Lc. lactis* subsp. *lactis* proteins while not being particularly abundant in milk, Godon et al. [1992; 1993] suggest the existence of a selective pressure for auxotrophy, meaning that maybe an intermediate of the BCAA pathway is toxic for the cell or perturbs the regulation of other pathways such as the anabolic pathway for pantothenate and the catabolic pathway for acetoin and 2,3-butanediol, which are both linked to the BCAA pathway, the former via α-ketoisovaleric acid, which is a precursor of valine, the latter via ALA, the common intermediate of leucine and valine biosynthesis [Umbarger and Davis, 1962].

The genes for the biosynthesis of BCAA in *L. lactis* subsp. *lactis* are organised in a large cluster, which is divided into two units. Both units are necessary for leucine biosynthesis, whereas only the second is needed for the synthesis of isoleucine and valine [Renault et al., 1995; Godon et al., 1992]. Chopin [1993] suggests that the two units form a single operon. This organisation in a single operon, in contrast to other bacteria where those genes are more scattered, allows a co-ordinated regulation of the expression of BCAA genes. It has been shown by several workers that the expression of BCAA genes is controlled by transcriptional attenuation [Renault et al., 1995; Godon et al., 1992; Chopin et al, 1993].

It is interesting to note that the gene encoding ALD is situated in the same operon as the BCAA genes. ALD transforms ALA, the first intermediate for the biosynthesis of leucine and valine, to acetoin and is positively controlled by the availability of leucine and possibly valine in the cell. ALD is a key enzyme in a new class of regulatory mechanisms, a metabolic shunt, which controls the flux of ALA towards biosynthesis or catabolism [Chopin, 1993; Renault et al., 1995].

This special regulatory mechanism makes it possible to isolate ALD negative mutants, which are of considerable interest for the production of lactic butter and quark since these mutants are unable to form acetoin from ALA, which can then be decarboxylated to diacetyl under the proper conditions. The isolation works on the basis that wild type *Lc. lactis* subsp. *lactis* cannot grow in the presence of leucine and simultaneous absence of valine, because high leucine concentrations in the cell activate ALD, which converts available ALA to acetoin rather than to leucine and valine. Since the cells need valine, they die in its absence. ALD negative mutants cannot produce acetoin from ALA enzymatically, implying that ALA is still available for valine biosynthesis. Therefore the mutants survive in a medium that contains leucine but not valine [Goupil et al., 1995; Chopin, 1993].
A comparison of the genomes of *Lc. lactis* subsp. *lactis* derived from milk and plants showed that the operon responsible for BCAA biosynthesis is present in the auxotrophic dairy strains but that some genes are inactive. The remaining active genes might have a role other than BCAA biosynthesis; this role cannot be essential though because deletion of the operon in prototrophic strains did not affect their viability [Godon et al. 1993]. One of those remaining active genes might be the gene encoding ALD, which is activated by BCAA in dairy strains of *Lc. lactis* subsp. *lactis* despite their inability to synthesise those amino acids [Monnet et al., 1994a].

Metabolic Engineering of Citrate Metabolism

Mutations occur frequently in bacteria and sometimes result in desirable properties in the mutant. McKay and Baldwin [1974] for example isolated a strain of *Lc. lactis* subsp. *lactis* which formed abnormally large colonies on agar. When examined more closely, it was discovered that the mutant grew as fast in milk and broth as the parent strain but was slower in acid production. It also consumed six times as much oxygen as the parent strain and produced large amounts of acetoin and some diacetyl. The reason for these abnormalities was an enzymatic defect; the mutant possessed only low amounts of LDH and was therefore unable to reduce pyruvate to lactic acid, which resulted in an excess of pyruvate in the cell. This mimics the situation in Cit^+ *Lc. lactis* subsp. *lactis*, which produce excess pyruvate from citrate on top of the pyruvate from sugar metabolism. Kuila and Ranganathan [1978] tried to induce mutations in Cit^+ *L. lactis* subsp. *lactis* using UV radiation. This resulted in two types of mutants; type I was a high diacetyl producer, whereas type II produced greater amounts of acid. Mutants of type I were LDH negative or at least impaired and therefore disposed of the excess pyruvate by producing more acetoin and

diacetyl. Some of the type I mutants also had an impaired acetoin dehydrogenase. Type II mutants showed a slight increase in LDH activity. The production of diacetyl and acetoin can also be manipulated by metabolic engineering. Gasson et al. [1996] suggested three sites, LDH, ALD and ALS genes, for manipulation. By eliminating LDH in a Cit$^-$ *Lc. lactis* subsp. *lactis,* they produced a strain that generated similar amounts of acetoin during sugar fermentation as Cit$^+$ *Lc. lactis* subsp. *lactis* growing on both sugar and citrate. Inactivation of the gene encoding ALD increased production of diacetyl by preventing decarboxylation of ALA to acetoin and therefore increasing the opportunity for its oxidative decarboxylation to diacetyl. Finally they took advantage of the fact that *Lc. lactis* subsp. *lactis* can produce different types of ALS. They substituted the ALS normally active in the diacetyl production pathway, which has a low affinity for pyruvate and therefore only works in situations of excess pyruvate, with an ALS that converts pyruvate to ALA during BCAA biosynthesis, whose affinity for pyruvate is higher. This enzyme is not transcribed when BCAA are present in the medium, but changing the promoter for the genes resulted in constitutive production of this type of ALS, therefore allowing ALS activity in the presence of BCAA and increasing the production of diacetyl and acetoin in several Cit$^-$ *Lc. lactis* subsp. *lactis* strains.

Measurement of Diacetyl and Acetoin

The first methods used to measure diacetyl and acetoin were gravimetric ones, which involved the formation of a nickel dimethylglyoxime complex between diacetyl and hydroxylamine. Acetoin had to be oxidised to diacetyl prior to the reaction [Michaelian and Hammer, 1935]. Newer methods were subsequently developed including colorimetry [Prill and Hammer, 1938; Westerfeld, 1945], polarography [Ferren et al., 1967], gas liquid chromatography [Thornhill and Cogan, 1984], and headspace gas chromatography [Monnet et al., 1994b].

The method usually used to measure acetoin, the Westerfeld procedure [1945], is not specific because acetoin is oxidised to diacetyl during the assay, and therefore separation of acetoin from diacetyl is required when both compounds are present. Two of the separation methods used are salting-out chromatography [Speckman and Collins,1968b] and steam distillation [Walsh and Cogan, 1974]. In the laboratory in which this study was carried out, steam distillation is the routine method used. The first 10ml fraction collected contains all the diacetyl and most of the acetoin, whereas the second 10ml fraction contains ~25% of the acetoin [Walsh and Cogan, 1974]. The method of Walsh and Cogan, which is a modification of the colorimetric,

Prill and Hammer method, and which is specific for diacetyl, is used to quantify diacetyl in the first fraction, while acetoin can be measured by the Westerfeld method [1945] in the second fraction.

A problem occurs if the mixture also contains ALA, since this compound is easily converted to diacetyl and acetoin by heat (e.g. steam distillation or gas chromatography), which can lead to false results. The breakdown during distillation can be reduced to 2% by adjusting the pH to 9.0 with NaOH prior to distillation [Veringa et al., 1984]. A reduction in the breakdown of ALA to diacetyl to 0.2% during distillation at pH 1.0 was reported by Cronin and Rispin [1996].

Measurement of Acetolactate

ALA is an intermediate in the bacterial production of acetoin and diacetyl. It is an unstable compound and easily decarboxylated, either oxidatively to diacetyl or non-oxidatively to acetoin [De Man and Pette, 1956].
On the one hand, this is a disadvantage in quantification but, on the other hand, it opens up the possibility of using decarboxylation as a means to measure ALA, i.e. the compound is measured as the difference in the levels of acetoin before and after decarboxylation. There are different ways to do this; one method uses heat to break down ALA [Umbarger and Browne, 1958; Jordan and Cogan, 1988], other methods use acids such as HCl or a combination of heat and acid [Veringa et al., 1984; Jordan and Cogan, 1995]. After those methods, the Westerfeld procedure [1945] can be used to quantify the amount of the acetoin produced by breakdown of ALA. In those methods the acetoin measured before decarboxylation is not the true amount of acetoin but the sum of acetoin and diacetyl. However, this is not normally a problem, as the levels of acetoin produced by cultures are much greater than those of diacetyl. Another method reported by Gollop et al. [1987], uses oxidative decarboxylation of ALA to diacetyl to measure ALA. This method requires quantitative oxidation of ALA to diacetyl which was obtained by heating in the presence of Fe^{2+} and Fe^{3+}. The resulting diacetyl was then separated by an air distillation and assayed by the method of Prill and Hammer [1938]. ALA was then quantified from the difference between a sample distilled in the presence and absence of Fe^{2+} and Fe^{3+}.

Manufacture of Butter

Two types of butter are produced, sweet cream butter and sour cream butter, which is also called lactic, cultured or ripened cream butter. The two types differ considerably in taste. The bland flavour of sweet cream butter originates in the flavour of the milk constituents, particularly the milk fat and the changes caused by the pasteurisation of the cream, whereas the flavour of lactic butter is dominated by the products formed by the starter organisms during fermentation of the cream, e.g. lactate, acetate and diacetyl. The starter is usually a mixed-strain culture of the L or DL type.

Lactic butter can be manufactured by two processes as shown in Figure 2. During the traditional process, the milk is separated and the resulting cream pasteurised. The cream, containing 35-40% fat, is then inoculated with the starter culture and incubated at 21°C until the pH reaches 4.5 to 4.8, when the fermentation is complete. The ripened cream is cooled to 5°C and churned. This results in butter and sour buttermilk. The uses for sour buttermilk are limited; in contrast, sweet buttermilk is much more useful as an ingredient in dairy products. It is obtained during churning of sweet cream in the production of sweet cream butter. So an alternative process for the production of lactic butter, the so-called NIZO process, was developed [Veringa et al., 1976].

The alternative method for the manufacture of lactic butter divides the process into three independent steps, namely the production of a lactic acid culture concentrate, the production of aroma compounds and the production of sweet cream butter [Veringa et al., 1976]. Lactic acid culture concentrate is produced from whey by fermentation with *Lb. helveticus* which produces large amounts of lactic acid. The whey culture is then ultrafiltrated and concentrated by evaporation [Veringa et al., 1976; Van den Berg, 1991]. The aroma compounds are produced by fermentation of milk with a particular culture. After the end of the fermentation the milk is cooled down to 5°C, lactic acid culture concentrate is added and the mixture is aerated for 15min to 2h [Van den Berg, 1991]. Lactic acid culture concentrate and aeration enhance the decarboxylation of ALA to diacetyl. In the alternative process, sweet cream is churned to the granule stage with the release of sweet buttermilk. After separating the butter granules from the buttermilk, the starter mixture together with the lactic acid culture concentrate are worked into the butter granules to obtain a product which cannot be distinguished from lactic butter made by the traditional process [Van den Berg, 1991].

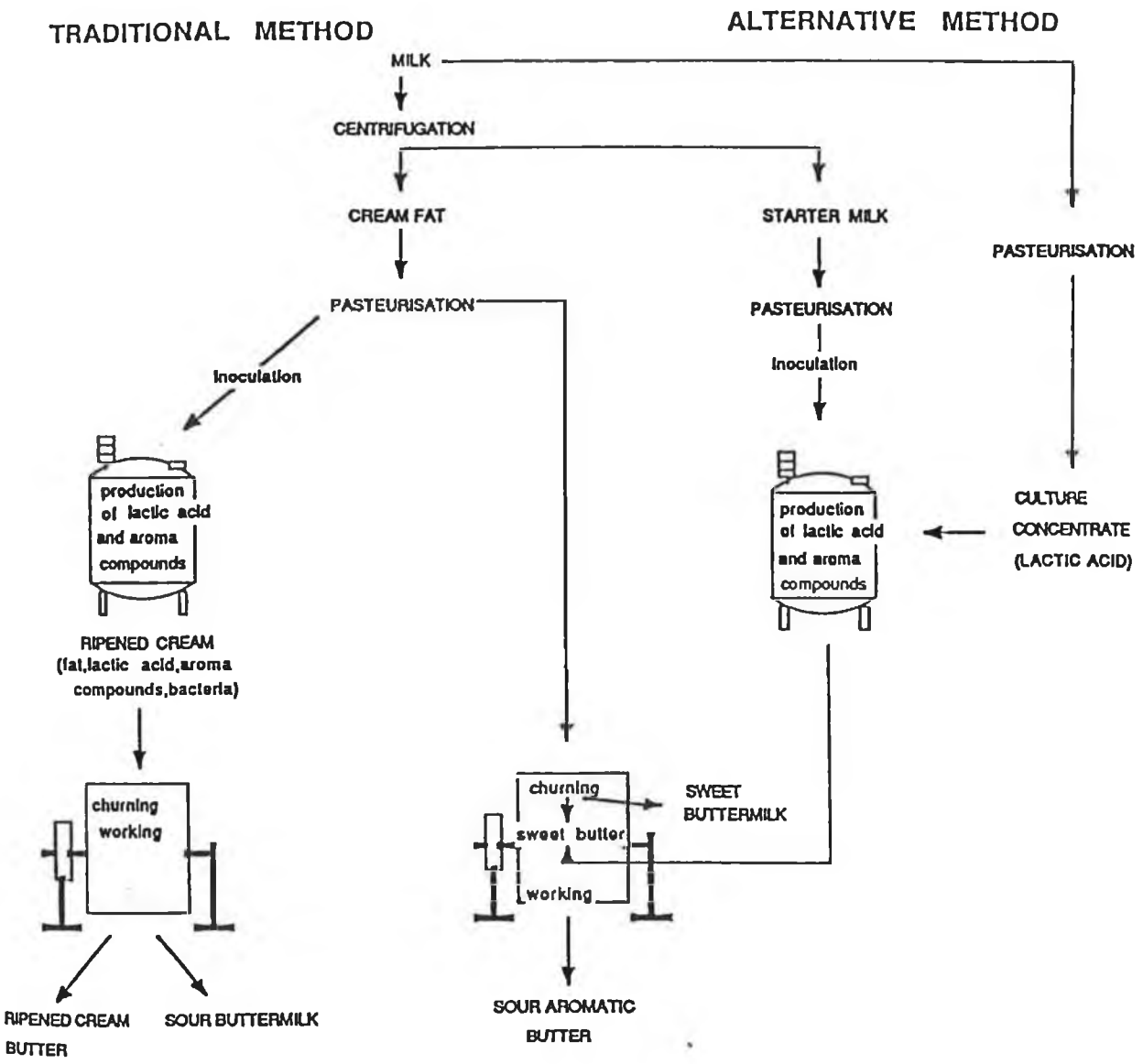

Fig. 2: Methods for the production of lactic butter [Veringa et al., 1976]

The new process has several advantages over the older process. The major advantage is the production of sweet rather than sour buttermilk as a by-product. Additionally, there are fewer oxidative defects on cold storage of the butter due to a lower copper content in the "sweet cream" lactic butter compared with traditionally produced lactic butter, and it is less likely to develop rancid flavour because of the lower content of free fatty acids in the sweet cream granules. Apart from that, the rheological properties of the butter can be better controlled because the choice of the most suitable temperature treatments in the sweat cream butter is wider. In addition, the optimum temperature for aroma production can be chosen and starter cultures with a temperature optimum unfavourable for the properties of cream can be used [Veringa et al., 1976]. A problem that can occur is that residual ALA, which has not been broken down during aeration of the starter/lactic acid mixture, is converted to acetoin rather than to diacetyl during storage.

The most common starter type for the production of butter is 4/25, which is a D culture containing Cit^+ *Lc. lactis* subsp. *lactis* as flavour producer. This strain lacks ALD and, as a result, overproduces ALA, which is then subsequently broken down to diacetyl during aeration at low pH. The starter also produces acetaldehyde, which gives an unwelcome flavour to the butter. Consequently the *Leuconostoc* containing culture (Fr19) is also added which is able to reduce acetaldehyde to ethanol which has no effect on flavour. The most important aroma compounds in this type of butter are diacetyl, and probably also acetate and lactate [Babel, 1944; Van den Berg, 1991].

Effect of Butter Cultures on Butter

The main purpose of the starter cultures in butter is to produce lactic acid and diacetyl. Lactic acid lowers the pH giving the butter a distinct acid taste while diacetyl is the aroma compound commonly associated with lactic butter. Diacetyl is produced by the so-called aroma bacteria, usually Cit^+ *Lc. lactis* subsp. *lactis*. Many strains of these bacteria grow poorly in milk producing little acid and aroma. The addition of acid though, especially citric acid, results in an increase in the production of diacetyl and acetoin, which indicates a kind of co-operation or "symbiosis" of the two types of bacteria present in butter starter cultures, i.e. the acid producers and the aroma producers [Kluyver, 1933]. Addition of synthetic diacetyl to butter results in an unsatisfactory harsh and unnatural flavour according to Babel and Hammer [1944].

Quark

Quark, also spelled quarg, to distinguish it from the subatomic particles, is a fresh, unripened soft cheese. It is related to such cheeses as cream cheese and Baker's cheese and is often confused with cottage cheese [Kosikowsky, 1977; Kroger, 1980; Sohal et al., 1988; Jelen and Renz-Schauen, 1989]. The greatest production of quark, which is called tvorog in Eastern Europe, is in Germany, although production of quark is spreading to other countries now due to its surprising versatility in cooking [Mann, 1987].

Quark consists essentially of coagulated, flocculated casein with a high water content. It is produced from milk by acid and/or rennet coagulation followed by a separation of the whey. Its composition varies, dependent on the composition of the milk. If it is made from skim milk it is almost fat free but it can contain up to 12% fat. The protein content varies from 14 to 18% and the main flavour component is diacetyl [Kosikowsky, 1977; Kroger, 1980].
The starter cultures used in its production are mixed DL cultures, containing mainly *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* as acid producers and Cit^+ *Lc. lactis* subsp. *lactis* and *Leuconostoc* as aroma producers [Law, 1981].

The production of quark can be divided into three steps, i.e. inoculation, incubation and separation. Pasteurised skim-milk is mixed with 1 to 2% starter culture and incubated at 20 to 22°C for low-temperature incubation (preferred) or at 25 to 30°C for high-temperature incubation. Sixty or ninety minutes after inoculation with the starter culture, rennet is added to enhance protein stabilisation. At this time the pH is about 6.3. At the end of the incubation period, usually 16 to 18h later, the coagulum will have reached a pH of 4.6 to 4.7. Traditionally, whey separation was achieved by cutting the curd into small cubes (10-15cm) and filling it into bags placed on drip tables. In the early 1960's a continuous separator was introduced to remove the whey from the broken coagulum. This modification of a classical dairy centrifuge opened the door for further developments and improvements [Kroger, 1980; Jelen and Renz-Schauen, 1989].
Several refinements were made to improve yield, shelf life and quality characteristics. One of the new procedures is the Centri-whey method, where the whey obtained in the quark manufacture is heated to 95°C, cooled and the denatured whey protein is removed with a self-cleaning separator and added to the milk for the next days processing. Other methods are the Lactal and the Ultrafiltration methods [Jelen and Renz-Schauen, 1989; Kroger, 1980].

At present a new strategy for the production of quark is being investigated. The new process is based on the same idea as the NIZO method for butter making, i.e. separation of acid production from flavour production. This would allow better control over the quality of the end product and production of consumer-tailored flavoured varieties.

A major problem of quark is its short shelf life of 14 days; this is mainly due to the growth of contaminants and the development of bitterness during storage. The contamination problem has been reduced recently by the use of the thermisation process during which the milk undergoes a high-temperature treatment. Bitterness is caused by the accumulation of bitter peptides from hydrolysis of the C-terminal end of ß-casein, which are formed during ripening. Bitterness can be reasonably reduced by decreasing the amount of rennet added. In contrast to cheese, the starter cultures in quark have almost no influence on bitterness and there is no correlation between microbial contaminants and bitterness [Sohal et al., 1988].
Another problem that can occur during the manufacture of quark is floating of the curd, due to CO_2 production from citrate metabolism. This can be avoided by making the quark with *Lc. lactis* subsp. *cremoris* or *Lc. lactis* subsp. *lactis* as lactic acid producers, then "dressing" it with cream cultured with Cit^+ *Lc. lactis* subsp. *lactis* as a separate source of diacetyl.
Flavour defects may occur when diacetyl is reduced by bacteria containing diacetyl reductase (acetoin dehydrogenase), which converts diacetyl into the flavourless compound acetoin. This can happen during manufacture as well as during storage of the finished product, but high diacetyl reductase starter cultures should be avoided [Law,1981].

AIMS AND OBJECTIVES

The aim of this study was to investigate the relationship between the breakdown of ALA and the production of acetoin and diacetyl, as well as to develop improved methods for the determination of ALA and diacetyl in a mixture of the three compounds.

Another objective was to screen strains of Cit^+ *Lactococcus lactis* subsp. *lactis* for diacetyl production and to investigate the effect of O_2 and various additives on the metabolism of selected strains.

MATERIALS AND METHODS

Bacteria

The bacteria used for screening were Cit^+ *Lc. lactis* subsp. *lactis* strains from the DPC collection of strains. The strains studied more closely were Cit^+ *Lc. lactis* subsp. *lactis* strains 999 and 1166, 1166M1, which is an acetolactate decarboxylase negative (ALD^-) mutant of 1166, mixed culture 4/25 and strain 4/25A, which is an ALD^- strain of Cit^+ *Lc. lactis* subsp. *lactis* isolated from mixed culture 4/25, and *Lb. casei* strain 4191 and its ALD^- mutant F207M3. Strains 999, 1166 and 4/25A were obtained from the DPC collection of strains; strain 1166M1, 4191 and F207M3 were obtained from the Centre de Recherche International André Gaillard, Yoplait, Ivry-sur-Seine, France. Mixed culture 4/25 is the starter culture used commercially for the production of lactic butter.

Media

Reconstituted Skim Milk (RSM)

RSM was prepared from skim milk powder at various concentrations and either heat-treated at 90°C for 30min or sterilised at 121°C for 5min.

Lactic acid concentrate

The lactic acid concentrate was obtained from NIZO, Netherlands. It has a pH of 3.1 and a lactic acid content of 150g/l. For certain experiments, lactic acid concentrate was added to RSM at a ratio of 3:2 to imitate conditions during the manufacture of lactic butter.

L-M17 Broth:

Tryptone	5g
Soytone	5g
Meat digest	5g
Yeast extract	2.5g
Ascorbic acid	0.5g
Magnesium sulphate	0.25g
Disodium-ß-glycerophosphate	19g

dissolved in 950ml distilled water. After autoclaving at 121°C for 15min and cooling to 50°C, 50ml of 10g/100ml lactose solution is added.

Litmus milk:

Skim milk powder	100g
Litmus solution	10ml
Distilled water	1000ml

dissolved in 1000ml of distilled water. The litmus milk was sterilised in 100ml Duran bottles at 121°C for 5min.

Measurement of Diacetyl

Diacetyl was measured by the method described by Walsh and Cogan [1974], which is a modification of the Prill and Hammer [1938] method.

Reagents:

1. Hydroxylamine: 17.5g hydroxylamine hydrochloride ($NH_2OH.HCl$) made up to 500ml with distilled water.
2. Acetone-phosphate: 38g $K_2HPO_4.3H_2O$ and 40ml acetone made up to 200ml with distilled water.

3. Alkaline tartrate: 80g NaKtartrate.$4H_2O$ and 24ml 35g/100ml NH_3 made up to 200ml with distilled water.
4. Ferrous sulphate: 5g $FeSO_4.7H_2O$ made up to 100ml with 1ml/100ml H_2SO_4.

Procedure:

A volume of sample was steam distilled using a Büchi steam distillation apparatus. The first and second 10ml of distillate were collected separately in graduated test tubes. The first 10ml were used for the determination of diacetyl and the second 10ml for the measurement of acetoin. All the diacetyl and most of the acetoin are present in the first 10ml; the second 10ml contain about 25% of the acetoin [Walsh and Cogan, 1974].

To 5ml of the first 10ml of distillate, 1ml of hydroxylamine was added. The sample was mixed and heated in a waterbath to 75°C for 20min. It was then cooled in air. Within 10min, 0.5ml of acetone phosphate were added. After mixing, 1.5ml of alkaline tartrate were added, the sample was mixed again and immediately 0.2ml of $FeSO_4$ were added. After 15-20min the A_{530} was read against a reagent blank.

Independent aqueous solutions of diacetyl (10mM) were stored at 4°C. A standard curve was obtained by distilling and analysing different concentrations of diacetyl in the same way as the samples. The standard curve was linear up to an A_{530} of at least 2. Once this had been established, it was only necessary to distill one standard in duplicate in subsequent tests.

Measurement of Acetoin

Acetoin was determined by the Westerfeld [1945] method.

Reagents:

1. 0.5g/100ml creatine
2. 2.5M NaOH
3. 5g/100ml 1-naphthol in 2.5M NaOH

Procedure:

To 5ml of the second 10ml of steam distillate, or an aliquot made up to 5ml with distilled water, 1ml creatine solution was added, followed by 1ml of 1-naphthol, and the sample was mixed. After exactly 60min in a waterbath at 21°C the A_{525} was read against a reagent blank.

Two independent aqueous stock solutions of acetoin (10mM) were stored at 4°C. A standard curve was obtained by distilling and assaying different concentrations of acetoin. The standard curve was linear up to an A_{525} of at least 1.25. Once this had been established, only one standard in duplicate was subsequently used at each analysis.

Measurement of Acetolactate (ALA)

Since ALA is easily decarboxylated to acetoin in acidic solutions, it can be determined from the difference in the concentration of acetoin before and after decarboxylation with HCl [Jordan and Cogan, 1995].

Reagents:

1. 0.5M HCl
2. 50mM phosphate buffer pH 6.5 or 125mM phosphate buffer pH 7.5
3. 0.5g/100ml creatine
4. 2.5M NaOH
5. 5g/100ml 1-Naphthol in 2.5M NaOH

Procedure:

To portion of the sample, usually 50 or 100µl of liquid sample or 50 or 100mg of solid sample, 0.4ml of 0.5M HCl were added to induce decarboxylation of ALA to acetoin. A second portion of sample was treated with 0.4ml of distilled water instead of HCl. Sufficient 50mM phosphate buffer was then added to each test tube to make up the volume to 5ml. This minimises autodecarboxylation of ALA to acetoin in the

water-treated sample. The samples were held at 4°C for 16 to 30h and acetoin was then determined according to the Westerfeld [1945] method as described above.
A modification had to be made for samples containing the lactic acid concentrate, which decreased the pH of the samples to 3.2. Distilled water was used instead of phosphate buffer for samples treated with HCl, and the concentration of phosphate buffer was increased to 125mM for samples treated with water, in order to maintain the correct pH for decarboxylation of ALA on the one hand and to minimise autodecarboxylation on the other.

Using this method, ALA and acetoin concentrations can be determined at the same time. The amount of ALA present in a sample is obtained by subtracting the concentration of acetoin in the water-treated sample from the concentration of acetoin in the HCl-treated sample, taking into account that only 84% of ALA is converted to acetoin by this procedure [Jordan and Cogan, 1995]. Since during the Westerfeld method acetoin is converted to diacetyl, any diacetyl present in a sample has to be determined separately by the Walsh and Cogan [1974] method and subtracted from the water-treated sample to obtain the true value for acetoin.

An alternative method for the determination of ALA was developed, based on the fact that ALA can be decarboxylated oxidatively to diacetyl under the influence of metal ions, low pH and heat.

Reagents:

1. 0.1ml/100ml H_2SO_4
2. Citric acid (0.2M)/Na_2HPO_4 (0.4M) buffer at pH 3.3
3. 10mM $CuSO_4$ in solution (2)

To 3ml of sample 5.5ml of buffer and 1.5ml of $CuSO_4$ solution were added; for samples with low diacetyl concentrations the volume was increased 3-fold (final Cu^{2+} concentration 1.5mM). The pH of this mixture was 3.5. The mixture was steam distilled, which resulted in complete oxidation of ALA to diacetyl (see results). The first 10ml of distillate were collected and assayed for diacetyl by the Walsh and

Cogan [1974] method. The amount of diacetyl present in the sample before oxidation was determined by steam distillation in water instead of the $CuSO_4$ solution at pH 0.8. A pH 0.8 was achieved by adding 6M H_2SO_4. ALA was then calculated as the difference in the diacetyl levels measured before and after oxidation.

An acetoin standard was tested with every set of samples assayed for ALA by the Jordan and Cogan [1995] method, whereas a diacetyl standard was used with samples assayed for ALA using the $CuSO_4$ method.

Measurement of Citrate

Citrate was determined by the method of Marier and Boulet [1958].

Reagents:

1. 5.56g/100ml trichloracetic acid (TCA)
2. Pyridine
3. Acetic anhydride

Procedure:

A 0.5ml sample was added to 4.5ml of the TCA. The mixture was shaken vigorously and left standing for at least 30min to desorb and solubilize any citrate attached to the casein. The extract was then centrifuged for 5min at 14000rpm in an Eppendorf microfuge. To 1ml of supernatant, 1.3ml of pyridine and 5.7ml of acetic anhydride were added. The test tubes were placed immediately in a water bath at 30°C for 30min to dissipate the heat developed in the mixture and allow uniform colour to develop. The A_{428} was read against a reagent blank within another 30min. A standard curve must be carried out at each analysis since the relationship between A_{428} and concentration is variable and curvilinear.

Measurement of L-Lactate and Acetate

A 2ml sample was added to 2ml of 10g/100ml TCA, mixed and left standing for at least 30min. The extract was centrifuged for 5min at 14000rpm in an Eppendorf microfuge. Lactate and acetate were measured in the supernatant using Boehringer enzymatic test kits. Samples treated that way decreased the pH of the buffer solution used in the test kits by 0.1 of a pH unit.

ALA Standard Curves

An ALA stock solution was obtained by hydrolysing 2-acetoxy-2-methyl-acetoacetate (ALA double ester) with two equivalents of freshly prepared 0.1M NaOH by mixing gently at room temperature for 30min. RSM (10g/100g) or a mixture of RSM and lactic acid concentrate were added to the hydrolysed ester to give concentrations from 0-10mM ALA. This stock solution was then used for standard curves.

The pH was adjusted with NaOH (6M) or lactic acid (10g/100ml) to give pH values from 3.3 to 8.0. Five or 20ml of sample were distilled and the first 10ml of steam distillate assayed for diacetyl.

Model System

The effects of oxygen, milk solids, temperature, pH, metal ions (Fe^{2+} and Cu^{2+}) and haemin on the breakdown of ALA to diacetyl and acetoin were studied in a model system. The model system consisted of a Braun fermenter containing RSM or the 3:2 mixture of RSM and lactic acid concentrate and 1.2mM ALA. The contents of the fermenter were stirred at 500rpm and, where the effect of O_2 was studied, the medium was sparged with O_2 (10psi). ALA, acetoin and diacetyl were monitored

over time. Samples for diacetyl were adjusted to pH 6.5 with NaOH before distillation.

Conversion Rates

Conversion rates of ALA to acetoin and diacetyl were determined by plotting the concentrations of either compound against ALA concentrations. The conversion rate was obtained by multiplying the slope of the linear regression line by 100.

Growth Experiments

Pure Cultures

Screening

One hundred and thirty four strains from the laboratory collection were screened for citrate utilisation and diacetyl production. The strains, stored at -80°C, were grown in L-M17 broth over night at 30°C. A few drops of the fully grown culture were then used to inoculate litmus milk, which was incubated at 30°C until clotted. RSM (10g/100ml) was inoculated (1ml/100ml) with the freshly clotted culture. Portion (40ml) of the inoculated milk was then transferred to a 500ml sterile Duran bottle and oxygenated with O_2 for 1min. The bottles were then tightly capped and incubated at 30°C for 16h. The remaining 60ml was used as a non-oxygenated control and incubated in the same way. After 16h incubation the cultures were iced down and assayed for diacetyl, citrate and pH. For the estimation of diacetyl 10ml, or 10g in case of a clotted sample, was distilled. The pH was not adjusted prior to distillation.

Growth kinetics

After the screening strains 999 and 1166 were selected for more detailed studies. Strains 999 and 1166 were examined for their growth characteristics and metabolite production under oxygenated and non-oxygenated conditions. The cultures, stored at -80°C, were grown overnight in L-M17 broth at 30°C. This broth culture was used

to inoculate RSM (100g/l), which was then incubated at 30°C until clotted. Heat treated RSM (100g/l) was inoculated at 1ml/100ml with the clotted milk culture, mixed well and distributed in 100ml volumes in 500ml Duran bottles. Half of the bottles were sparged with O_2 for 3min and then tightly closed. The other half were used as non-oxygenated controls.

The effects of leucine and valine (10mM) on strains 999 and 1166, and the effects of metal ions (Fe^{++}, Cu^{++}, 100μM) and haemin (10μM) on strain 999 were studied under both oxygenated and non-oxygenated conditions.

Strains 1166 and 1166M1 were also studied under 0%, 21% and 100% O_2. The cultures were prepared as described above and sparged with O_2, air or N_2 for 6min. O_2 concentrations were measured using a Knick O_2 meter.

The bottles were incubated at 30°C in a water bath until the pH reached 4.7. At regular intervals bottles were taken out, iced down and assayed for ALA, acetoin, diacetyl, citrate, lactate, acetate and pH.

Comparison of the two methods for the determination of ALA

To compare the $CuSO_4$ method for the determination of ALA with the Jordan and Cogan [1995] method, strains 4/25A, 1166, 1166M1 and 4191M3 were grown as described above. Samples were taken at regular intervals and analysed for ALA with the $CuSO_4$ method and the Jordan and Cogan [1995] method.

Mixed Culture 4/25

Laboratory trials

Effect of solids

Mixed culture 4/25 was stored in the commercial containers at -20°C. Heat-treated RSM (16, 19 and 23g solids/100g) was inoculated with the culture following the instructions on the container. The culture was split into 50ml volumes in 250ml bottles and incubated at 21°C. The pH was monitored continuously using the

CINAC hardware and software (INRA, Grignon, France) and samples were taken at regular intervals for citrate, ALA, diacetyl and acetoin. ALA and diacetyl were determined with the $CuSO_4$ method.

Effect of temperature

The culture was grown at 23°C for 18h or until the pH dropped below 5.0. The pH was monitored continuously using the CINAC hardware and software (INRA, Grignon, France). At the end of growth, a sample was taken for citrate, ALA, acetoin and diacetyl. The culture was then divided into three separate fermenters. Lactic acid culture concentrate was added (ratio culture to lactic acid culture concentrate 2 to 3) and the cultures were stirred at 11, 23 and 30°C for 2h. Samples were taken at regular intervals and analysed for ALA, acetoin and diacetyl. ALA was determined by the Jordan and Cogan [1996] method.

Commercial trials

Culture 4/25 was grown for 18h at 23°C in 1000litres RSM with solid concentrations ranging from 17 to 23g/100ml in three commercial plants. Samples were taken prior to the addition of lactic acid concentrate and subsequently during aeration of the mixture at various temperatures, and analysed for ALA, acetoin and diacetyl. The samples taken prior to the addition of lactic acid concentrate were also analysed for citrate. Aeration times varied from 35min in plant C over 53min in plant A to 2.5h in plant B.

Quark

Quark samples with varying ALA concentrations were obtained from the Centre de Recherche International André Gaillard, Yoplait, Ivry-sur-Seine, France. In order to monitor ALA breakdown during storage at 4°C, samples were analysed in triplicate for ALA and diacetyl using the new method during storage at 4°C.

RESULTS

PART I

ALA Standard Curves

The effects of volume (5 and 20ml), pH (pH 3.3-8.0) and ALA concentration (0-10mM) on the conversion of ALA to diacetyl during distillation in RSM (10g solids/100g) or a mixture of RSM (10g solids/100g) and lactic acid concentrate were investigated. The results are shown in Tables 1 and 2. In RSM, the distillation volume had no effect on the conversion of ALA to diacetyl if the pH was >5.0. Below pH 5.0, conversion increased with decreasing pH and was greater when 5ml instead of 20ml were distilled. The ALA concentration seemed to have an effect on the breakdown during distillation; breakdown was higher in standard curves from 0 to 2.5mM than in standard curves from 0 to 10mM (Table 1). When the individual standard curves were examined more closely, it appeared that the breakdown was not linear over the concentration range from 0 to 10mM, with higher breakdown rates occurring at lower ALA concentrations. An example of a standard curve at pH 6.0 is shown in Figure 3.

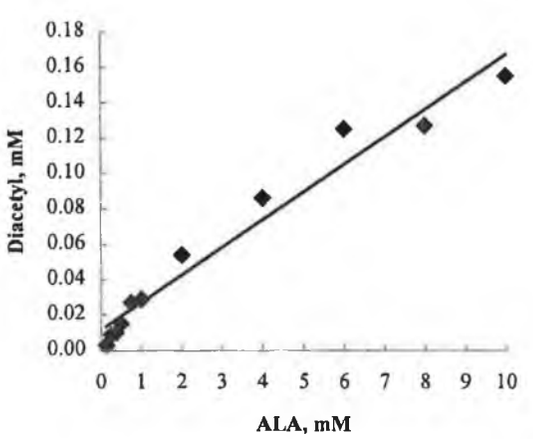

Fig. 3: Standard curve for ALA in RSM at pH 6.0. 20ml was distilled

In a mixture of RSM and lactic acid concentrate, the distillation volume did not appear to have as great an effect on ALA breakdown (Table 2). As in the case of RSM alone, breakdown rates were greater at lower pH values. In these experiments, the regression lines were linear with r^2 values >0.90.

The results from the ALA standard curves were difficult to reproduce from one day to another, although the same procedure was followed each time. It was evident, however, that the pH at which a sample is distilled has a major effect on ALA breakdown during distillation, with higher breakdown rates occurring at low pH, and that the breakdown of ALA is not linear over the concentration range from 0 to 10mM.

Table 1: Breakdown of ALA to diacetyl during distillation in RSM

Volume distilled (ml)	ALA (mM)	pH	n	Average Breakdown (%)	sd
5	0-1.2	3.3	1	38.6	
5	0-1.2	4.5	4	16.0	4.43
5	0-1.2	5.5	3	7.91	3.38
5	0-1.2	6.8	3	4.92	0.82
5	0-10	4.5	2	4.32	0.66
20	0-2.5	3.3	1	11.3	
20	0-2.5	4.3	1	16.0	
20	0-2.5	4.5	16	9.95	5.77
20	0-2.5	4.7	3	9.50	2.10
20	0-2.5	4.9	2	8.28	3.82
20	0-2.5	5.0	5	7.35	2.88
20	0-2.5	6.0	7	6.24	1.11
20	0-2.5	7.0	6	4.27	1.67
20	0-2.5	8.0	4	5.31	1.33
20	0-10	4.5	9	5.90	2.69
20	0-10	4.7	2	7.89	0.76
20	0-10	4.9	2	4.30	0.51
20	0-10	5.0	5	4.10	1.12
20	0-10	6.0	5	2.51	1.28
20	0-10	7.0	4	3.30	1.20
20	0-10	8.0	2	4.36	0.18

Table 2: Breakdown of ALA to diacetyl during distillation in RSM and lactic acid concentrate

Volume distilled (ml)	ALA (mM)	pH	n	Average Breakdown (%)	sd
5	0-2	3.3	4	37.6	4.29
5	0-2	4.5	1	29.4	
5	0-2	6.5	3	8.33	0.63
5	0-2	9.0	1	15.2	
20	0-2	3.3	1	46.3	
20	0-2	4.0	3	29.1	8.88
20	0-2	4.5	3	9.85	3.35
20	0-2	6.0	2	4.67	0.00
20	0-2	6.5	4	7.21	1.84
20	0-2	7.0	2	6.87	2.17

Model System

Effect of oxygen

The medium used for the oxygenation experiments was a mixture of RSM (16g solids/100g or 19g solids/100g) and lactic acid concentrate. The pH of the mixture was 3.3. The mixture was held at 21°C, stirred and sparged with O_2 and the conversion of ALA to acetoin and diacetyl was compared to a non-oxygenated control.

The first aeration trials resulted in conversion rates of ALA to diacetyl of 44.1% (16g solids/100g RSM) and 35.4% (19g solids/100g RSM), which were considerably lower than in the non-oxygenated controls which showed conversion rates of 52.6 and 55.7%, respectively (Table 3). The unexpected lower conversion rates in the oxygenated system were due to the fact that some diacetyl was carried out from the fermenter by the air leaving the system. In order to quantify the amount of diacetyl that was lost this way, the air leaving the system was led through either one or two bottles each filled with 1 litre of distilled water. Adding the amount of diacetyl measured in the first bottle of water to the diacetyl in the system increased conversion rates from 44.1 to 67.7% in the milk with the lower solids concentration (16g solids/100g) and from 35.4 to 72.3% in RSM with 19g solids/100g. Adding a

second bottle containing 1 litre of water to trap diacetyl leaving the first bottle did not increase the recovery. Conversion of ALA to acetoin in the lower concentration of RSM tested (16g solids/100g) was lower in the oxygenated system than in the control, 12.9% compared to 30.1%. At the higher level of milk solids (19g solids/100g) there was no difference in the average breakdown of ALA to acetoin when the system was oxygenated. Addition of the conversion rates of ALA to diacetyl and acetoin show that >80% of the ALA added was recovered as diacetyl and acetoin.

Table 3: Effect of O_2 on conversion (%) of ALA[a] to diacetyl and acetoin in a model system

	Conversion (%) to					
	Diacetyl			Acetoin		
	average	sd	n	average	sd	n
RSM 16g solids/100g						
non-oxygenated RSM	52.6	8.25	5	30.1	1.98	7
oxygenated RSM	44.1	8.72	5	12.9	8.56	5
oxygenated RSM + 1L of water	67.7	6.47	2			
RSM 19g solids/100g						
non-oxygenated RSM	55.7	9.24	5	24.0	13.1	5
oxygenated RSM	35.4	2.48	2	24.5	3.62	2
oxygenated RSM + 1L of water	72.3	0.00	1			
oxygenated RSM + 2×1L of water	73.8	4.02	2			

[a] measured by the Jordan and Cogan [1995] method

Effect of milk solids

The conversion of ALA to acetoin and diacetyl was studied in RSM (13, 16, 19 and 23g solids/100g) and lactic acid concentrate, mixed in a ratio 3:2, at 21°C. The results are shown in Table 4. The solids level of the milk did not affect the conversion of ALA to acetoin and diacetyl to any great extent. Breakdown to diacetyl was greater than breakdown to acetoin on all occasions.

Table 4: Effect of concentration of milk solids on the conversion of ALA[a] to acetoin and diacetyl at 21°C

Milk (g/100g)		Conversion (%) to Acetoin	Diacetyl	ALA used (%)	k_{ALA} (h^{-1})	n
13	average	21.8	69.0	56.6	0.2652	6
	sd	8.22	8.65	3.11	0.0340	
16	average	30.1	56.3	63.0	0.3422	7
	sd	1.98	9.11	8.38	0.0354	
19	average	24.0	55.7	64.9	0.3352	5
	sd	13.1	9.24	7.14	0.0773	
23	average	25.5	54.5	53.9	0.2597	4
	sd	8.84	3.84	2.43	0.0190	

[a] measured by the Jordan and Cogan [1995] method

During these experiments it became apparent, that the breakdown of ALA over time was not linear, but followed an exponential function, implying that breakdown was a first order reaction:

$$c_{ALA} = a \times e^{-k \times t}$$

where c_{ALA} is the ALA concentration (mM), a the coefficient, k the specific breakdown rate (h^{-1}) and t the time (h).

The specific breakdown rate, k, is also shown in Table 4; it increased from $0.2652h^{-1}$ in RSM of 13g solids/100g to $0.3352h^{-1}$ in RSM of 19g solids/100g, but dropped again to $0.2597h^{-1}$ in RSM of 23g solids/100g.

Effect of temperature

The effect of temperature on the breakdown of ALA was studied over a temperature range from 7 to 37°C in RSM of 16g solids/100g and lactic acid concentrate, mixed in a ratio of 3:2. Conversion rates to acetoin and diacetyl, and specific breakdown rates are shown in Table 5.

Table 5: Effect of temperature on breakdown of ALA[a]

Temperature (°C)		Conversion (%) to Acetoin	Conversion (%) to Diacetyl	k_{ALA} (h^{-1})	n
7	average	0.71	27.7	0.0896	2
	sd	0.26	2.01	0.0103	
13	average	26.2	34.9	0.1376	2
	sd	2.45	1.79	0.0164	
18	average	17.5	38.0	0.1834	2
	sd	8.63	9.68	0.0378	
21	average	30.5	47.9	0.3422	7
	sd	9.59	10.5	0.0354	
23	average	38.5	34.7	0.3291	2
	sd	2.09	0.04	0.0312	
26	average	27.6	51.1	0.6026	3
	sd	12.9	4.87	0.0263	
30	average	45.9	48.9	1.0869	3
	sd	3.93	3.36	0.3592	
37	average	50.1	48.0	2.5574	3
	sd	6.80	4.48	0.5097	

[a] measured by the Jordan and Cogan [1995] method

The specific rate of ALA breakdown (k_{ALA}) increased with increasing temperature with the exception of a small decrease at 23°C. The same was more or less true for the conversion of ALA to acetoin whereas conversion to diacetyl decreased slightly at temperatures >26°C.

The relationship between k_{ALA} and temperature is non-linear, but can be expressed as an exponential function (Fig. 4). An Arrhenius plot of the relationship between k_{ALA} and temperature was linear. This plot is used in thermodynamics to determine the activation energy (E_A) of a chemical reaction:

$$E_A = -m \times R$$

where E_A is the activation energy, m is the slope and R is the general gas constant [8.314J/(mol×K)].

The activation energy for the breakdown of ALA was 82.7kJ/mol or 19.8kcal/mol.

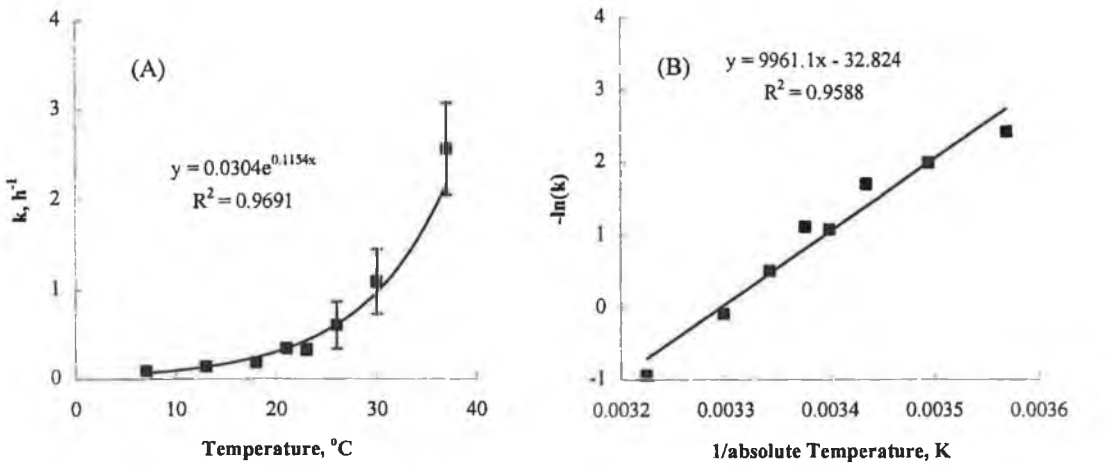

Fig. 4: (A) Effect of temperature on the specific breakdown rate, k, of ALA, with error bars. (B) An Arrhenius plot of the same data.

Effect of pH

Preliminary trials on the breakdown of ALA as a function of pH were carried out in 250ml Duran bottles filled with a mixture of RSM (16g solids/100g) and water (ratio 3:2) containing 1.2mM ALA over a pH range from 4.0 to 6.5. The pH was adjusted with lactic acid (3.33M) in Trial 1 and HCl (1M) in Trial 2, to determine if the type of acid influenced the rate of breakdown. The bottles were stirred on a multi-stirrer unit in a water bath at 21°C. ALA breakdown did not appear to follow a first order reaction except at pH 4.0. Correlation coefficients (r^2) for the higher pH values were low, because of low breakdown rates (Table 6). Specific breakdown rates were determined and plotted against pH and resulted in an exponential curve (Fig. 3). The type of acid used did not significantly influence ALA breakdown.

A third trial was carried out in a fermenter using the same reaction mixture, which was stirred at 500rpm at 21°C; the pH ranged from 3.0 to 6.0 and was adjusted with HCl (1M). At pH 3.0, 3.5 and 4.0, the correlation coefficients of an exponential curve fit for ALA breakdown over time were >0.97, indicating excellent agreement.

At pH values above 4.0, little breakdown occurred and correlation coefficients were below 0.6 (Table 6). The relationship between k_{ALA} and pH was exponential, but k_{ALA} values were slightly lower than in Trials 1 and 2 (Fig. 5).

Table 6: Specific breakdown rates (k_{ALA}) and correlation coefficients (r^2) for ALA[a] breakdown at varying pH values

	Trial 1		Trial 2		Trial 3	
pH	k_{ALA} (h^{-1})	r^2	k_{ALA} (h^{-1})	r^2	k_{ALA} (h^{-1})	r^2
3.0					0.2646	0.9796
3.5					0.1367	0.9787
4.0	0.0912	0.9449	0.0975	0.8615	0.0587	0.9722
4.5	0.0504	0.8056	0.0564	0.8401	0.0219	0.5739
5.0	0.0442	0.8424	0.0438	0.7966	0.0137	0.3726
5.5	0.0265	0.6855	0.0398	0.8957		
6.0	0.0195	0.6998	0.0120	0.5469	0.0377	0.5263
6.5	0.0207	0.6824	0.0075	0.4000		

[a] measured by the Jordan and Cogan [1995] method

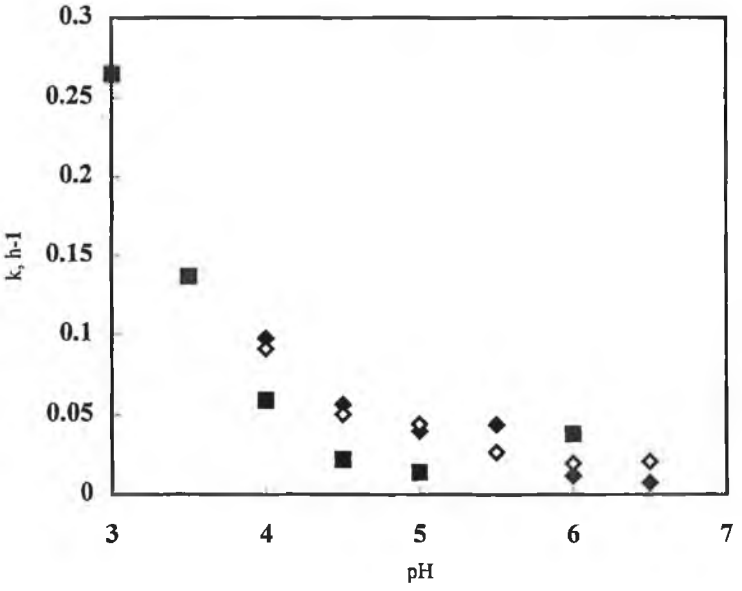

Fig. 5: Effect of pH on the specific breakdown rate of ALA (k); the pH was adjusted with lactic acid in Trial 1 (◇), and in Trial 2 (◆) and Trial 3 (■) with HCl.

Effect of metal ions and haemin

The effects of $CuSO_4$ (0.1, 0.2 and 2mM), $FeCl_3$ (0.2mM) and haemin (0.01 and 0.1mM) on the breakdown of ALA to diacetyl were studied in RSM (13g solids/100g) at pH 6.5 at 21°C. Figure 6 shows a typical result.

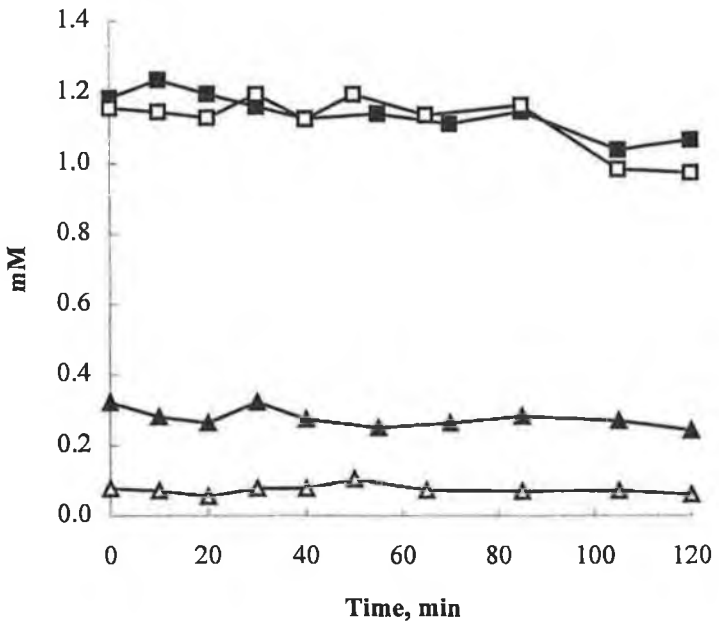

Fig. 6: ALA breakdown (■, □) and diacetyl production (▲, △) in RSM (13g solids/100g) at pH 6.5 at 21°C in the presence (full symbols) and absence (open symbols) of 0.2mM $CuSO_4$.

Over a period of 2h little or no breakdown of ALA occurred in the presence and absence of Cu^{2+}. However, ~4 times more diacetyl was detected in the presence of Cu^{2+} than in the control. Breakdown of ALA to diacetyl was instantaneous and diacetyl levels did not increase above the initial value. This indicates that ALA was converted to diacetyl during measurement rather than in the fermenter, most likely during distillation, due to the combined effect of heat and the oxidising agent. Similar results were obtained for $FeCl_3$ and haemin. Breakdown of ALA to diacetyl for all additives is shown in Table 7.

Table 7: Breakdown[a] of ALA[b] to diacetyl in the presence of various additives

Additive	Concentration (mM)	n	Breakdown (%)	sd
Haemin	0.01	5	9.3	1.80
	0.10	4	26.7	4.18
$CuSO_4$	0.10	1	21.1	
	0.20	3	22.7	3.92
	2.00	1	67.3	
$FeCl_3$	0.20	2	11.3	0.94
Control	/	4	5.6	0.59

[a] Breakdown was calculated from the values at time 0
[b] ALA was measured by the Jordan and Cogan [1995] method

Breakdown was greatest in the presence of 2mM $CuSO_4$ (67.3%), followed by 0.1mM haemin (26.7%). There was only a small difference between the breakdown rates in the presence of 0.1 and 0.2mM $CuSO_4$ (21.1% and 22.7%); breakdown was similar when 0.01mM haemin and 0.2mM $FeCl_3$ where used (9.25% and 11.3%). In the control, 5.6% of ALA was converted to diacetyl.

Development of a New Method for the Determination of ALA

An alternative method for the determination of ALA, previously described by Gollop et al. [1987], involves the oxidative decarboxylation of ALA to diacetyl by heating the sample for 10min at 80°C in the presence of 0.15mM each of $FeCl_3$ and $FeSO_4$ followed by "air" distillation at 60°C. When this method was investigated, using steam distillation at 100°C, only 15.7% of the ALA was recovered as diacetyl. The pH of the mixture of RSM and $FeCl_3/FeSO_4$ was 5.5 instead of 4.0 as recommended by Gollop et al. [1987]. The effect of different concentrations of acid and Fe was investigated. The results (Table 8) showed considerable variation with the highest breakdown (48.7%) at pH 0.8 in the presence of 3.5mM each of Fe^{2+} and Fe^{3+}. In addition, some of the regression coefficients of diacetyl on ALA were low, indicating poor reproducibility.

Table 8: Breakdown of ALA to diacetyl during steam distillation at various pH in the presence of iron

Final Fe concentration (mM)	Acid	Final acid concentration (mM)	pH	n	Slope[a]	r^2
0.15 Fe^{2+} 0.15 Fe^{3+}	HCl H_2SO_4	1.5 1.5	5.5	5	0.157	0.837
0.15 Fe^{2+} 0.15 Fe^{3+}	H_2SO_4	7.14	3.5	5	0.360	0.728
0.15 Fe^{2+}	H_2SO_4	7.14	3.5	5	0.316	0.967
0.15 Fe^{3+}	H_2SO_4	7.14	3.5	5	0.437	0.922
3.5 Fe^{2+} 3.5 Fe^{3+}	HCl	350	0.8	5	0.487	0.999

[a] Regression of diacetyl on ALA

The results in the model system (Table 7) show that $CuSO_4$ has a greater effect on the breakdown of ALA to diacetyl than $FeCl_3$. To find the optimum concentration of $CuSO_4$, ALA (2mM and 1mM) in RSM (10g/100g) was distilled in the presence of 0 to 7mM $CuSO_4$ (in 18.8mM H_2SO_4) at pH 3.5. The results show that at $CuSO_4$ concentrations >1mM, the conversion of ALA to diacetyl was essentially 100% (Fig. 7). A concentration of 1.5mM $CuSO_4$ was chosen as the optimum.

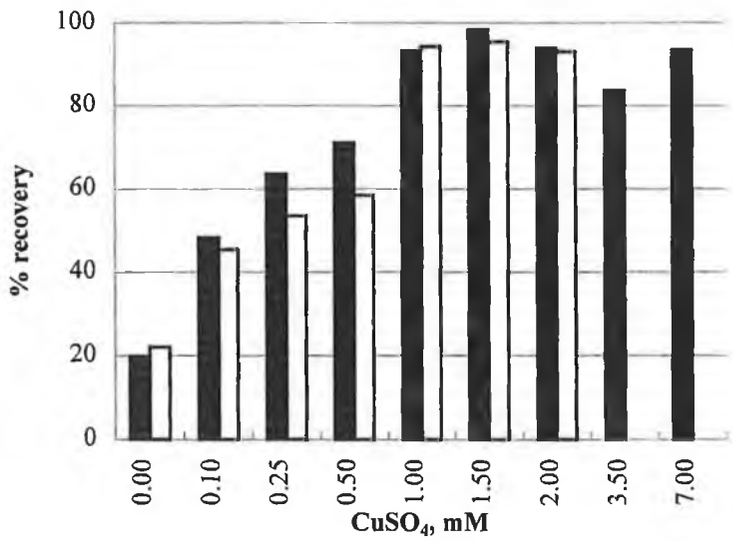

Fig. 7: Recovery of ALA as diacetyl at a range of $CuSO_4$ concentrations; closed bars: 1mM ALA, open bars: 2mM ALA

Standard curves containing 0.25-4mM ALA were distilled in the presence and absence of 1.5mM $CuSO_4$ (in 18.8mM H_2SO_4) at pH 6.5, 3.5 and 0.8. The pH attained by the mixture of RSM (normal pH 6.5) and $CuSO_4$ in H_2SO_4 was 3.5. The other pH values were obtained by adjusting with 2.5M NaOH and 6M H_2SO_4 to obtain pH values of 6.5 and 0.8, respectively. Transformation of ALA to diacetyl was greatest at pH 3.5 in the presence of $CuSO_4$ (Fig. 8). In addition it was linear and reproducible; the error bars show the standard deviation of 10 trials. At ALA concentrations >4mM breakdown rates decreased due to incomplete recovery of the large amounts of diacetyl in the first 10ml of steam distillate. The lowest breakdown occurred at pH 0.8 in the absence of $CuSO_4$. Regression analysis for all conditions is shown in Table 9. It was concluded that ALA should be determined at pH 3.5 in the presence of 1.5mM $CuSO_4$, in which complete oxidative decarboxylation of ALA to diacetyl occurs, and diacetyl at pH 0.8 in the absence of $CuSO_4$, where <2% oxidative decarboxylation of ALA to diacetyl occurs.

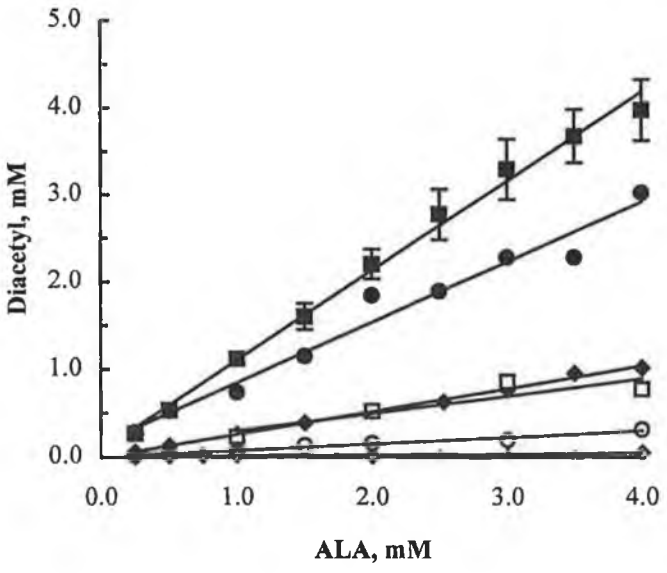

Fig. 8: ALA standard curves at pH 6.5 (●, ○), 3.5(■, □) and 0.8 (◆, ◇), in the presence (closed symbols) and absence (open symbols) of 1.5mM $CuSO_4$

Table 9: Effect of pH and Cu_2SO_4 on the conversion of ALA to diacetyl

	+$CuSO_4$			-$CuSO_4$		
pH	n	slope	r^2	n	slope	r^2
0.8	9	0.260	0.996	6	0.0103	0.782
3.5	9	1.025	0.994	5	0.1970	0.805
6.5	9	0.690	0.970	7	0.0749	0.973

Changing the pH from 3.5 to 3.0 in the presence of 1.5mM $CuSO_4$ decreased the conversion of ALA to diacetyl by ~10%. At pH 4.0, breakdown of ALA to diacetyl remained at 98%, but it dropped by 20%, when the pH was increased to 5.0 (Fig. 9).

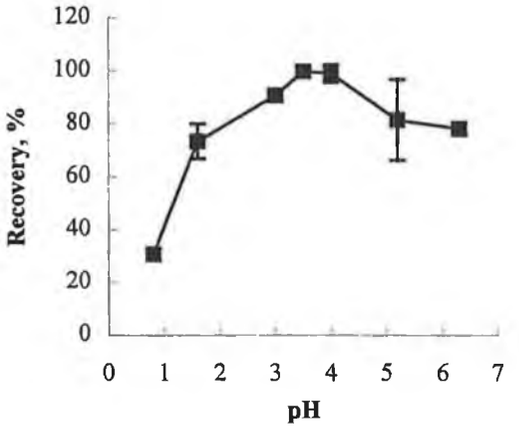

Fig. 9: Breakdown of ALA to diacetyl in the presence of 1.5mM $CuSO_4$ at different pH values. Error bars show the standard deviation of three trials.

As the pH of the mixture of sample and $CuSO_4$ appeared to be fairly critical, replacement of the H_2SO_4 with a buffer was investigated. A citric acid (0.2M)/Na_2HPO_4 (0.4M) buffer at pH 3.3 resulted in a pH of the mixture of RSM (normal pH 6.5) and $CuSO_4$ of 3.5. This buffer also resulted in 100% conversion of ALA to diacetyl (results not shown) and worked well over the range of pH values expected in a typical growth curve for Cit^+ *Lc. lactis* subsp. *lactis* (~6.6 to ~4.7). A mixture of 7ml buffer and 3ml RSM at pH 6.6 resulted in a pH of 3.54, whereas mixing 7ml of buffer and 3ml of RSM at pH 4.7 resulted in a pH of 3.47.

When ALA standard curves were repeated using the citric acid/Na_2HPO_4 buffer and substituting a mixture of Fe^{2+} and Fe^{3+} (both 1.5mM) for Cu^{2+}, recovery of ALA as diacetyl was 96.3%, indicating that the type of transition element is probably not too critical under the conditions chosen (data not shown).

Experiments were conducted to determine if the ALA, which was not converted to diacetyl, was converted to acetoin. Standard curves of ALA were distilled in the presence and absence of 1.5mM $CuSO_4$ at pH 0.8, 3.5 and 6.5, and diacetyl measured by the Walsh and Cogan [1974] procedure in the first 10ml of distillate and acetoin by the Westerfeld [1945] procedure in the second 10ml of distillate. In the presence of Cu^{2+}, ALA was preferentially decarboxylated to diacetyl at pH 3.5 and 6.5 and to acetoin at pH 0.8, whereas in the absence of Cu^{2+}, ALA was transformed to acetoin (Fig. 10). As expected, transformation to diacetyl was 102% at pH 3.5 in the presence of Cu^{2+} (Table 10). The sum of diacetyl and acetoin found after distillation at both pH 3.5 and 6.5 in the presence of Cu^{2+} was greater than the amount of ALA initially present in the distillation flask (124% recovery). This problem did not occur at pH 0.8 in both the absence and presence of Cu^{2+}, where recovery of ALA was 95% and 96%, respectively, and at pH 3.5 and 6.5 in the absence of Cu^{2+}, where recovery of ALA was 99.1% and 84.4%, respectively. The overestimation of the sum of acetoin and diacetyl in the presence of Cu^{2+} was not due to interference of Cu^{2+} with the assays for acetoin and diacetyl, since the separate measurements of diacetyl and acetoin themselves were unaffected by the presence of 1.5mM $CuSO_4$. Also, no acetoin was transformed to diacetyl during distillation and measurement (data not shown).

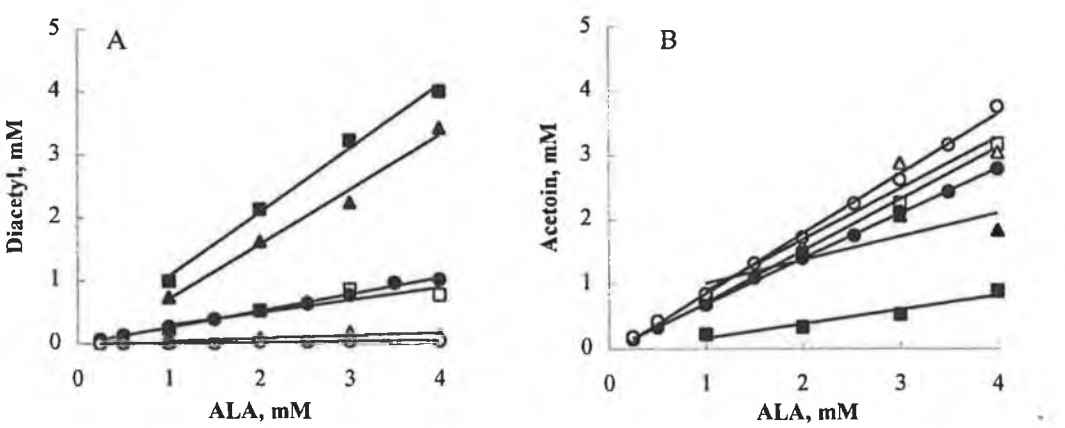

Fig. 10: Transformation of ALA to diacetyl (A) and acetoin (B) at pH 0.8 (●, ○), 3.5 (■, □) and 6.5 (▲, △) in the presence (closed symbols) and absence (open symbols) of 1.5mM $CuSO_4$ (diacetyl was measured in the first 10ml of distillate; acetoin was measured in the second 10ml of distillate).

Table 10: Conversion of ALA to diacetyl and acetoin at pH 0.8, 3.5 and 6.5 in the presence and absence of 1.5mM $CuSO_4$.

	+$CuSO_4$				-$CuSO_4$			
	Diacetyl		Acetoin		Diacetyl		Acetoin	
pH	slope	r^2	slope	r^2	slope	r^2	slope	r^2
0.8	0.260	0.996	0.702	0.999	0.017	0.879	0.931	0.998
3.5	1.015	0.993	0.220	0.939	0.197	0.805	0.794	0.935
6.5	0.869	0.985	0.366	0.744	0.043	0.672	0.801	0.996

To find out, what caused the overestimation, diacetyl and acetoin (0.5-4mM) were distilled at pH 3.5 and 6.5, and two 10ml fractions of each were collected. All fractions were assayed for diacetyl and acetoin, respectively, using absolute (undistilled) standards for the calculations. The results (Tables 11) were analysed by linear regression.

Table 11: Recovery of acetoin and diacetyl standards after distillation at pH 3.5 and 6.5

	pH 3.5				pH 6.5			
	Acetoin		Diacetyl		Acetoin		Diacetyl	
Fraction	slope	r^2	slope	r^2	slope	r^2	slope	r^2
1	0.4569	0.989	0.841	0.997	0.3624	0.999	0.850	0.999
2	0.2440	0.999	0.041	0.966	0.2279	0.999	0.038	0.970

Recovery of diacetyl standards was 84% in the first fraction and 4% in the second fraction, while 46% and 24% of the acetoin standards was recovered in the first and second fraction, respectively. When ALA is distilled in the presence of Cu^{2+}, it preferentially breaks down to diacetyl. The majority of this diacetyl comes over into the first 10ml of distillate, but ~4% comes over into the second 10ml of distillate (data not shown). The diacetyl in the second 10ml fraction of distillate, which is usually used for the determination of acetoin, reacts in the same way as acetoin with the Westerfeld [1945] reagents. Therefore, diacetyl that has already been accounted for in the first 10ml fraction by using a distilled standard, is calculated a second time as acetoin, and since only 24% of the acetoin standard comes over in the second 10ml fraction, the concentration of diacetyl in that fraction is actually multiplied ~4-fold. This explains the overestimation of diacetyl and acetoin from ALA in the

presence of Cu^{2+} at pH 3.5 and 6.5, where ALA is preferentially transformed to diacetyl and therefore large amounts of diacetyl are present.

Comparison of $CuSO_4$ Method and Jordan and Cogan [1995] Method

The method usually used for the determination of ALA is the Jordan and Cogan [1995] method, which is carried out on undistilled samples. To determine how the $CuSO_4$ method compared with this method, the production of ALA by two ADC negative strains of *L. lactis* subsp. *lactis*, 4/25A and 1166M1, and one ADC^- strain of *Lb. casei*, 4191M3, and strain 1166, which is the ADC positive parent of strain 1166M1, was monitored using both methods. Figure 11 shows the correlation of the two methods. The r^2 value was 0.958, indicating excellent agreement between both methods. The $CuSO_4$ method overestimated ALA by 5.7% compared to the Jordan and Cogan [1995] method. However, the latter method is not accurate if large amounts of acetoin and small amounts of ALA are present and since measurement of ALA by the $CuSO_4$ method results in 100% conversion of ALA to diacetyl it is concluded the $CuSO_4$ method is more reliable.

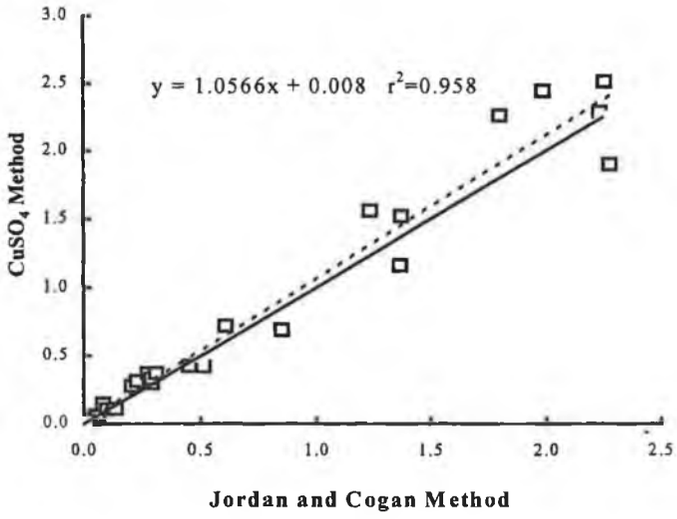

Fig. 11: Regression analysis of the $CuSO_4$ method on the Jordan and Cogan [1995] method. The dotted line is the regression line and the complete line is the expected line if both methods gave identical results.

Mixed Culture 4/25

Commercial trials I

Trials were carried out in three commercial plants producing butter according to the NIZO process. Samples were taken from the culture tank before addition of the lactic acid concentrate and assayed for citrate, ALA, acetoin and diacetyl. The results are shown in Table 12. In plants A and C, high solids levels were used to grow the culture and the culture did not use the citrate completely, whereas in both trials in plant B effectively all the citrate was utilised during the ~18h incubation period. Little or no ALA was detected in plants B and C, both of which had high concentrations of acetoin (5.5-10.2mM). The failure to detect ALA was possibly due to the Jordan and Cogan [1995] method used in which small amounts of ALA cannot be detected in the presence of high concentrations of acetoin. Diacetyl concentrations varied, but were generally low (0.07-0.22mM).

Table 12: Concentrations of the important parameters of culture 4/25 in 3 plants after overnight growth and before addition of lactic acid concentrate

	Plant A	Plant B		Plant C
		Trial	Trial	
Citrate used, %	67	94	99	72
ALA, mM	ND[b]	0.32	0.61	0
Acetoin, mM	ND	5.50	6.22	10.2
Diacetyl, mM	ND	0.07	0.15	0.22
Solids[a], g/100g	21	17	17	23

[a] from plant records
[b] not determined

After the lactic acid concentrate was added, the mixture was aerated for different lengths of time in the different plants, and samples were taken for ALA, acetoin and diacetyl during the aeration period. The temperature at which the mixture of culture and lactic acid concentrate was aerated was generally low (<15°C). It can be seen in Figures 12a, b and c, that the concentrations of the three compounds did not change from their initial level during the aeration period, except for one erratic point in Trial 1 in plant B, which was probably the result of an error in measurement. ALA

concentrations were 1mM in plant B, 2mM in plant A and 4.7mM in Plant C. Acetoin concentrations were ~0.5mM in plant A and ~2.5mM in plants B and C. Diacetyl concentrations were generally low, but never increased beyond 0.5mM.

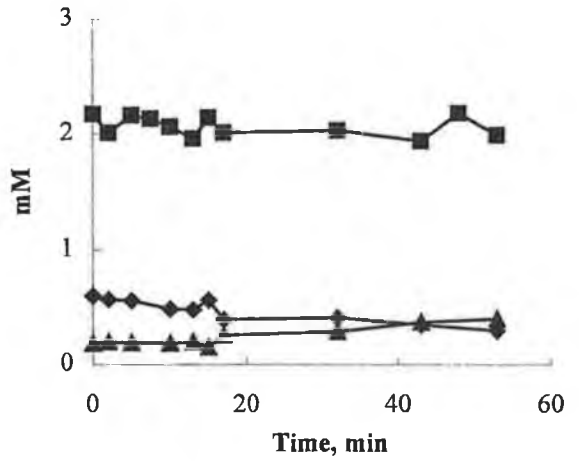

Fig. 12a: Breakdown of ALA (■) and production of acetoin (◆) and diacetyl (▲) by mixed culture 4/25 in plant A

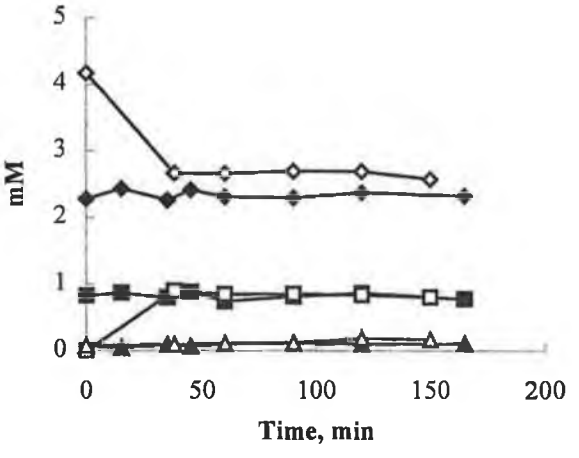

Fig. 12b: Breakdown of ALA (■) and production of acetoin (◆) and diacetyl (▲) by mixed culture 4/25 in plant B (1: open symbols, 2: closed symbols).

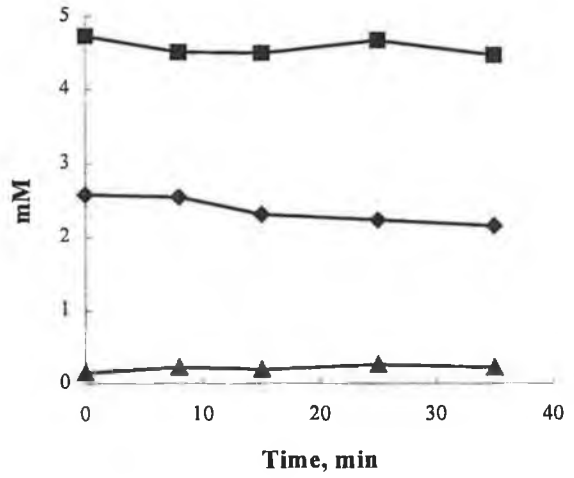

Fig. 12c: Breakdown of ALA (■) and production of acetoin (◆) and diacetyl (▲) by mixed culture 4/25 in plant C.

Laboratory trials

Effect of milk solids

Mixed culture 4/25 was grown in RSM (16, 19 and 23g solids/100g) at 21°C. pH, ALA, acetoin, diacetyl and citrate were monitored over time. Increasing solids concentrations slowed down the decrease in pH, probably due to the greater buffering capacity of the higher milk solids concentrations. ALA and acetoin production were slightly slower in the milk with the highest solids concentration, whereas diacetyl production was virtually unaffected. Citrate was utilised at similar rates in the three milks, but the initial level of citrate increased with increasing milk solids (Fig. 13a, 13b, 13c).

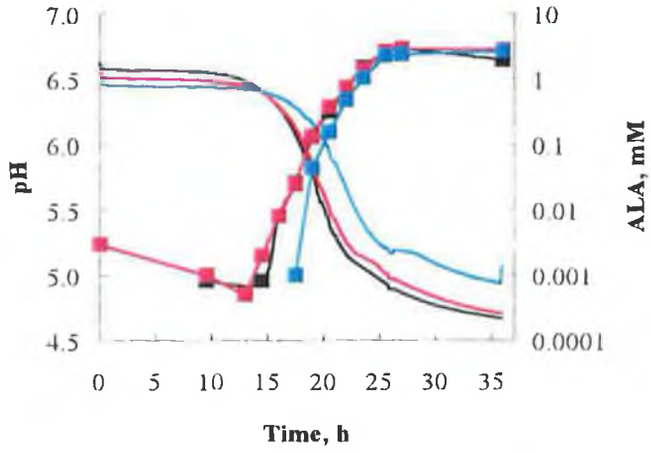

Fig. 13a: Effect of milk solids on pH (no symbol) and ALA production (■) of mixed culture 4/25; 16 (black), 19 (red) and 23 (blue) g solids/100g.

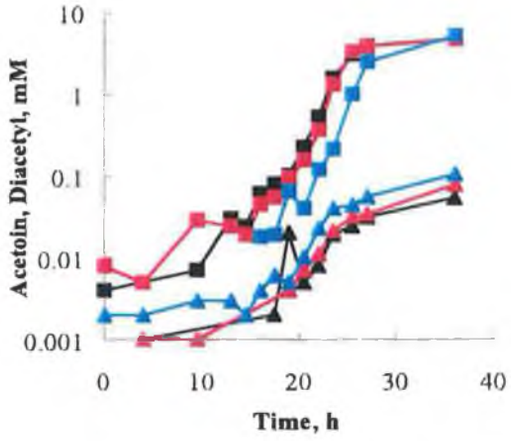

Fig 13b: Effect of milk solids on acetoin (■) and diacetyl (▲) production of mixed culture 4/25; 16 (black), 19 (red) and 23 (blue) g solids/100g.

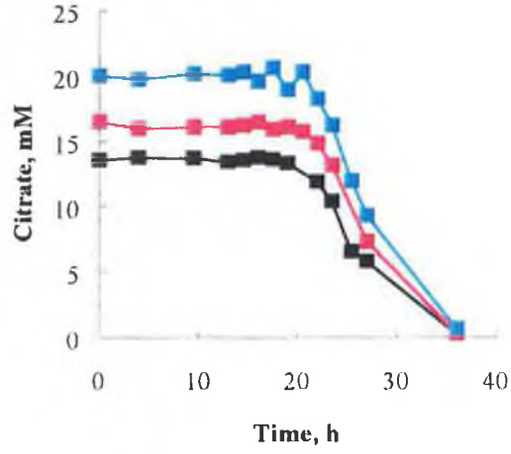

Fig. 13c: Effect of milk solids on citrate utilisation; 16 (black), 19 (red) and 23 (blue) g solids/100g.

Effect of temperature

Mixed culture 4/25 was incubated at 23°C in RSM (16g solids/100g) until it decreased the pH of the milk to <5.0. Table 13 shows citrate utilisation and ALA, acetoin and diacetyl production of the mixed culture 4/25 after ~18h of growth in three trials. Most of the citrate initially present in the milk was used. The culture produced similar amounts of acetoin and diacetyl in three trials and more acetoin than diacetyl was produced. The amount of ALA produced varied between the three trials.

Table 13: Citrate utilisation and ALA[a], acetoin and diacetyl production of mixed culture 4/25

	Trial 1	Trial 2	Trial 3
Citrate used (%)	84	85	95
ALA[a] (mM)	1.57	0.59	3.65
Acetoin (mM)	4.30	3.75	3.96
Diacetyl (mM)	0.11	0.16	0.21

[a] measured by the Jordan and Cogan method

After ~18h, the cultures were divided into three fermenters, lactic acid concentrate was added in the ratio 3:2 and ALA breakdown and acetoin and diacetyl production were monitored for 2h at 11, 23 and 30°C. ALA and acetoin were determined by the Jordan and Cogan [1996] method. Figure 14 shows the results of the three trials. The amount of ALA breakdown varied in the three trials but the trends were the same. The breakdown was greatest at 30°C, followed by 23°C, and no breakdown occurred at 11°C. The acetoin levels did not change much during the 2h period at 11 or 23°C but at 30°C a small increase from the initial value occurred. At 11°C, the diacetyl level did not increase significantly from the initial concentration (0.2-0.4mM), at 23°C it reached 1.4 to 1.8mM, while at 30°C it reached ~2mM. Conversion of ALA to diacetyl varied from 61 to 70% at 23°C and from 58 to 66% at 30°C. With the exception of trial 3, conversion of ALA to diacetyl was lower at 30°C than at 23°C, because at the higher temperature some ALA was converted to acetoin (Fig. 15).

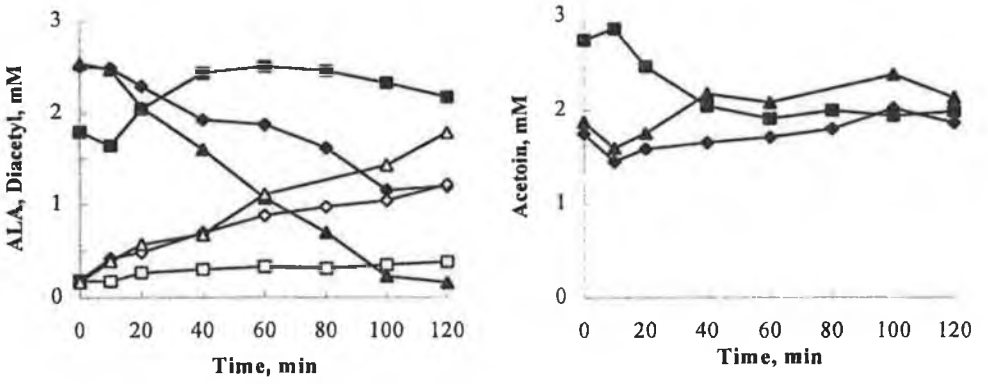

Fig. 14a: Breakdown of ALA (closed symbols) and production of diacetyl (open symbols) and acetoin (closed symbols) at 11 (■), 23 (◆) and 30°C (▲) in Trial 1.

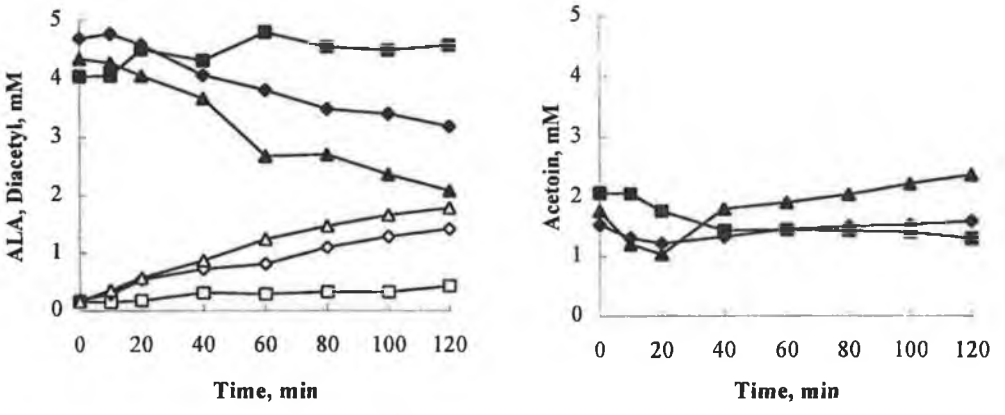

Fig. 14b: Breakdown of ALA (closed symbols) and production of diacetyl (open symbols) and acetoin (closed symbols) at 11 (■), 23 (◆) and 30°C (▲) in Trial 2.

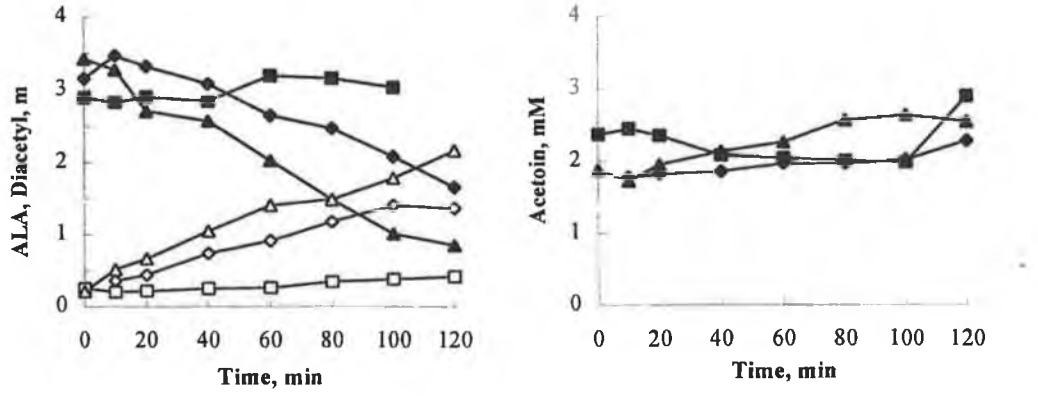

Fig. 14c: Breakdown of ALA (closed symbols) and production of diacetyl (open symbols) and acetoin (closed symbols) at 11 (■), 23 (◆) and 30°C (▲) in Trial 3.

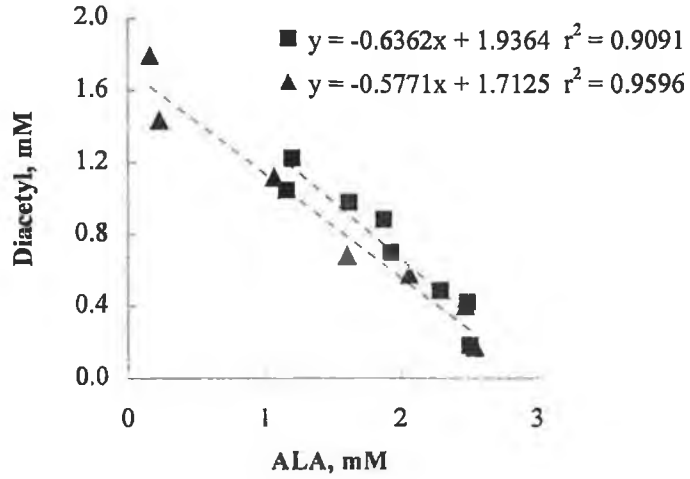

Fig. 15a: Breakdown rates of ALA to diacetyl at 23°C (■) and 30°C (▲) in Trial 1.

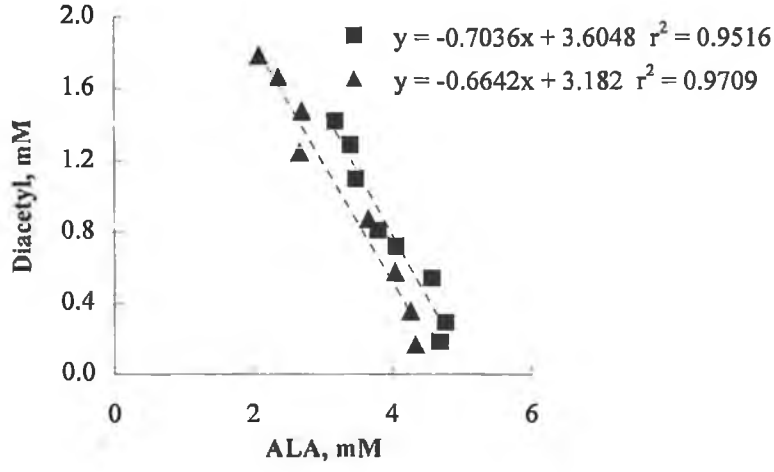

Fig. 15b: Breakdown rates of ALA to diacetyl at 23°C (■) and 30°C (▲) in Trial 2.

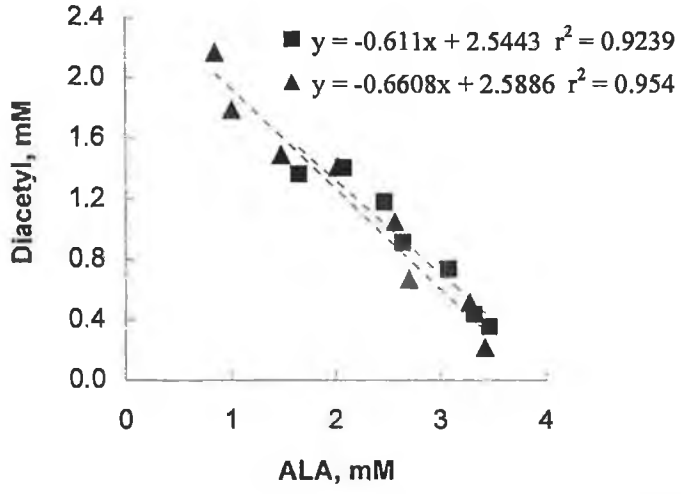

Fig. 15c: Breakdown rates of ALA to diacetyl at 23°C (■) and 30°C (▲) in Trial 3.

Commercial trials II

Effect of temperature

Trials were carried out in a commercial plant producing butter according to the NIZO process. The culture was grown in RSM containing 17g/100g solids. Samples were taken from the culture tank before addition of the lactic acid concentrate and assayed for citrate, ALA, acetoin and diacetyl. ALA was measured by the $CuSO_4$ method, diacetyl by distillation at pH 0.8 and acetoin by the Westerfeld [1945] procedure. The results are shown in Table 14.

Table 14: Concentrations of the important parameters of culture 4/25 after overnight growth and before addition of lactic acid concentrate.

	Trial 1	Trial 2
Citrate used (%)	99.9	94.1
ALA[a] (mM)	1.98	1.43
Acetoin (mM)	3.24	2.20
Diacetyl (mM)	0.003	0.040

[a] measured by $CuSO_4$ method

After the lactic acid concentrate was added, the temperature of the mixture of culture and lactic acid concentrate was increased to ~35°C by pumping the mixture through a heat exchanger. Significant increases in ALA breakdown and diacetyl production began as soon as the temperature reached 20°C (Figure 16), but diacetyl production ceased after 60min in Trial 1 and continued to increase slowly in Trial 2 up to 120min. ALA was converted to diacetyl at a rate of 60 and 64% in Trials 1 and 2 respectively (Fig. 17). Decarboxylation was almost complete in 120min and there was no increase in the levels of acetoin. The culture was diluted with lactic acid concentrate in the ratio 3:2. This resulted in an apparent increase in the initial level of ALA for which there is no obvious explanation. There was an immediate increase in the initial levels of diacetyl. This was probably due to the low pH (3.5) which is attained on addition of the lactic acid concentrate (Table 14 and Fig. 16).

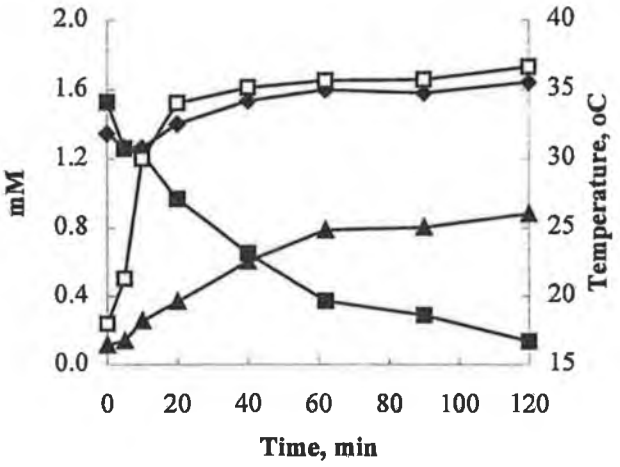

Fig. 16a: Breakdown of ALA (■) and production of acetoin (◆) and diacetyl (▲) after addition of the lactic acid concentrate in Trial 1. The temperature (□) of the mixture is also shown.

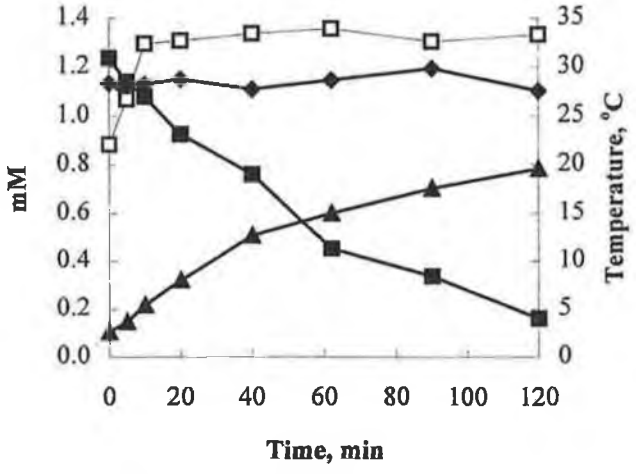

Fig. 16b: Breakdown of ALA (■) and production of acetoin (◆) and diacetyl (▲) after addition of the lactic acid concentrate in Trial 2. The temperature (□) of the mixture is also shown.

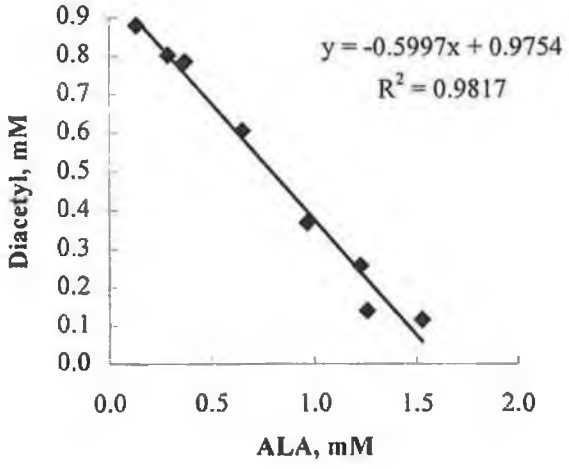

Fig. 17a: Breakdown rate of ALA to diacetyl in Trial 1.

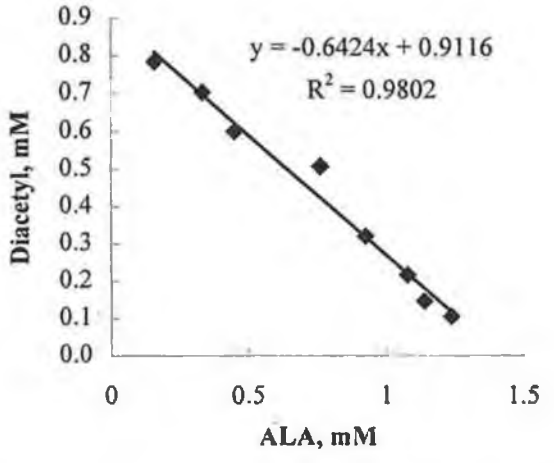

Fig. 17b: Breakdown rate of ALA to diacetyl in Trial 2.

Quark

Six quark samples with varying ALA concentrations were analysed for ALA and diacetyl with the $CuSO_4$ method over a period of ~3 weeks storage at 4°C. The results are shown in Figure 18. For practical reasons it was not possible to measure the levels at day 0. ALA decreased during storage in all samples, whereas diacetyl increased, with the exception of sample Z, the control. Most ALA was contained in sample M, followed by A, H, D and Y in decreasing order. The standard deviation between triplicate samples was <6%. The rates of ALA breakdown in samples Y, D, H, A and M were 6.9, 11.3, 14.6, 16.9 and 16.8μmol/L/day respectively. Regression of the diacetyl values on the ALA values in each sample gave r^2 values of >0.94 and conversion rates of 50, 44, 40, 37 and 32% for samples Y, D, M, A and H respectively.

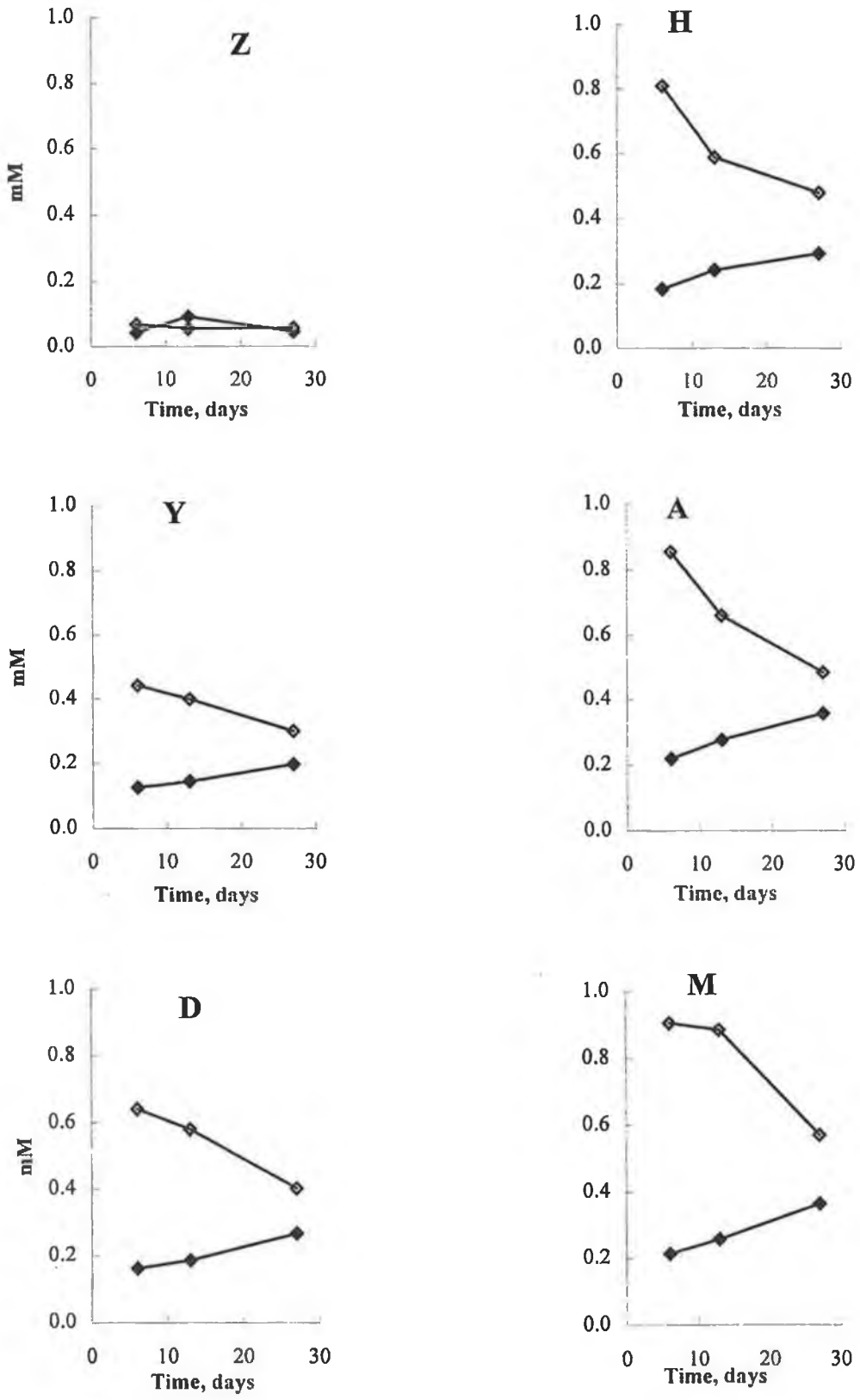

Fig. 18: Effect of storage at 4°C on ALA and diacetyl in quark. Closed symbols represent diacetyl, open symbols ALA.

PART II

Growth Experiments

Screening

One hundred and thirty four strains of Cit^+ *Lc. lactis* subsp. *lactis* were screened for citrate utilisation and diacetyl production under oxygenated and non-oxygenated conditions. Table 16 summarises the results of those strains which produced >0.2mM diacetyl under oxygenated conditions. This cut-off point was chosen, because it was the amount of diacetyl produced by strain 4/25A, the ADC^- strain used commercially for the production of diacetyl in the manufacture of lactic butter. Oxygenated cultures produced 2.3 to 20 times more diacetyl than non-oxygenated cultures. All strains grew slightly slower under oxygenated than under non-oxygenated conditions. Two strains, 999 and 1166, were selected for more detailed studies, since they produced the greatest amounts of diacetyl.

Effect of oxygenation on strains 999 and 1166

Oxygenation of strains 999 and 1166 for 3min before growth in RSM slowed down the subsequent rate of pH decrease, lactate production and citrate utilisation compared with non-oxygenated control cultures (Fig. 19). In strain 999, the rate of acetate production decreased slightly under oxygenated conditions. Acetate production was not determined for strain 1166. Both strains produced ~10 times more diacetyl in oxygenated than in non-oxygenated cultures, whereas acetoin production was unaffected by oxygen. Both strains produced inconsistent amounts of ALA in oxygenated and non-oxygenated cultures. This is due to problems with the determination of ALA by the Jordan and Cogan [1996] method. When ALA concentrations are close to or less than 10% of acetoin concentrations, negative values for ALA can be obtained which explains the erratic patterns of ALA production.

Table 15: Citrate utilisation and diacetyl production by Cit^+ lactococci under oxygenated and non-oxygenated conditions after 16h at 30°C in 10% RSM

	Oxygenated			Non-oxygenated			
DPC Strain No	pH	% Citrate utilised	Diacetyl mM	pH	% Citrate utilised	Diacetyl mM	Increase Fold
4/25 A	5.63	73	0.199	5.65	99	0.082	2.40
1166	4.76	100	0.422	4.51	100	0.033	12.79
999	4.74	100	0.416	4.57	100	0.026	16.00
990	5.28	100	0.408	4.98	100	0.047	8.68
1006	4.70	100	0.297	4.53	100	0.044	6.75
2392	5.48	100	0.295	5.03	100	0.033	8.94
1165	4.65	100	0.289	4.51	100	0.039	7.41
1002	4.64	100	0.287	4.52	100	0.046	6.24
1003	4.64	100	0.286	4.51	100	0.042	6.81
2345	4.99	100	0.284	4.89	100	0.039	7.28
2342	4.91	100	0.282	4.83	100	0.031	9.10
1008	4.74	100	0.281	4.59	100	0.014	20.07
1007	4.66	100	0.28	4.51	100	0.049	5.71
938	4.95	100	0.276	4.72	100	0.036	7.67
2311	5.43	100	0.276	5.10	100	0.022	12.55
2337	4.87	100	0.269	4.71	100	0.033	8.15
1005	4.65	100	0.268	4.51	100	0.044	6.09
2346	5.04	100	0.267	4.90	100	0.031	8.61
2382	5.15	100	0.267	4.93	100	0.028	9.54
2351	5.05	100	0.265	4.93	100	0.037	7.16
2315	5.73	100	0.264	5.38	100	0.026	10.15
2393	4.88	100	0.262	4.67	100	0.042	6.24
2288	4.92	100	0.259	4.70	100	0.023	11.26
2349	5.04	100	0.258	4.98	100	0.031	8.32
2332	4.72	100	0.257	4.58	100	0.031	8.29
918	4.41	100	0.255	4.34	100	0.031	8.23
2327	5.89	99	0.254	5.54	100	0.022	11.55
2280	4.96	100	0.251	4.71	100	0.025	10.04
2380	5.23	100	0.251	4.74	100	0.027	9.30
2287	5.00	100	0.249	4.73	100	0.018	13.83
2391	5.27	100	0.246	4.78	100	0.094	2.62
2339	4.89	100	0.245	4.71	100	0.03	8.17
2322	5.71	100	0.244	5.47	100	0.057	4.28
2328	4.89	100	0.242	4.73	100	0.027	8.96
1004	4.64	100	0.241	4.51	100	0.046	5.24
937	5.25	100	0.239	4.97	100	0.041	5.83
2310	5.83	96	0.237	5.50	100	0.027	8.78

Table 15 continued

	Oxygenated			Non-oxygenated			
DPC Strain No	pH	% Citrate utilised	Diacetyl mM	pH	% Citrate utilised	Diacetyl mM	Increase Fold
2316	5.77	100	0.233	5.53	100	0.03	7.77
1160	4.65	100	0.231	4.51	100	0.025	9.24
2286	4.94	100	0.226	7.74	100	0.027	8.37
2313	5.83	99	0.226	5.48	100	0.032	7.06
2272	5.57	100	0.224	5.32	100	0.048	4.67
2279	4.91	100	0.224	4.67	100	0.024	9.33
2395	4.83	100	0.224	4.63	100	0.099	2.26
2338	4.94	100	0.222	4.73	100	0.035	6.34
2312	6.05	86	0.221	6.01	84	0.036	6.14
2307	5.60	10	0.214	5.28	100	0.026	8.23
2308	5.62	100	0.211	5.4	100	0.051	4.14
2333	4.97	100	0.211	4.76	100	0.027	7.81
2383	4.82	100	0.211	4.67	100	0.08	2.64
2329	4.92	100	0.209	4.79	100	0	
925	5.90	83	0.207	5.72	100	0.045	4.60
2334	4.75	100	0.2	4.68	100	0.068	2.94
2340	5.01	100	0.2	4.77	100	0.029	6.90

Effect of leucine and valine on strains 999 and 1166

The addition of 10mM leucine to an oxygenated culture of strain 999 in RSM resulted in a decrease in lactate production and citrate utilisation (Fig. 20). The rate of pH decrease was also retarded. Acetoin, diacetyl and acetate production were only affected to a small extent and no ALA was detected.

Under non-oxygenated conditions, the effect of leucine was similar, except that ALA was detected. In the absence of leucine, ALA concentrations were erratic, due to the limitations of the Jordan and Cogan [1996] method described earlier.

Leucine (10mM) seemed to have an inhibitory effect on the rate of pH decrease, lactate production and citrate utilisation by strain 1166 under oxygenated conditions, but when the experiment was repeated and only pH measured, this effect disappeared, indicating that there was a problem with the growth of the culture shown in Figure 21. Slightly more acetoin was produced in the absence of leucine;

diacetyl production was unaffected and ALA was only detected in the first two hours of incubation.

Under non-oxygenated conditions, citrate utilisation by strain 1166 was slightly slower in the presence of leucine than in its absence, but the pH decrease and lactate, acetoin and diacetyl production were virtually unaffected. Small amounts of ALA were detected both in the presence and absence of leucine (Fig. 21).

Strain 999 grew, produced lactate and utilised citrate more slowly in the presence of 10mM valine than in its absence, under oxygenated and non-oxygenated conditions (Fig. 22). The rates of acetoin and diacetyl production were slightly less in the presence of valine than in its absence under both oxygenated and non-oxygenated conditions, but final concentrations were similar. The rate of acetate production was almost unaffected under oxygenated conditions in the presence of valine, but decreased under non-oxygenated conditions.

Addition of valine (10mM) to a culture of strain 1166 did not affect growth, utilisation of citrate and production of lactate, acetoin, diacetyl and acetate under oxygenated and non-oxygenated conditions. No ALA was detected (Fig. 23).

Effect of $CuSO_4$ on strain 999

There was no effect of $CuSO_4$ (0.1mM) on the production of lactate and acetoin and utilisation of citrate by strain 999 under both oxygenated and non-oxygenated conditions (Fig. 24). Diacetyl concentrations were marginally higher in the presence of $CuSO_4$; ALA concentrations were very low and therefore the amounts detected using the Jordan and Cogan [1996] method were erratic.

Effect of $FeSO_4$ on strain 999

Similarly $FeSO_4$ (0.1mM) had little if any effect on growth or any of the metabolites measured under both oxygenated and non-oxygenated conditions (Fig. 25).

Effect of haemin on strain 999

Addition of haemin (10μM) to an oxygenated culture of strain 999 had little effect on growth, citrate utilisation, and acetate and lactate production. Diacetyl production was faster at the beginning of growth in the presence of haemin, but the final diacetyl levels were similar in both the presence and absence of haemin. Acetoin production increased in the presence of haemin. ALA was only detected in the absence of haemin during the first 3h of incubation.

Under non-oxygenated conditions similar results were obtained, but diacetyl production was higher in the presence than in the absence of haemin. ALA was detected only in the absence of haemin, but the concentrations were erratic (Fig. 26).

Effect of oxygen concentration on 1166 and 1166M1

The effect of O_2 concentration, 0% (N_2), 21% (air) and 100% (O_2), on strains 1166 and 1166M1, an ALD^- mutant of 1166, was studied (Fig. 27). In both strains, citrate utilisation and the decrease in pH was more rapid, and hence growth was also faster, when the cultures were grown under N_2, followed, in turn, by air and O_2. In both strains, there was little effect of the three gases on acetoin production, except that lower amounts were produced by the mutant compared to the parent. ALA was only produced by the mutant and was unaffected by exposure to the gas except at the end of growth. Diacetyl production increased with increasing O_2 concentrations, but the increase was smaller in the mutant than in the parent culture. In the parent culture O_2 (100%) caused a ~12fold increase in diacetyl compared to cultures grown under N_2 or air. Air had little effect on diacetyl synthesis, when compared to cultures grown under N_2. The amounts of diacetyl produced were higher in the mutant than in the parent strain at all O_2 concentrations.

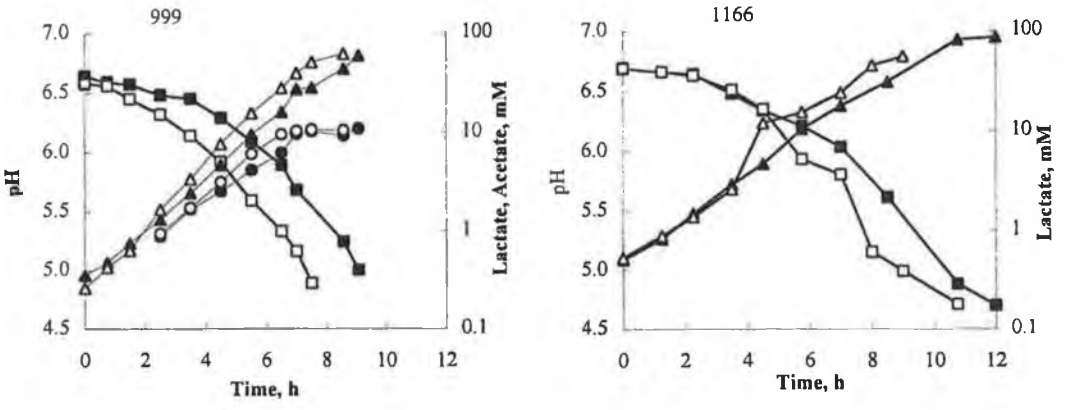

Fig. 19a: Effect of O_2 on pH (■, □) and lactate (▲, △) and acetate (●, ○) production by strains 999 and 1166 in RSM (10g/L); closed symbols represent oxygenated cultures, open symbols non-oxygenated cultures.

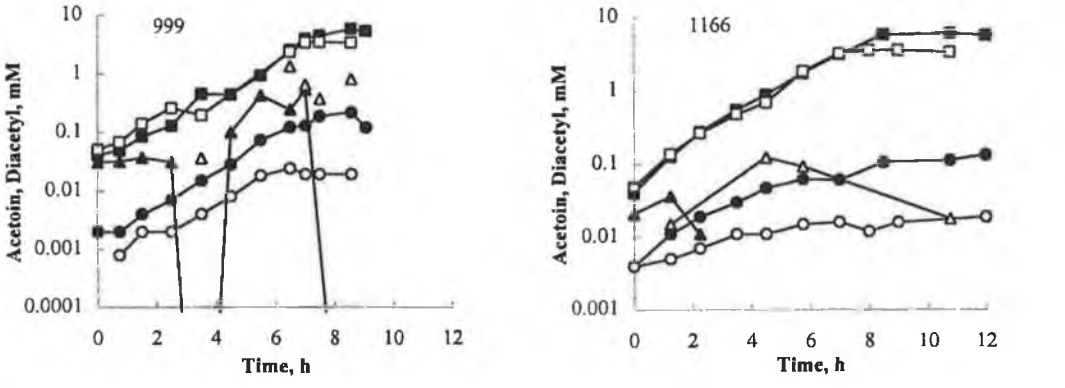

Fig. 19b: Effect of O_2 on acetoin (■, □), ALA (▲, △) and diacetyl (●, ○) production by strains 999 and 1166 in RSM (10g/L); closed symbols represent oxygenated cultures, open symbols non-oxygenated cultures.

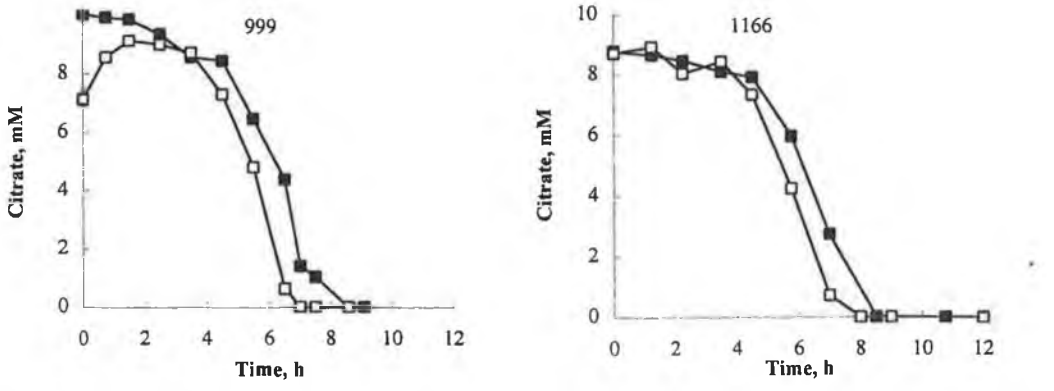

Fig. 19c: Effect of O_2 on citrate utilisation by strains 999 and 1166 in RSM (10g/L); closed symbols represent oxygenated cultures, open symbols non-oxygenated cultures.

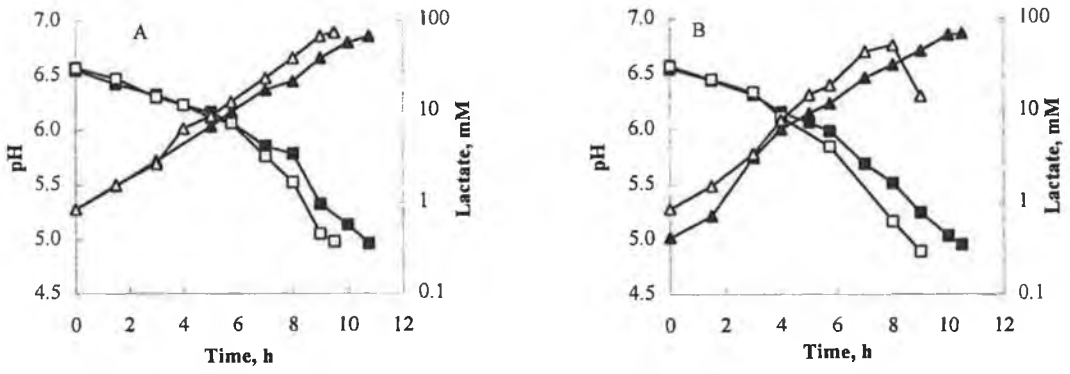

Fig. 20a: Effect of leucine on pH (■, □) and lactate (▲, △) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of leucine, open symbols its absence.

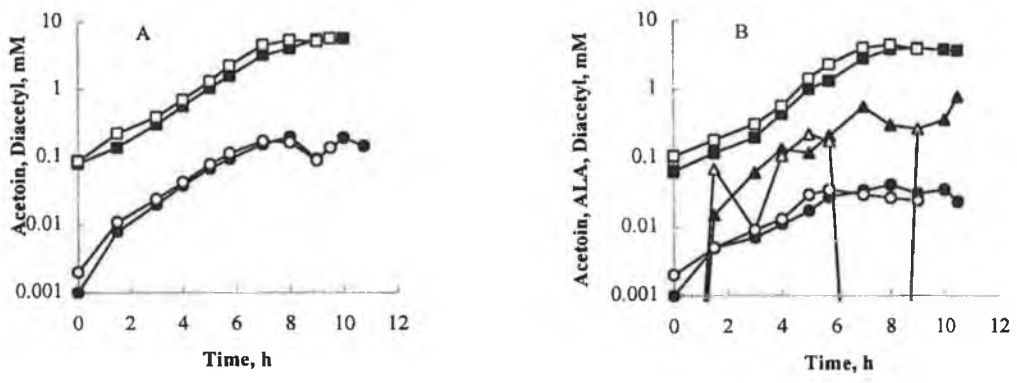

Fig. 20b: Effect of leucine on acetoin (■, □), ALA (▲, △) and diacetyl (●, ○) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of leucine, open symbols its absence.

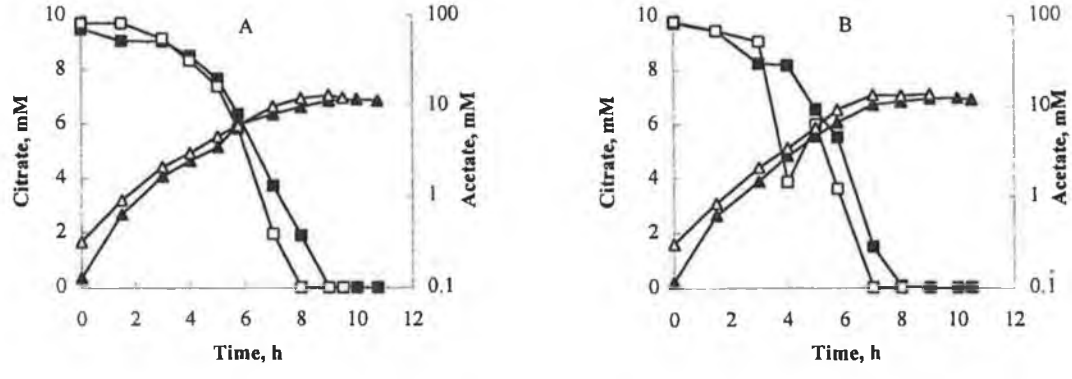

Fig. 20c: Effect of leucine on citrate utilisation (■, □) and acetate production (▲, △) by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of leucine, open symbols its absence.

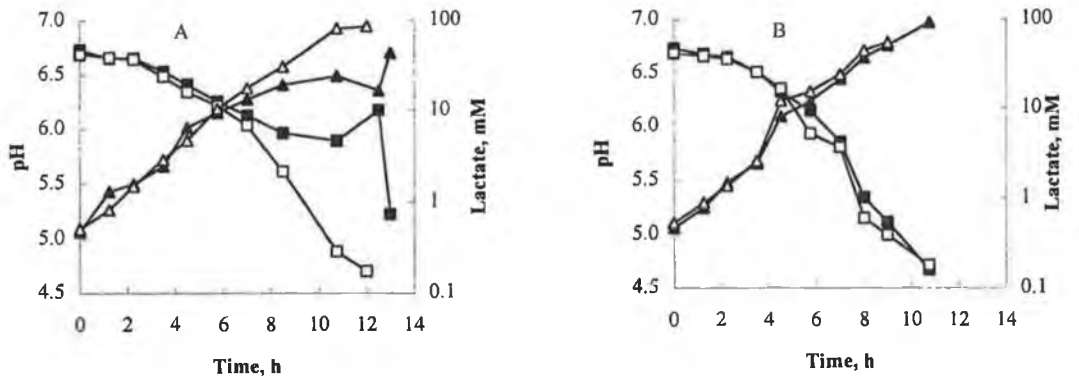

Fig. 21a: Effect of leucine on pH (■, □) and lactate (▲, △) production by strain 1166 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of leucine, open symbols its absence.

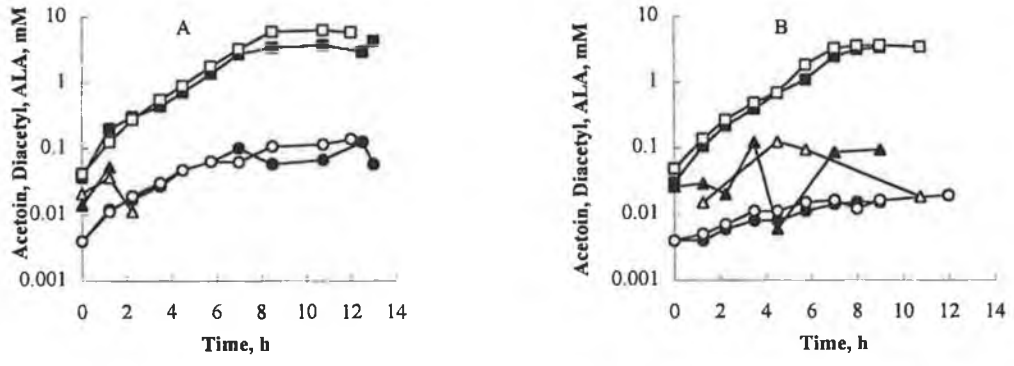

Fig. 21b: Effect of leucine on acetoin (■, □), ALA (▲, △) and diacetyl (●, ○) production by strain 1166 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of leucine, open symbols its absence.

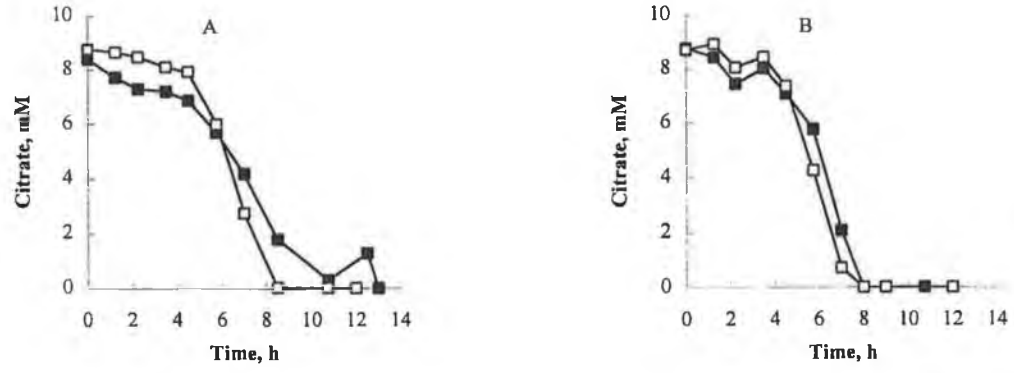

Fig. 21c: Effect of leucine on citrate utilisation by strain 1166 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of leucine, open symbols its absence.

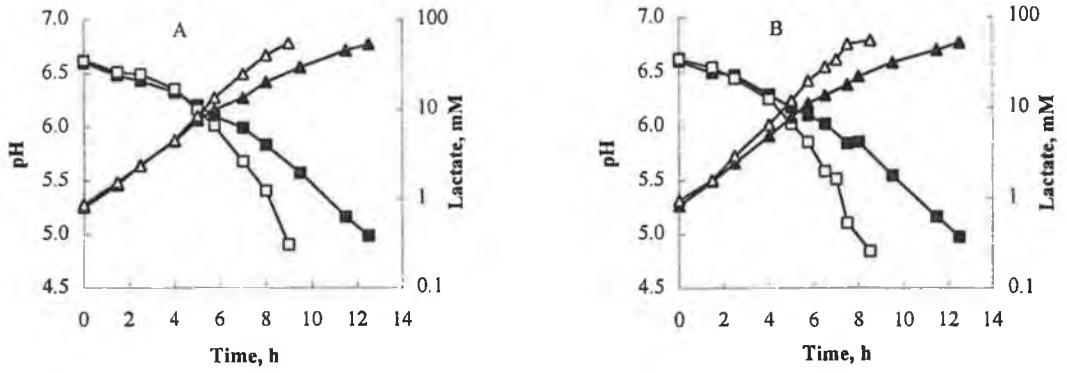

Fig. 22a: Effect of valine on pH (■, □) and lactate (▲, △) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of valine, open symbols its absence.

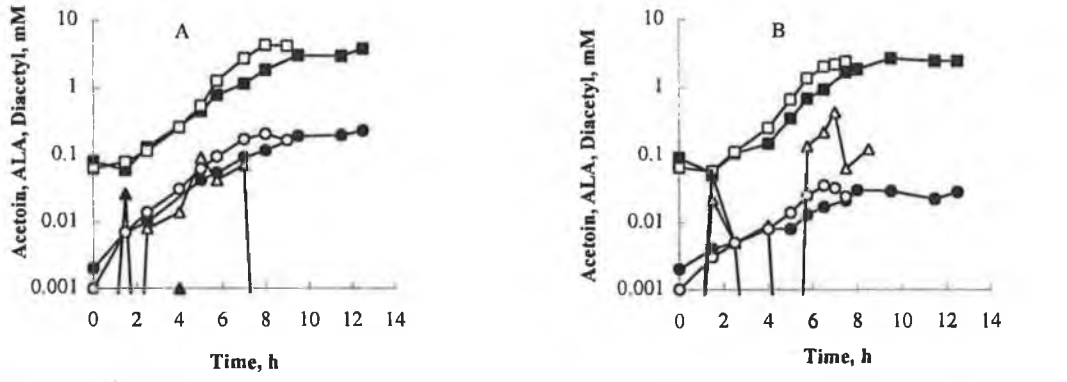

Fig. 22b: Effect of valine on acetoin (■, □), ALA (▲, △) and diacetyl (●, ○) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of valine, open symbols its absence.

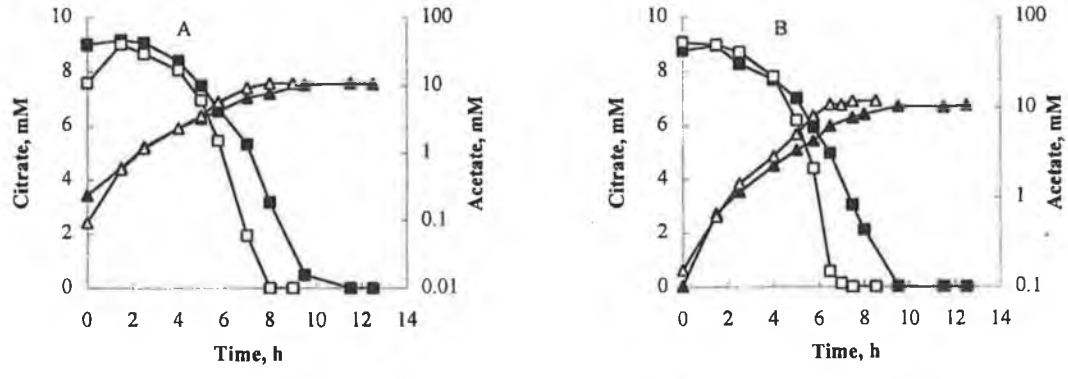

Fig. 22c: Effect of valine on citrate utilisation (■, □) and acetate production (▲, △) by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of valine, open symbols its absence.

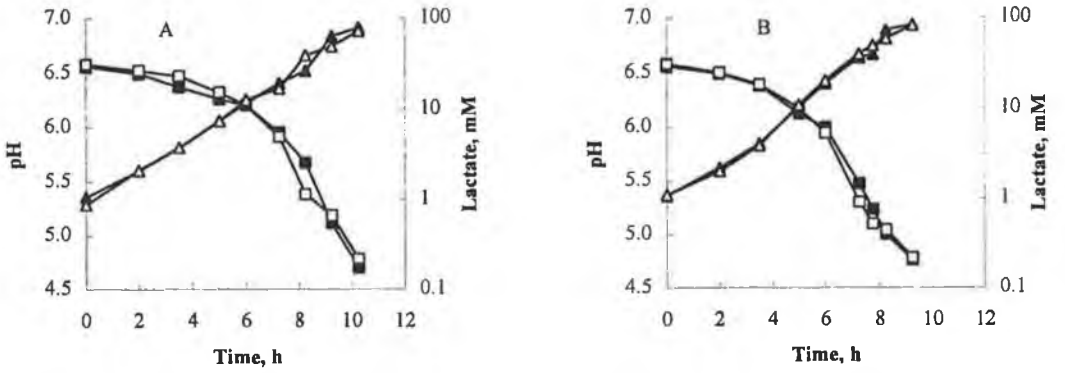

Fig. 23a: Effect of valine on pH (■, □) and lactate (▲, △) production by strain 1166 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of valine, open symbols its absence.

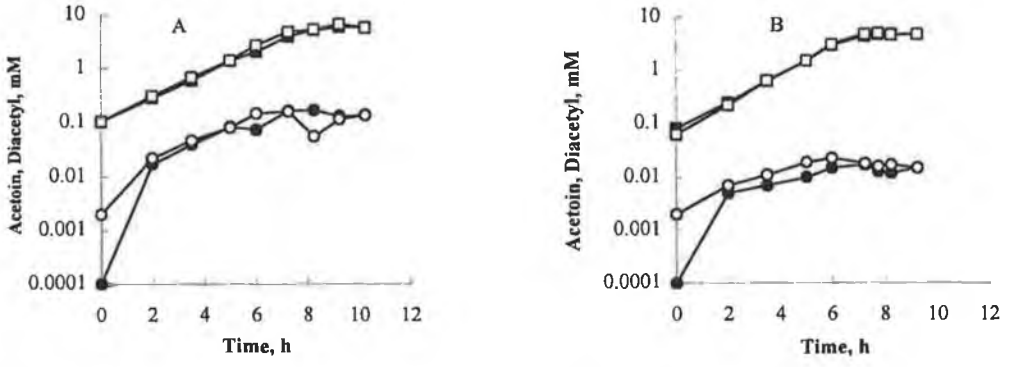

Fig. 23b: Effect of valine on acetoin (■, □) and diacetyl (●, ○) production by strain 1166 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of valine, open symbols its absence.

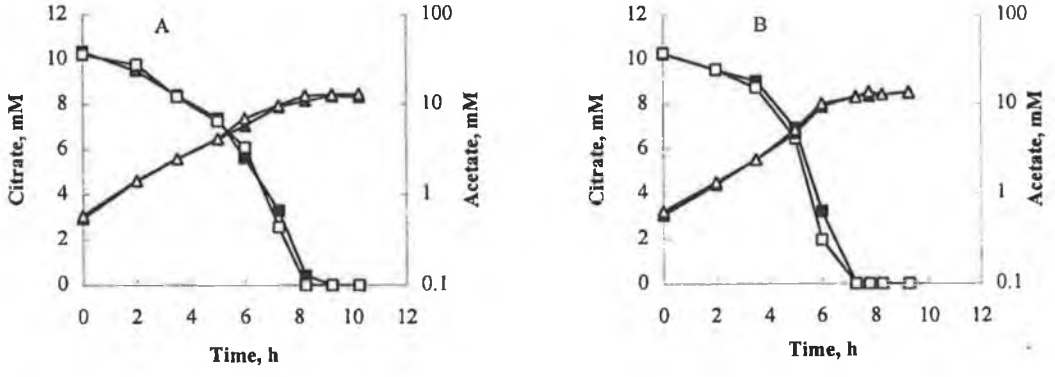

Fig. 23c: Effect of valine on citrate utilisation (■, □) and acetate production (▲, △) by strain 1166 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of valine, open symbols its absence.

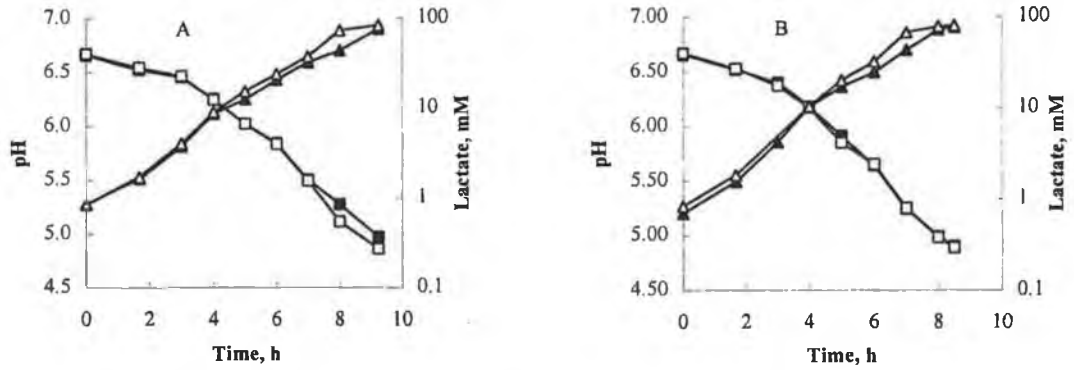

Fig. 24a: Effect of $CuSO_4$ on pH (■, □) and lactate production (▲, △) by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of $CuSO_4$, open symbols its absence.

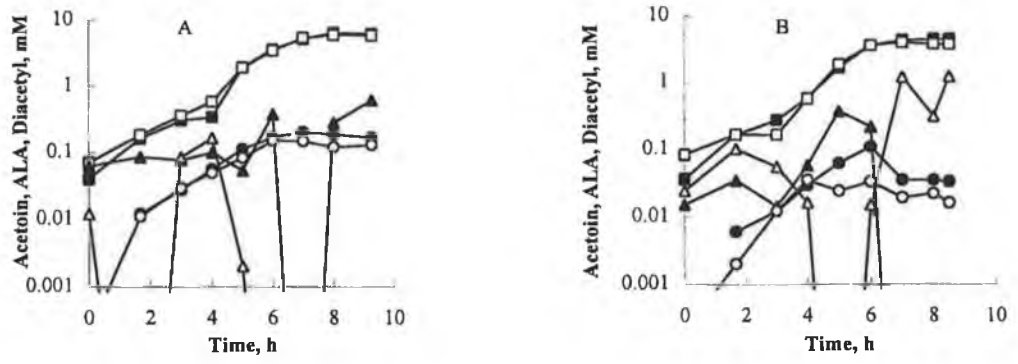

Fig. 24b: Effect of $CuSO_4$ on acetoin (■, □), ALA (▲, △) and diacetyl (●, ○) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of $CuSO_4$, open symbols its absence.

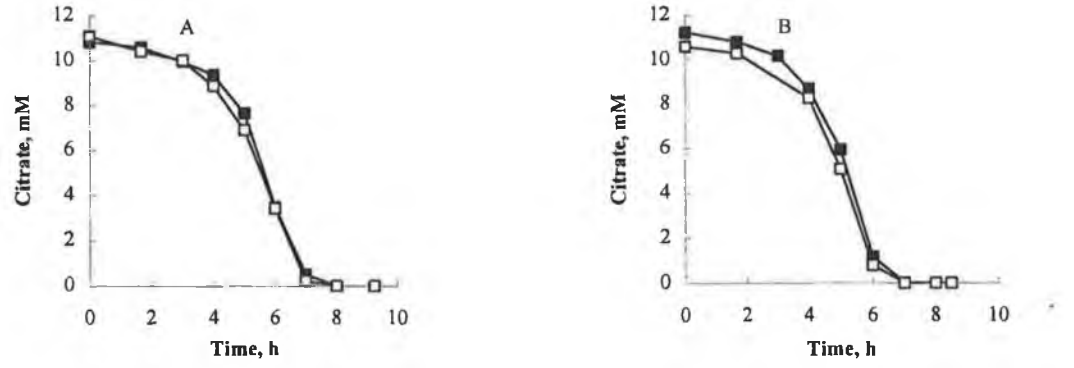

Fig. 24c: Effect of $CuSO_4$ on citrate utilisation (■, □) by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of $CuSO_4$, open symbols its absence.

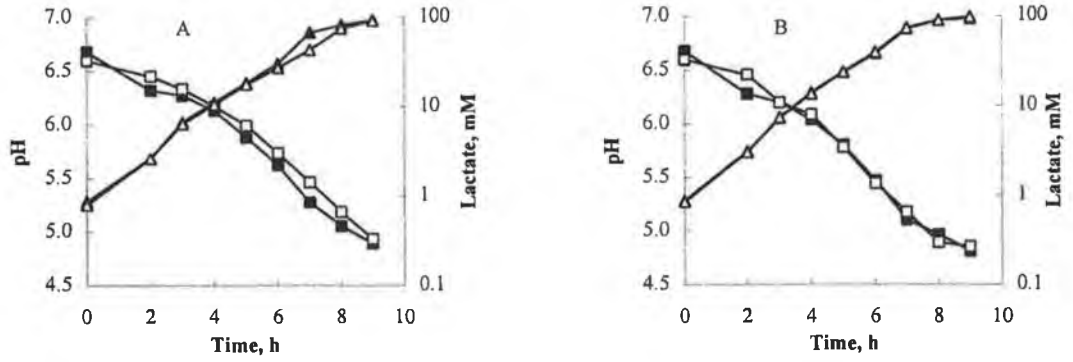

Fig. 25a: Effect of $FeSO_4$ on pH (■, □) and lactate (▲, △) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of $FeSO_4$, open symbols its absence.

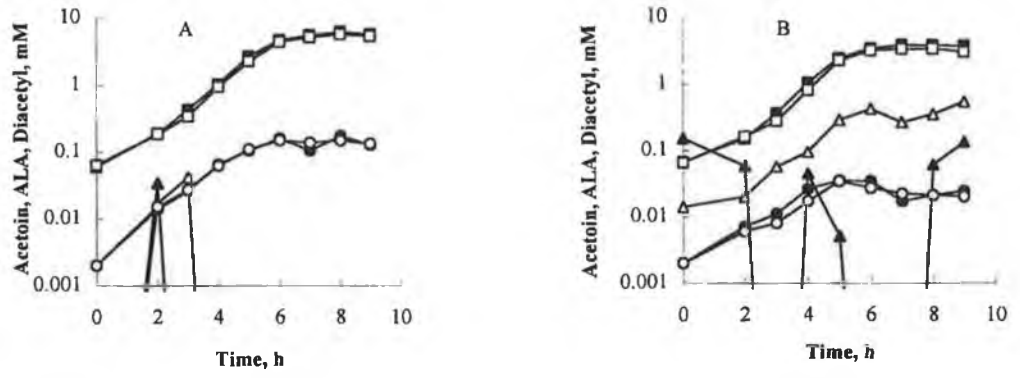

Fig. 25b: Effect of $FeSO_4$ on acetoin (■, □), ALA (▲, △) and diacetyl (●, ○) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of $FeSO_4$, open symbols its absence.

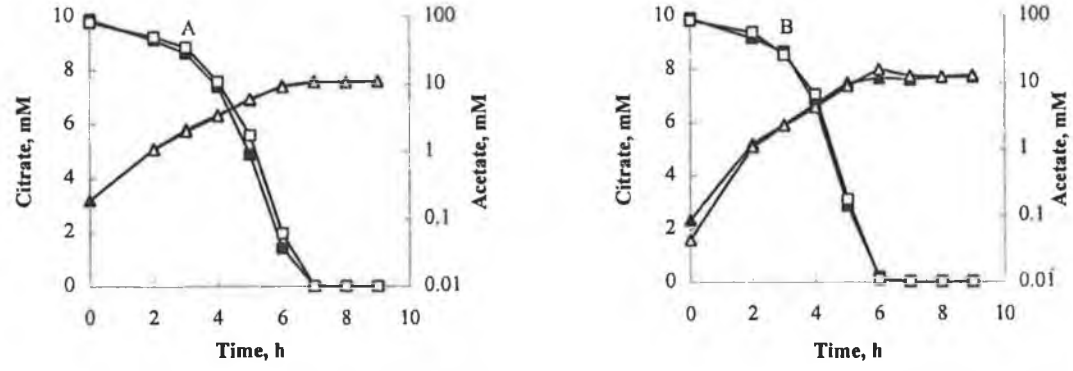

Fig. 25c: Effect of $FeSO_4$ on citrate utilisation (■, □) and acetate production (▲, △) by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of $FeSO_4$, open symbols its absence.

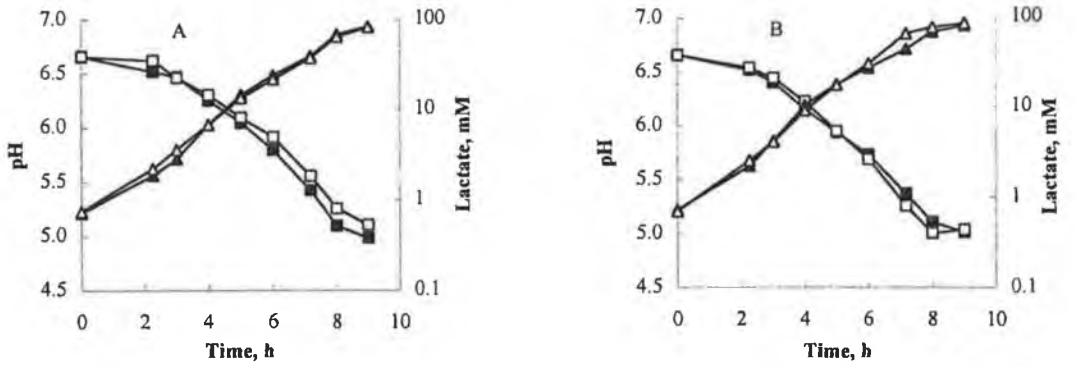

Fig. 26a: Effect of haemin on pH (■, □) and lactate (▲, △) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of haemin, open symbols its absence.

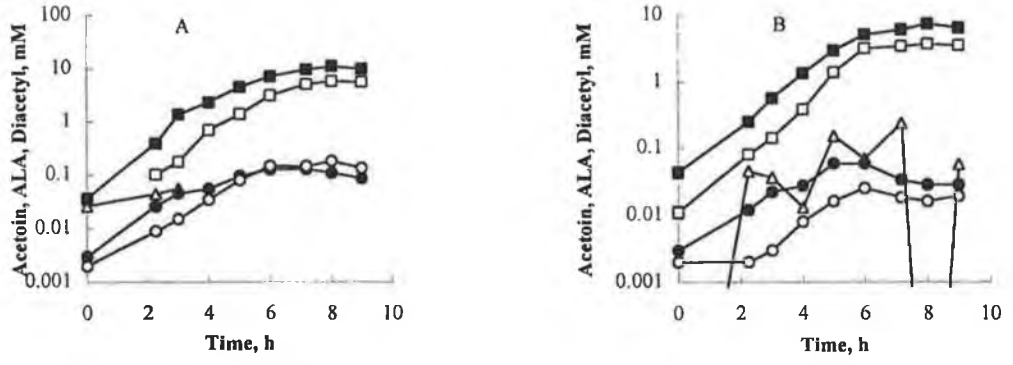

Fig. 26b: Effect of haemin on acetoin (■, □), ALA (▲, △) and diacetyl (●, ○) production by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of haemin, open symbols its absence.

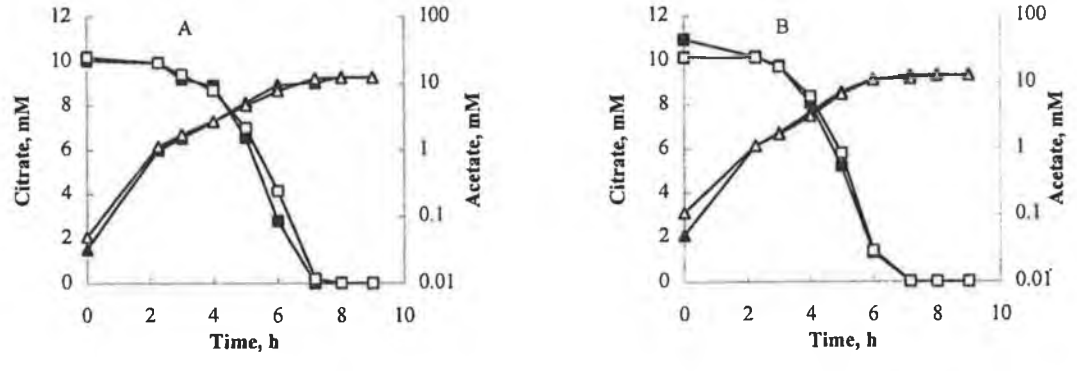

Fig. 26c: Effect of haemin on citrate utilisation (■, □) and acetate production (▲, △) by strain 999 in RSM (10g/L) under oxygenated (A) and non-oxygenated (B) conditions; closed symbols represent the presence of haemin, open symbols its absence.

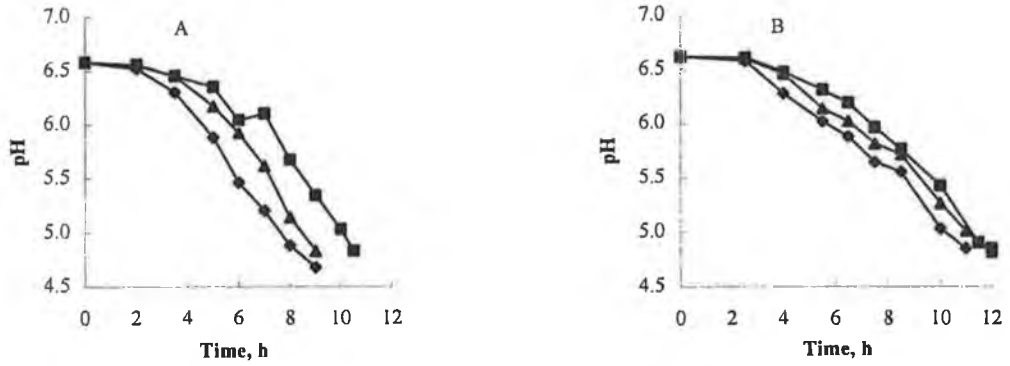

Fig. 27a: Effect of O_2 (■), air (▲) and N_2 (◆) on pH of strain 1166 (A) and 1166M1 (B) in RSM (10g/L).

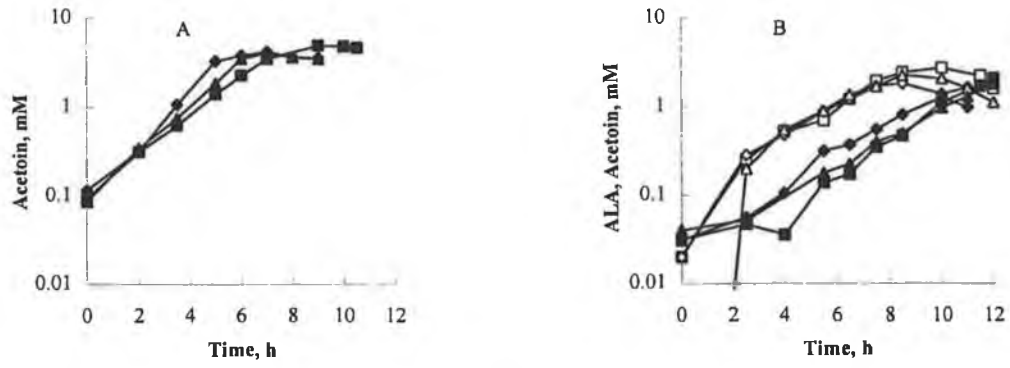

Fig. 27b: Effect of O_2 (■, □), air (▲, △) and N_2 (◆, ◇) on acetoin (closed symbols) and ALA (open symbols) production of strain 1166 (A) and 1166M1 (B) in RSM (10g/L).

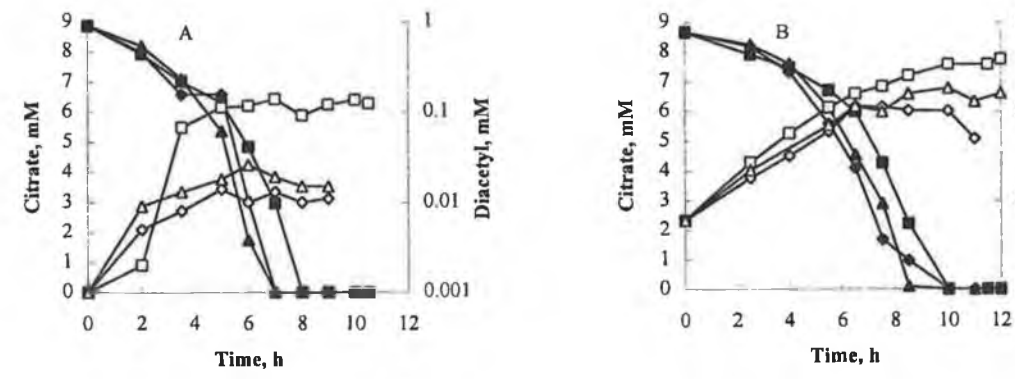

Fig. 27c: Effect of O_2 (■, □), air (▲, △) and N_2 (◆, ◇) on citrate utilisation (closed symbols) and diacetyl production (open symbols) of strain 1166 (A) and 1166M1 (B) in RSM (10g/L).

DISCUSSION

Part I

Diacetyl is an important flavour compound in many fermented dairy products, especially lactic butter, quark, cultured buttermilk and cottage cheese. It is produced from ALA by oxidative decarboxylation; non-oxidative or enzymatic decarboxylation of ALA results in the production of acetoin, which is not important in determining flavour. During the manufacture of lactic butter by the NIZO process, ALA is produced by the strain of Cit^+ *Lc. lactis* subsp. *lactis*, present in mixed culture 4/25. Because ALA is easily decarboxylated in acid solutions, especially in the presence of heat, it can interfere with the determination of acetoin and diacetyl.

Initial experiments in this study were therefore aimed at determining the effect of pH on the decarboxylation of ALA to diacetyl during distillation. Although the reproducibility of the ALA standard curves was very poor, the results (Tables 1 and 2) clearly show that distillation at pH 6.0 results in minimal breakdown of ALA to diacetyl. The rates increase considerably at pH values <6.0 and slightly at pH values >6.5, suggesting that in measuring diacetyl, the pH of samples should be adjusted to pH 6.0 to 6.5 before distillation, to reduce the decarboxylation of ALA to diacetyl. This pH is lower than the pH of 9.0 recommended by Veringa et al. [1984]. These results also contrast with those obtained by Jordan [1987], who stated that during distillation at pH values as low as pH 4.5, <5% breakdown of ALA to diacetyl occurred. The reasons for these contradictory results are not clear, but, in the case of Veringa et al. [1984], could be due to the type of distillation apparatus used. In the present study, ALA standard curves were carried out in the same way and using the same distillation apparatus as in the study of Jordan [1987]. There is no apparent explanation for the differences in breakdown found in Jordan's [1987] and this study. There was no significant difference in the breakdown of ALA to diacetyl between samples made up in milk and samples made up in a mixture of milk and lactic acid concentrate, when both were adjusted to the same pH (Tables 1 and 2),

indicating that the pH is one of the main factors determining the breakdown of ALA. The results also show that the conversion of ALA to diacetyl during distillation is not linear over the concentration range used (0 to 10mM ALA), with greater breakdown occurring at lower ALA concentrations. Breakdown was reasonably linear from 0 to 2mM ALA (Fig. 3). The non-linearity of ALA breakdown does not pose a problem as far as cultures of Cit^+ *Lc. lactis* subsp. *lactis* are concerned, because these cultures produce ALA in concentrations that lie at the lower and, therefore, reasonably linear part of the standard curve. Later in this study, a paper by Cronin and Rispin [1996] was discovered, which reported rates of breakdown of ALA to diacetyl as low as 0.2% after distillation at pH 1.0. Cronin and Rispin's [1996] results were confirmed in this study with breakdown rates of ~1% after distillation at pH 0.8 (Table 9). Therefore, distillation at pH 0.8 is more suitable than distillation at pH 6.0 to 6.5 for the determination of diacetyl in samples containing ALA.

Once the conditions for minimal breakdown of ALA to diacetyl were determined, a model system was set up to monitor the conversion of synthetic ALA to diacetyl and acetoin over time in milk and a mixture of milk and lactic acid concentrate under various conditions. This model system mimicked the situation which occurs in the manufacture of lactic butter, where the mixture of culture and lactic acid concentrate is aerated to enhance the breakdown of ALA to diacetyl. The parameters studied in the model system were the levels of O_2 and milk solids, the temperature, the pH and the addition of metal ions (Fe^{2+} and Cu^{2+}) and haemin. O_2 unexpectedly decreased the amounts of diacetyl produced from ALA, compared to a control which was just stirred. This was due to the oxygenation method used. Trapping the exiting O_2 in water showed that about 30 to 50% of the total amount of diacetyl produced was recovered. Little acetoin was lost through aeration, because 80 to 98% of the ALA added to the system was recovered as diacetyl plus acetoin. As a result of these experiments, it was decided to incorporate O_2 into the medium by stirring alone. This is sufficient for the small volumes used at laboratory scale but at industrial scale, where bigger volumes are involved, aeration by stirring would probably result

in incorporation of insufficient O_2 into the milk, which would then lead to insufficient conversion of ALA to diacetyl.

In the NIZO process for the manufacture of lactic butter, it is recommended that mixed culture 4/25 is grown in milk with a solids concentration of 16g solids/100g. The reason for this is, that higher solids inhibit the growth of the starter culture and therefore the production of ALA [Veringa et al., 1976; Van den Berg, 1991]. Experiments in the model system were set up, to determine if different concentrations of solids affected ALA breakdown. No significant effect on the breakdown of ALA to acetoin and diacetyl was found.

ALA breakdown was a first order reaction and its specific breakdown rate increased as the absolute temperature increased (Fig. 4A). An Arrhenius plot of this data (Fig. 4B) was linear and the activation energy was 19.8kcal/mol, which agrees with the 25kcal/mol found by Monnet et al. [1994c]. The specific rate of ALA breakdown decreased with increasing pH (Fig. 3), which also agrees with the results of Monnet et al. [1994c]. The type of acidulant (i.e. lactic acid or HCl) did not affect the rate of ALA breakdown.

In many cultured dairy foods, the level of diacetyl required to give organoleptically acceptable products is low (1-5mg/kg). Therefore, it is important to be able to detect the level of ALA in a product post-manufacture, in order to determine the potential of the product to develop diacetyl from ALA during storage, particularly where ALD^- mutants, which produce up to 3mM ALA, are used. Monnet et al. [1997] have shown, that the rate of degradation of ALA decreases with decreasing temperature. However, our results (Fig. 18) show that even when quark is stored at 4°C, ALA is spontaneously decarboxylated to diacetyl during storage at a rate of 30-50%. In quark and other dairy products, produced with starter cultures containing Cit^+ *Lc. lactis* subsp. *lactis*, ALA concentrations can be low compared to acetoin concentrations, which makes the determination of ALA by the Jordan and Cogan [1995] method nearly impossible, because the difference between samples treated

with HCl (ALA decarboxylated to acetoin) and samples treated with water (no decarboxylation of ALA) is smaller than the variation between duplicate samples.

To overcome this problem, a new method for the measurement of ALA was developed, in which ALA was oxidatively decarboxylated to diacetyl, rather than to acetoin. Gollop et al. [1987] used a mixture of 0.15mM each of Fe^{2+} and Fe^{3+} in combination with low pH and heat to achieve decarboxylation of ALA to diacetyl. The mechanism of metal ion catalysed formation of diacetyl from ALA is not clear. Gollop et al. [1987] thought that a complex between the enediol, formed after decarboxylation of the ALA and a metal ion-O_2 complex was involved. Initial experiments with the method of Gollop et al. [1987] did not result in a satisfactory conversion of ALA to diacetyl. This could have been due to the use of steam distillation rather than 'air' distillation as recommended by Gollop et al. [1987]. The results with the model system (Table 7) showed that Cu^{2+} was a better 'oxidiser' of ALA than Fe^{2+}. Distilling a sample containing ALA in the presence of 1.5mM Cu^{2+} at pH 3.5 resulted in 100% conversion of ALA to diacetyl (Table 9), and these results provided the basis for the new method for measuring ALA. At pH values <3.0 and >4.0, significant reductions in the amounts of diacetyl produced from ALA were found. Thus the pH at which the samples are distilled is quite important and the use of a citric acid/phosphate buffer gave the same result as adjusting the pH to 3.5 with H_2SO_4. Use of the buffer is more desirable in practice because it will ensure better control of the pH. Substituting 1.5mM each of Fe^{2+}/Fe^{3+} for the copper, resulted in ~100% breakdown of ALA to diacetyl also. This result shows that the type of metal ion is not important as long as the other conditions (heat, low pH, metal ion concentration) are optimal for ALA breakdown. By distilling a sample at pH 0.8 in the absence of Cu^{2+}, the true level of diacetyl in the sample can be determined. The difference in the sample in which all the ALA is converted to diacetyl and the true diacetyl level, is the ALA concentration. The $CuSO_4$ method overestimated ALA by 5.7% compared to the Jordan and Cogan [1995] method. However, due to the limitations of the latter method, this difference is considered not to be significant. The reason that the $CuSO_4$ method for measuring ALA is superior to those methods based on conversion of ALA to acetoin, is due to the small

amounts of diacetyl (<0.06mM) relative to the large amounts of acetoin (~4mM) produced by Cit^+ *Lc. lactis* subsp. *lactis*.

Trials were carried out in three commercial plants producing butter according to the NIZO process. Either low levels or no ALA were detected before addition of the lactic acid concentrate, probably due to the high levels of acetoin present in the samples. These analyses were carried out by the Jordan and Cogan [1995] method. When the lactic acid concentrate was added, ALA was detected, but no ALA breakdown occurred and, therefore, no diacetyl was produced during aeration (Fig. 12). This could be due to the low temperature of the mixture of starter and lactic acid concentrate in all three plants. Citrate was not used completely before addition of the lactic acid concentrate where milk with solids concentrations >17g/100g was used (Table 12). It is recommended by Van den Berg [1991] to use milk with 16g solids/100g to grow the 4/25 starter culture, since higher milk solids inhibit its growth. Therefore, laboratory scale trials were carried out, in which the growth of the culture in RSM containing 16, 19 and 23g solids/100g at 21°C was compared (Fig. 13a, b, c). As expected, the rate of pH decrease was slower in the milk with higher solids levels, probably due to higher buffering capacities. Surprisingly, ALA and acetoin production were not affected by the milk solids level to any great extent. One would have expected that increased levels of both compounds would be produced in the milk with the higher solids levels because of the increased levels of citrate. The reason why this does not happen is unclear. Diacetyl production was slightly greater at the highest level of solids. This may be due to higher levels of ions which could decarboxylate ALA.

Breakdown of ALA increases with increasing temperature (Fig. 4). Laboratory trials were carried out, to determine if an increase in temperature of the mixture of culture 4/25 and lactic acid concentrate would increase ALA breakdown and, therefore, diacetyl production. As expected, there was no ALA breakdown at 11°C (Fig. 14), which is close to the temperature at which commercial plants aerate the mixture of starter culture and lactic acid concentrate. ALA breakdown occurred at 23°C but was greater at 30°C. Similar results were obtained for diacetyl. Regression analysis of

diacetyl on ALA showed good correlation coefficients (Fig. 15). Acetoin levels were unaffected by the increase in temperature.

Due to these results, further industrial trials were carried out, in which the temperature at which the mixture of culture and lactic acid concentrate was aerated was raised to 30 to 37°C. As expected, this increase in temperature accelerated ALA breakdown and consequent diacetyl production; little acetoin was produced from ALA at the higher temperatures (Fig. 16) The conversion rates of ALA to diacetyl (~60%) corresponded well to the results obtained in the laboratory trials at 30°C (Fig. 15 + 17).

Part II

Bassit et al. [1993] showed that growing Cit^+ *Lc. lactis* subsp. *lactis* under oxygenated conditions increased the level of diacetyl produced. This was confirmed for all 134 strains of Cit^+ *Lc. lactis* subsp. *lactis* in the present study (Table 15). Two strains, 999 and 1166, were selected for more detailed study, because, under oxygenated conditions, they produced twice the amount of diacetyl as strain 4/25A. The latter strain was chosen as the reference strain, because it is a natural ALD^- mutant, and produces high amounts of ALA, which cannot be enzymatically converted to acetoin but which can be oxidatively decarboxylated to diacetyl under the right conditions.

In Cit^+ *Lc. lactis* subsp. *lactis*, ALD is positively controlled by the three branched-chain amino acids, leucine, valine and isoleucine, implying that in the presence of any of these amino acids, more acetoin would be produced than in their absence [Monnet et al., 1994a]. However, with strains 999 and 1166 there was no significant difference in the amount of acetoin or diacetyl produced in the presence of leucine or valine under either oxygenated or non-oxygenated conditions (Fig. 20, 21, 22, 23). The levels of leucine and valine used were ~2500 times higher than the levels present in the milk. In this part of the study the determination of ALA was a problem

occasionally, because the method used to detect ALA [Jordan and Cogan, 1995] often resulted in negative values. In this method, ALA is detected as the difference in the total level of acetoin determined after decarboxylation with HCl and the amount of 'free' acetoin. Negative values can be obtained if low levels of ALA and high levels of acetoin are present.

Kaneko et al. [1987, 1990] reported, that growing Cit^+ *Lc. lactis* subsp. *lactis* in RSM in the presence of metal ions (Cu^{2+}, Fe^{2+}, Fe^{3+} and Mo^{6+}) or haemin increased the production of diacetyl during growth, with Cu^{2+} being the most effective. The results in the present study show that synthetic ALA is converted to diacetyl during distillation, when metal ions (Cu^{2+}, Fe^{2+}) or haemin are present in the sample, with haemin and Cu^{2+} being more effective than Fe^{2+}. This suggests that the results of Kaneko et al. [1987, 1990] could be due to an artefact of the method of analysis used by them, i.e. head space gas chromatography after heating to 80°C for 30min. The diacetyl that was measured could have been produced from ALA during measurement, rather than show a true effect of the metal ions and haemin on product formation. Unfortunately, ALA was not measured in the studies of Kaneko et al. [1987, 1990]. In the present study, Cu^{2+}, Fe^{2+} and haemin did not significantly increase diacetyl production further in the oxygenated cultures; whereas in the non-oxygenated cultures, diacetyl production was increased to some extent but only in the presence of haemin (Fig. 24, 25, 26). This increase was genuine and not caused by distillation in the presence of haemin, because no ALA, which could be converted to diacetyl, was detected in the cultures grown in the presence of haemin.

The effect of different O_2 levels (0%, 21% and 100%) on ALA, acetoin and diacetyl production by Cit^+ *Lc. lactis* subsp. *lactis* strains 1166 and 1166M1, its ALD^- mutant, was studied (Fig. 27). ALD^- mutants should produce more ALA and diacetyl at the expense of acetoin, since the enzymatic decarboxylation of ALA to acetoin is prevented. The mutant decreased the pH at a slower rate than the parent, possibly because its acid producing ability was partially damaged by the procedure used to create the mutant [Monnet et al. 1997]. No ALA was produced by the parent strain, whereas the mutant, as expected, produced high amounts of ALA. Less

acetoin was produced by the mutant than by the parent. The acetoin produced by the mutant cannot be due to ALD activity and must therefore be due to chemical decarboxylation. Increasing O_2 concentrations inhibited the growth of both the parent and the mutant, as indicated by the lower rates of pH decrease, and, therefore, also slowed down citrate utilisation. ALA production was unaffected by increasing O_2 levels. The conversion of ALA to diacetyl was promoted by increasing O_2 concentrations in both the parent and the mutant, but the increase was bigger in the parent than in the mutant. Acetoin production was unaffected by different levels of O_2.

In conclusion, it has been shown that breakdown of ALA to acetoin and particularly to diacetyl can be influenced by pH, temperature, oxidising agents and heat and that it is a first order reaction. These results were used to develop a new method for the detection of ALA, in which the combined effects of low pH, high temperature and Cu^{2+} were used to obtain maximum breakdown of ALA to diacetyl. It was also found that, under the conditions used commercially for the production of lactic butter, no breakdown of ALA occurred and no diacetyl was produced. Increasing the temperature during the aeration of the mixture of starter culture and lactic acid concentrate to ~35°C was shown to increase diacetyl production and reduce ALA concentrations in the finished product to zero. Experiments with quark showed considerable breakdown of ALA to diacetyl during storage at 4°C and, therefore, possible undesirable changes in flavour during storage. Growth of Cit^+ *Lc. lactis* subsp. *lactis* strains was not significantly influenced by the addition of leucine, valine, Cu^{2+}, Fe^{2+} or haemin, whereas O_2 increased diacetyl production. Studies with an ALD^- mutant of 1166 showed the expected increase in ALA and diacetyl production.

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