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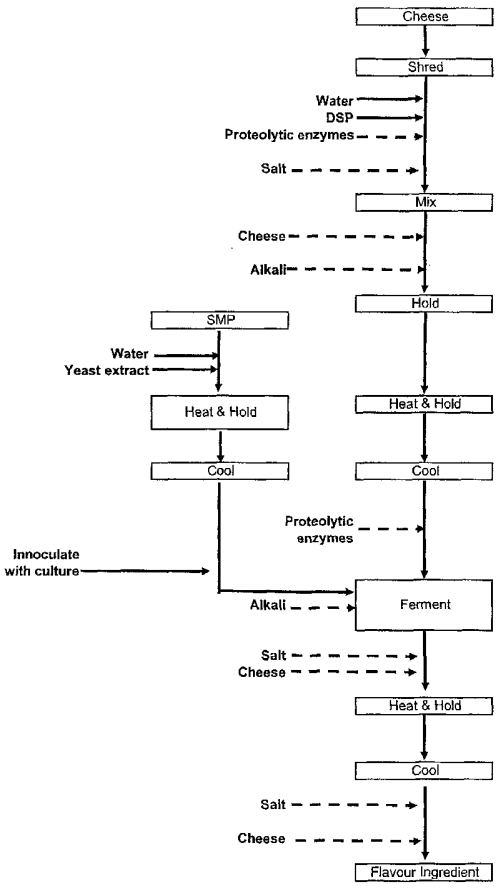

(57) Abstract: The present invention relates to a method of manufacturing a cheese flavour ingredient and an ingredient so prepared. The method comprises subjecting a protein in water mixture to proteolysis by a protease enzyme and to fermentation with a physiologically acceptable bacterium of one of the genera Enterococcus, Staphylococcus or Pseudomonas capable of producing a cheese flavour ingredient. The cheese flavour ingredient of the invention has a mixture of flavours and may be used as a component of balanced cheese-like flavour compositions. The present invention also relates to bacterial strains useful in the preparation of such an ingredient, these being Enterococcus faecium, Enterococcus faecalis, Enterococcus casselifavus, Staphylococcus simulans and Pseudomonas putida

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Cheese Flavour Ingredient and Method of its Production

Technical Field

This invention relates to a method of manufacturing a cheese flavour ingredient and an ingredient so prepared. It is also relates to bacterial strains useful in the preparation of such an ingredient.

Background to the Invention

The expression "cheese flavour ingredient" means an ingredient from a protein source that is a component of a mixture of ingredients that have a cheese flavour. The cheese flavour ingredient may or may not itself have a cheese flavour.

Cheese is a biologically dynamic food in which flavour develops as a result of the activity of the enzymes and cultures that occur in the cheese. Flavour develops in the cheese as the enzymes and cultures trapped in the curd act on the milk substrates - caseins, fats, and residual lactose (Crow *et al,* 1993). The initial ripening reactions are hydrolytic, generating peptides and amino acids from milk caseins by the action of proteases and free fatty acids from milk fat by the action of esterases and lipases. The later reactions during cheese ripening are more complex. Amino acids, free fatty acids and lactate are the primary substrates and the flavour reactions involve, amongst other transformations, the dynamic interaction of deamination reactions, transferase reactions, and synthetic reactions (McSweeny *et al*, 2000).

Often cheese flavours take months and years to develop. Flavour development can be greatly accelerated using an enzyme modified cheese (EMC) process. In an EMC process, selected enzymes (proteases and lipases) are added to a cheese curd that is typically slurried with water. Intense flavours develop over a period of hours to days (Kilkawley *et al*, 1998). An EMC process is described in US patent 3,765,905. EMC processes often involve both proteolysis of dairy proteins and lipolysis of dairy fats. Examples of such EMC processes are disclosed in international patent application WO 99/63834, US patent 5,455,051 and European patent application EP 1 053 689.

However, often these flavour enhancing processes amplify particular groups of flavourful compounds, such as short chain fatty acids, and peptides of a particular molecular weight and

as a consequence, the products of these EMC processes lack the balanced flavour profile of a cheese that has been ripened naturally.

Object of the Invention

It is an object of this invention to go some way towards overcoming this disadvantage or at least to offer the public a useful choice.

Summary of the Invention

Accordingly, the invention may be said broadly to consist in a process for the manufacture of a cheese flavour ingredient which comprises the steps of:

(a) forming a protein in water mixture,

(b) adding a protease enzyme to said mixture to establish a proteolysis reaction,

(c) adding a bacterial culture to said mixture to establish a fermentation reaction, said bacterial culture comprising a physiologically acceptable bacterium capable of producing a cheese flavour ingredient selected from the group comprising *Enterococcus, Staphylococcus* and *Pseudomonas* bacteria,

(d) maintaining said mixture under microaerophilic or anaerobic conditions at a temperature within the range of about 20-50°C at a pH within the range of about 5.0 to 8.0 for a time period of from about 20 to 100 hours, and

(e) terminating said reactions and recovering the cheese flavour ingredient produced.

Preferably, said protein comprises a dairy protein. More preferably, said dairy protein comprises casein. Preferably said mixture comprises a fat, protein and water emulsion. In a preferred embodiment said mixture comprises cheese and water.

Alternatively, said mixture comprises on or more of cheese curd, ripened cheese, mature cheese, milk solids, reconstituted whole milk powder, milk protein concentrate, a casein, a milk protein or a non-dairy protein, milk fat or a non-dairy oil or fat.

Preferably said mixture comprises total solids in the range of 5% (w/w) to 50% (w/w) of the emulsion.

Preferably, said protease enzyme is a peptidase or a proteinase or a combination of a peptidase and a proteinase.

In one embodiment, said proteinase is protease A "Amano" (Amano Enzymes) and said peptidase is Neutrase (Novo Nordisk).

In another embodiment said proteinase is Flavorpro 192P (Biocatalysts) and said peptidase is Promod 215P (Biocatalysts).

In one embodiment the method comprises maintaining the emulsion at a temperature within the range of 20-60°C to continue said proteolysis reaction before beginning step (c). Preferably, said proteolysis reaction is conducted at a temperature of from 40-50°C. Most preferably, said proteolysis reaction is conducted at a temperature of 43°C.

In one embodiment said proteolysis reaction is conducted for some 2 to 24 hours before beginning step (c). Most preferably, said proteolysis reaction is conducted for about 4 hours.

In one embodiment said proteolysis reaction is terminated by heating the reaction mixture to 80-100°C for from 3 to 30 minutes before beginning step (c). Most preferably, said proteolysis reaction is terminated by raising the temperature of the reaction mixture to about 93°C for about 15 minutes.

In one embodiment, said mixture is cooled after being heated to terminate said proteolysis reaction.

Preferably, further protease is added after said cooling step.

In one embodiment steps (b) and (c) are allowed to proceed at the same time.

In one embodiment, said bacterium is *Enterococcus faecium* B9642, AGAL accession number NM01/24754.

In another embodiment said bacterium is *Enterococcus faecium* B9645, AGAL accession number NM01/24755.

In another embodiment said bacterium is *Enterococcus faecaelis* B9509, AGAL accession number NM01/24757.

In another embodiment said bacterium is *Enterococcus casseliflavus* B9518, AGAL accession number NM01/24753.

In another embodiment said bacterium is *Staphylococcus simulans* B9646, AGAL accession number NM01/24756.

In another embodiment said bacterium is *Pseudomonas putida* B9647, AGAL accession number NM01/24752.

Preferably, said fermentation reaction is conducted at a temperature of from 30 to 45°C.

Most preferably, said fermentation reaction is conducted at a temperature of 40°C.

Preferably, said fermentation reaction is conducted at a pH of from 6.3 to 6.5.

Preferably, said fermentation reaction is conducted for from 30 to 72 hours.

More preferably, said fermentation reaction is conducted for between about 50 and about 65 hours. Most preferably, said fermentation reaction is conducted for about 53 hours or about 64 hours.

In one embodiment cheese and/or salt may be added during formation of the mixture, where required, to provide a desired solid contents and salt level in any final product. In another embodiment additional cheese and salt may be added immediately before said fermentation reaction is terminated. In another embodiment, cheese and/or salt may be added part way through said fermentation reaction.

Preferably, said fermentation reaction is terminated by heating to a range of 80°C to 100°C and holding for a time of 3 to 30 minutes.

Most preferably, said fermentation reaction is terminated by heating to a temperature of about 93°C and holding for a time of about 15 minutes.

In one embodiment salt is added to said reaction mixture after said fermentation reaction has been terminated.

In another embodiment, cheese is added to said reaction mixture after said fermentation reaction has been terminated.

In one embodiment, the cheese flavour ingredient produced by said fermentation reaction is dried.

Preferably, said ingredient is dried by spray drying.

One embodiment of the invention comprises a cheese flavour ingredient produced by the method of the invention. Another embodiment of the invention comprises a food product comprising a cheese flavour ingredient of the invention. Preferably said food product comprises a product selected from the group comprising natural cheese, processed cheese, analogue cheese, foods comprising natural cheese, processed cheese or analogue cheese, sauces, dips, snacks, biscuits, soups, pizza, and cheese and savoury flavoured foods.

The invention may also be said broadly to consist in the bacterial strain *Enterococcus faecium* B9642, AGAL accession number NM01/24754.

Alternatively, the invention consists in the bacterial strain *Enterococcus faecium* B9645, AGAL accession number NM01/24755.

In another embodiment said bacterium is *Enterococcus faecaelis* B9509, AGAL accession number NM01/24757.

In another embodiment said bacterium is *Enterococcus casseliflavus* B9518, AGAL accession number NM01/24753.

In another embodiment said bacterium is *Staphylococcus simulans* B9646, AGAL accession number NM01/24756.

In another embodiment said bacterium is *Pseudomonas putida* B9647, AGAL accession number NM01/24752.

Another embodiment of the invention comprises a food product comprising a biologically pure culture of the invention.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

Brief Description of the Figure

The invention may also be more fully understood by having reference to the accompanying drawing wherein:

Figure 1 is a flow diagram of a preferred embodiment of the invention.

Detailed Description of Preferred Embodiments

In the preferred embodiment a protein in water mixture is prepared. Preferably, the protein is a dairy protein source such as cheese. Preferably a cheese, such as a Cheddar or Gouda, is shredded and water is added together with an emulsifying agent such as disodium phosphate (DSP) to form an emulsion. Preferably, the total solids in the mixture range between 5% (w/w) to 50% (w/w).

The preferred emulsifying salt is disodium phosphate (DSP). It is added until its concentration is up to 5% w/w. A preferred concentration is around 1.2% w/w. Other emulsifying food grade salts such as other phosphates and citrate salts may be used.

In addition, food grade emulsifying gums or other food grade emulsifying compounds may be used to assist in the formation of the emulsion.

If necessary, the pH of the mixture may be adjusted to within a range of pH 6.3 to 6.5 by adding a food grade base (alkali).

The reaction mixture is preferably heated to inactivate microbes in the mixture. A preferred temperature is about 93°C for approximately 15 minutes. The total time elapsed from initiating the process to this first heating step is preferably about 60 minutes to ensure adequate mixing and pH stabilisation.

The mixture is preferably then cooled to about 40°C.

The protease enzymes may be added before or after the heating step. In a preferred embodiment, about 0.1% (w/w) of the protease enzymes are added to the mixture. If the protease enzymes are added before the heating step then preferably the heating step also serves to terminate proteolysis.

The preferred protease enzymes which are added to the mixture are a blend of proteinase and peptidase enzymes. Protease A "Amano" (Amano Enzymes) and Neutrase (Novo Nordisk) are exemplary of a blend which can be employed. Another blend is Flavorpro 192P (Biocatalysts) protease and Promod 215P (Biocatalysts) peptidase.

In one embodiment, once the proteolytic enzymes are incorporated, the mixture is preferably held at a reaction temperature of about 20 to 60°C, preferably about 40°C, for about at least 10 minutes until the mixture is readily stirrable.

In another embodiment the proteolysis reaction and the fermentation reaction are allowed to proceed at the same time.

A bacterial culture of *Enterococcus faecium* B9642 (as defined below) is added to the mixture and cultured with minimal agitation, to minimise air entrapment. Strict anaerobic conditions are not required as the metabolic activity of the added enterococcal culture will ensure that dissolved oxygen levels are minimal during the course of the fermentation. However, the reaction is carried out under microaerophilic or anaerobic conditions.

A fermentation reaction is thereby established. The temperature is maintained at approximately 20 to 50°C, preferably about 40°C. If necessary the pH is maintained within a

range of 6.3 to 6.5 by adding a food grade base. This may be done continuously or at intervals up to about 12 hours.

The fermentation reaction is continued for 20 to 100 hours until the desired ingredient has been produced. A preferred fermentation time is about 50 to 65 hours, about 50 to 54 hours or about 60 to 64 hours.

In some cases it is desirable to adjust the solids content of the fermentation mixture by adding additional starting material, such as grated cheese prior to terminating the fermentation. Salt may be added at the same time or at any other time during the fermentation.

The fermentation step is terminated by heating the mixture. In one embodiment the proteolysis reaction is also terminated at this point. A preferred temperature is 93°C for approximately 15 minutes to inactivate the culture and its enzymes. Salt content is adjusted to give a final concentration of 5% in the aqueous phase. The solids content may be adjusted when desired by the addition of grated cheese.

The flavour ingredient is in the deactivated reaction mixture. This may be used directly or it may be dried by spray drying, for example, for further use.

The culture which is added to the ferment in the flow diagram of Figure 1 is preferably made up as follows. Skim milk powder (SMP) is reconstituted as a 10% aqueous solution to which 0.1% (w/w) yeast extract is added. The medium is then sterilised by heating to preferably 120°C for typically 15 minutes.

The medium is then cooled to 30 to 45°C prior to inoculation. A culture of the bacterium *Enterococcus faecium* B9642 is added under sterile inoculation conditions. The inoculated medium is incubated for 20 to 24 hours to allow for culture growth. During growth of the culture the pH is adjusted to maximise cell densities above 10^9/ml. The prepared culture may either be stored frozen at -20°C or stored chilled at 5°C until required, or added immediately to the fermentation vessel at typically 4% (w/w) inoculation level to initiate the fermentation. Other means known in the art of producing a culture are also useful herein.

The flavour ingredient recovered after completion of the fermentation has a concentrated mixture of flavours (derived chiefly from the products of amino acid fermentations). A panel comprising practitioners experienced in assessing the taste attributes of cheeses and cheese

flavour concentrates was used to assess the product diluted in a bland white sauce. The white sauce was made from mixing and heating 125g of sauce powder (5g salt, 120g standard white flour and 500g cream powder) blended with 450 g of water. The ingredient was usually diluted in the range of 1 to 20%, typically at 10%, to produce strong flavours that enable batch-to-batch comparison. The flavour was described as having a strong smear flavour, often described as a dirty socks flavour. The other main flavours described were cooked, salty, savoury flavours with sulphury notes. The flavour of the ingredient was compared with two other commercial ingredient flavour concentrates in different dilutions in the white sauce. The ingredient was found to be comparable to, but to have a higher potency than the two commercial concentrates derived by the forced maturation of smear ripened cheese curd.

The flavour ingredient can be used in a range of food applications either on its own or in combination with other flavouring agents. In general terms the ingredient is diluted sufficiently so that the smear flavour note is not detected but rather the ingredient provides flavour balance and enhances the impact of other flavour components. In most applications the flavour ingredient is used in the range of 0.1% to 2%. It may be used to enhance the flavour of natural cheese, processed cheese and analogue cheese foods. It can be used in sauces and dips, for example in a fondue recipe (14% Cheddar cheese, 23% cream, 47% milk, 9% butter, 4% flour, 2% other flavours) the 1% addition of the flavour ingredient improves the savoury and cheesy taste. It can be used in other foods requiring a cheese like flavour component such as snacks, biscuits, soups, pizza or other cheese or savoury flavoured food.

The bacterium used in the fermentation step according to the invention is of one of the genera *Enterococcus, Staphylococcus* and *Pseudomonas*.

Enterococcus is the preferred genus for this fermentation. The genus *Enterococcus* was proposed originally for gram positive diplococci of intestinal origin (Schleifer and Kilpper-Balz, 1984). The genus can be distinguished from other gram-positive, catalase-negative cocci by their ability to grow between 10°C and 45°C, in 6.5% NaCl, at high pH (pH 9.6) (Hardie *et al*, 1997). While some strains of *Enterococcus* have been associated with opportunistic secondary infections in humans (Franz *et. al.,* 1999), these are of a type not found in foods. In fact, several strains are used as probiotics to confer health benefits when they are consumed and many strains are used for the positive contribution they make to food fermentations that include cheeses and other fermented milk products (Franz *et al*, 1999).

Enterococci occur in a number of cheese varieties but are particularly associated with the cheese produced in southern Europe. They may occur in numbers ranging from 10^4 - 10^6 CFU/g in some ripened cheeses, including Emmental. The predominant enterococcal isolates from cheese are *E. faecalis* and *E. faecium*. The beneficial contribution of *Enterococci* to flavour in cheese has been attributed to the metabolic activity of the cultures, particularly the spectrum of proteolytic enzymes (Centeno *et al*, 1996) and esterase enzymes (Tsakalidou *et al*, 1993) and the wide ranging aromatic flavour compounds produced by the cultures.

Enterococcus faecium B9642 is a preferred bacterial strain. It is stored in the culture collection of the Dairy Research Institute, Palmerston North, New Zealand as culture number B9642. It is also deposited in the culture collection of the Australian Government Analytical Laboratory (AGAL), 1 Suakin Street, Pymbal, NSW 2073, Australia as accession number NM01/24754.

Enterococci are Gram-positive, catalase negative cocci mostly in pairs or chains. They produce lactic acid as the major end product of fermentation and do not form gas. Enterococci can be distinguished from other streptococci by their ability to grow at 10 and 45°C, grow in 6.5% sodium chloride, grow at pH 9.6 and to survive heating at 60°C for 30 minutes. They posses group D antigen (Lancefield serological typing scheme).

Further characteristics of the *Enterococcus* strains used in this invention are set out below in Table 1.

Table 1. Preferred *Enterococcus* strains have the following characteristics.

Test		*E. faecium* B9642	*E. faecium* B9645	*E. faecalis* B9509	*E. casseliflavus* B9518
Microscopic appearance		oval cocci	oval cocci	oval cocci	oval cocci
Gram stain		+	+	+	+
Catalase		-	-	-	-
Growth at:	10°C	+	+	+	+
	45°C	+	+	+	+
	55°C	-	-	-	-
NH_3 from arginine		+	+	+	nd*
Acid from:	L-Arabinose	±	±	-	+
	D-Arabinose	-	-	-	-
	Glucose	+	+	+	+

Lactose	+	+	+	+
Maltose	+	+	+	+
Mannose	+	+	+	+
Melezitose	+	+	+	±
Melibiose	-	-	+	+
Raffinose	-	-	+	+
Ribose	+	+	+	+
Sorbitol	-	-	+	+
Sucrose	+	+	+	-
Trehalose	+	+	+	+

* nd = not determined

A culture of *Staphylococcus* has also been used in this invention. Staphylococci are Gram-positive, catalase positive, facultative anaerobic, non-motile cocci, 0.5 – 1.0 μm in diameter, dividing in more than one plane to form pairs and clusters. Staphylococci can be distinguished from other micrococcaceae by their ability to grow anaerobically and being oxidase negative. The major end product of fermentation anaerobically is lactate. Characteristics of the *S. simulans* B9646 strain are set out in Table 2 below.

Table 2. Characteristics of *S. simulans* B9646.

Test		***S. simulans* B9646**
Microscopic appearance		Cocci
Gram stain		+
Catalase		+
Growth at:	10°C	-
	15°C	+
	45°C	+
	55°C	-
NH_3 from arginine		+
Acid from:	Fructose	+
	Glucose	+
	Lactose	+
	Maltose	-
	Mannitol	+
	Mannose	+
	Raffinose	-
	Ribose	-
	Sucrose	-
	Trehalose	+
	Turanose	-

A culture of *Pseudomonas* has also been used in this invention. Pseudomonads are Gram-negative, catalase positive, usually oxidase positive, straight or curved rods motile by polar flagella. Their metabolism is respiratory, never fermentative, using oxygen as the terminal electron acceptor. Species in the genus *Pseudomonas* can be distinguished from other members of the pseudomonadaceae family because they do not require growth factors, do not grow at pH 3.6, do not form flocks with dendritic outgrowths and do not produce xanthomonadins. The characteristics of *P. putida* 9647 are set out in Table 3 below.

Table 3. Characteristics of *P. putida* 9647.

Test		*P. putida* B9647
Microscopic appearance		rod
Gram stain		-
Oxidase		+
Growth at:	4°C	+
	37°C	+
	45°C	-
NH_3 from arginine		+
Acid from:	D-Arabinose	-
	Citrate	+
	Fructose	+
	Gluconate	+
	Glucose	+
	Maltose	+
	Mannitol	+
	Mannose	+
	Rhamnose	-
	Sucrose	-
	Trehalose	-

The deposit details for the strains in each of Tables 1 to 3 are set out in Table 4.

Table 4. Bacterial Strains and Deposit Details.

Strain	AGAL Accession No.	Date
Enterococcus faecium B9642	NM01/24754	23/11/2001
Enterococcus faecium B9645	NM01/24755	23/11/2001
Enterococcus faecalis B9509	NM01/24757	23/11/2001
Enterococcus casseliflavus B9518	NM01/24753	23/11/2001
Staphylococcus simulans B9646	NM01/24756	23/11/2001
Pseudomonas putida B9647	NM01/24752	23/11/2001

The invention consists in the foregoing and also envisages constructions of which the following gives examples.

Example 1

A protein in water mixture comprising a cheddar cheese was formed and then incubated with protease enzymes in a first incubation and then incubated with protease enzymes and a culture of *Enterococcus faecium* B9642 in a second incubation.

Ingredients for the first incubation: 500 kg of grated commercial New Zealand Cheddar cheese; 12 kg of food grade disodium phosphate dissolved in 50 L hot water; 600 g proteolytic enzymes (protease A "Amano" and Neutrase in equal amounts) dissolved in 10 L water at 43°C; 250L water at 43 to 45°C; 50% (w/w) solution of food grade NaOH.

Ingredients for the second incubation: 400 kg grated Cheddar cheese; 600 g proteolytic enzymes (protease A "Amano" and Neutrase in equal amounts) dissolved in 5 L sterile water; 26kg of *Enterococcus faecium* B9642 culture; 50% (w/w) solution of food grade NaOH; 19.5 kg cheese salt.

First incubation:

1. The water was placed in a tank at a temperature of about 53°C so addition of the cold cheese would bring it down to about 43°C. The dissolved disodium phosphate was

added and mixed into the water. Approximately half the grated cheese was added while stirring over about 15 to 20 minutes.

2. Freshly made protease solution was added, the mixture was stirred for about 5 minutes and then the remaining grated cheese added over about 10 minutes. The temperature was set to 43°C and the mixture incubated for about 4 hours. After about 3.5 hours NaOH (2.4 L) was added to take the pH to 6.5.

3. After 4 hours incubation at 43°C, the solution was heated to about 93°C and held for 15 minutes before cooling to about 40°C.

Second incubation:

4. A further 600 mL NaOH solution was added to the mixture to take the pH to about 6.4-6.6. The proteolytic enzyme solution and 26 kg of the B9642 culture was added. The reaction mixture was held at 40°C with gentle stirring for about 53 hours. The pH was checked and adjusted to 6.4 to 6.6 if necessary.

5. At the end of the 53 hour incubation the grated cheese and cheese salt was added with mixing and the mixture heated to 93°C and held for 15 minutes.

6. The ingredient was packed at 85°C in 20 L pails with plastic liners.

Example 2

A protein in water mixture comprising a Gouda cheese source was formed in a first incubation then incubated with protease enzymes and a culture of *Enterococcus faecium* B9642 in a second incubation.

Ingredients for the first incubation: 440 kg of grated brine salted commercial New Zealand Gouda cheese; 10.5 kg disodium phosphate dissolved in 75 L hot water; 3 kg of cheese salt; 325 L water at 43 to 45°C; 50% (w/w) solution of NaOH.

Ingredients for the second incubation; 760 kg grated Cheddar cheese; 1320 g proteolytic enzymes (protease A "Amano" and Neutrase in equal amounts) dissolved in 5 L sterile water; 26 kg of B9642 culture; 50% (w/w) solution of NaOH; 37 kg cheese salt.

First incubation

1. The water was placed in a tank at about 53°C so the cold cheese would bring it down to about 43°C. The dissolved disodium phosphate was added with stirring following by approximately half the grated cheese over about 15 to 20 minutes. The mixture was stirred throughout the process.

2. The remaining grated cheese was added over 10 minutes. The temperature was set to about 43°C and the mixture incubated for 30 minutes. During this incubation, the cheese salt and 3.5 L NaOH (pH stable at 6.4 – 6.6) were added.

3. The solution was heated up to about 93°C and held for about 15 mins before cooling to about 37°C.

Second incubation

4. The pH was adjusted to pH 6.4-6.6 if necessary and the temperature set to about 40°C. The proteolytic enzyme solution was added and the mixture stirred for about 10 minutes. Then 26 kg of B9642 was added and the mixture held at about 40°C with intermittent stirring for 64 hours. At end of the 64 hour incubation the grated cheese and cheese salt was added with mixing. Then the reaction mixture was heated to 93°C and held for 15 minutes.

5. The ingredient was packed in 20 L pails with plastic liners at 85°C.

The final flavour ingredient from each example had the same sensory profile described above (a strong smear flavour, often described as a dirty socks flavour; other main flavours described

were cooked, salty, savoury flavours with sulphury notes) when evaluated in a white sauce application as described above.

The above describes some preferred embodiments of the present invention and indicates several possible modifications but it will be appreciated by those skilled in the art that other modifications can be made without departing from the scope of the invention.

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Tsakalidou E, Manolopoulou E, Tsilibari V, Georgalaki M, and Kalanzopolous G (1993). Esterolytic activities of *Enterococcus* durans and *Enterococcus* faecium strains isolated from Greek cheese. *Netherlands Milk and Dairy Journal,* 47, 145-150.

What we claim is:

1. A method for the manufacture of a cheese flavour ingredient comprising the steps of:

(a) forming a protein in water mixture,

(b) adding a protease enzyme to said mixture to establish a proteolysis reaction,

(c) adding a bacterial culture to said mixture to establish a fermentation reaction, said bacterial culture comprising a physiologically acceptable bacterium capable of producing a cheese flavour ingredient selected from the group comprising *Enterococcus, Staphylococcus* and *Pseudomonas* bacteria,

(d) maintaining said mixture under microaerophilic or anaerobic conditions at a temperature within the range of about 20-50°C at a pH within the range of about 5.0 to 8.0 for a time period of from about 20 to 100 hours, and

(e) terminating said reactions and recovering the cheese flavour ingredient produced.

2. A method as claimed in claim 1 wherein said mixture comprises a fat, protein and water emulsion.

3. A method as claimed in claim 1 or 2 wherein said protein comprises a dairy protein.

4. A method as claimed in claim 3 wherein said dairy protein comprises casein.

5. A method as claimed in claim 1 wherein said mixture comprises a mixture of cheese and water.

6. A method as claimed in claim 1 wherein said mixture comprises one or more of cheese curd, ripened cheese, mature cheese, milk solids, reconstituted whole milk powder, milk protein concentrate, casein, milk protein, non-dairy protein, milk fat, and a non-dairy oil or fat.

7. A method as claimed in any one of the preceding claims wherein said mixture comprises total solids in the range of 5% (w/w) to 50% (w/w) of the mixture.

8. A method as claimed in any one of the preceding claims wherein said protease enzyme comprises a peptidase or a proteinase or a combination of a peptidase and a proteinase.

9. A method as claimed in any one of the preceding claims comprising maintaining the emulsion at a temperature within the range of 20-60°C to continue said proteolysis reaction before beginning step (c).

10. A method as claimed in claim 9 wherein said proteolysis reaction is conducted at a temperature of from about 40-50°C.

11. A method as claimed in claim 9 wherein said proteolysis reaction is conducted at a temperature of about 43°C.

12. A method as claimed in claim 9 wherein said proteolysis reaction is conducted for about 2 to 24 hours.

13. A method as claimed in claim 9 wherein said proteolysis reaction is conducted for about 4 hours.

14. A method as claimed in any one of the preceding claims wherein said proteolysis reaction is terminated before beginning step (c).

15. A method as claimed in claim 14 wherein said proteolysis reaction is terminated by heating the reaction mixture to 80-100°C for from 3 to 30 minutes.

16. A method as claimed in claim 14 wherein said proteolysis reaction is terminated by raising the temperature of the reaction mixture to about 93°C for about 15 minutes.

17. A method as claimed in any one of claims 14 to 16 wherein said mixture is cooled after being heated to terminate said proteolysis reaction.

18. A method as claimed in claim 17 wherein further protease is added after said cooling step.

19. A method as claimed in any one of claims 1 to 13 wherein said steps (b) and (c) are allowed to proceed at the same time.

20. A method as claimed in any one of the preceding claims wherein said bacterium comprises *Enterococcus faecium* B9642, AGAL accession number NM01/24754.

21. A method as claimed in any one of the preceding claims wherein said bacterium comprises *Enterococcus faecium* B9645, AGAL accession number NM01/24755.

22. A method as claimed in any one of the preceding claims wherein said bacterium comprises *Enterococcus faecaelis* B9509, AGAL accession number NM01/24757.

23. A method as claimed in any one of the preceding claims wherein said bacterium comprises *Enterococcus casseliflavus* B9518, AGAL accession number NM01/24753.

24. A method as claimed in any one of the preceding claims wherein said bacterium comprises *Staphylococcus simulans* B9646, AGAL accession number NM01/24756.

25. A method as claimed in any one of the preceding claims wherein said bacterium comprises *Pseudomonas putida* B9647, AGAL accession number NM01/24752.

26. A method as claimed in any one of the preceding claims wherein said fermentation reaction is conducted at a temperature of from about 30 to 45°C.

27. A method as claimed in any one of the preceding claims wherein said fermentation reaction is conducted at a temperature of about 40°C.

28. A method as claimed in any one of the preceding claims wherein said fermentation reaction is conducted at a pH of from about 6.3 to 6.5.

29. A method as claimed in any one of the preceding claims wherein said fermentation reaction is conducted for from about 30 to 72 hours.

30. A method as claimed in any one of the preceding claims wherein said fermentation reaction is conducted for between about 50 hours and about 65 hours.

31. A method as claimed in any one of the preceding claims wherein said fermentation reaction is conducted for about 53 hours or about 64 hours.

32. A method as claimed in any one of the preceding claims wherein additional cheese and/or salt are added immediately before said fermentation reaction is terminated.

33. A method as claimed in any one of the preceding claims wherein cheese and/or salt is added part way through said fermentation reaction.

34. A method as claimed in any one of the preceding claims wherein said fermentation reaction is terminated by heating to a range of about 80°C to 100°C and holding for a time of about 3 to 30 minutes.

35. A method as claimed in any one of the preceding claims wherein said fermentation reaction is terminated by heating to a temperature of about 93°C and holding for a time of about 15 minutes.

36. A method as claimed in any one of the preceding claims wherein salt is added to said reaction mixture after said fermentation reaction has been terminated.

37. A method as claimed in any one of the preceding claims wherein cheese is added to said reaction mixture after said fermentation reaction has been terminated.

38. A method as claimed in any one of the preceding claims comprising a further step wherein the cheese flavour ingredient is dried.

39. A method as claimed in claim 37 wherein said ingredient is dried by spray drying.

40. A cheese flavour ingredient produced by the method claimed in any one of claims 1 to 38.

41. A food product comprising a cheese flavour ingredient as claimed in claim 39.

42. A food product as claimed in claim 40 wherein said food product comprises a product selected from the group comprising natural cheese, processed cheese, analogue cheese,

foods comprising natural cheese, processed cheese or analogue cheese, sauces, dips, snacks, biscuits, soups, pizza, and cheese and savoury flavour foods.

43. A biologically pure culture of *Enterococcus faecium* B9642, AGAL accession number NM01/24754.

44. A biologically pure culture of *Enterococcus faecium* B9645, AGAL accession number NM01/24755.

45. A biologically pure culture of *Enterococcus faecaelis* B9509, AGAL accession number NM01/24757.

46. A biologically pure culture of *Enterococcus casseliflavus* B9518, AGAL accession number NM01/24753.

47. A biologically pure culture of *Staphylococcus simulans* B9646, AGAL accession number NM01/24756.

48. A biologically pure culture of *Pseudomonas putida* B9647, AGAL accession number NM01/24752.

49. A food product comprising a biologically pure culture as claimed in any one of claims 32 to 47.

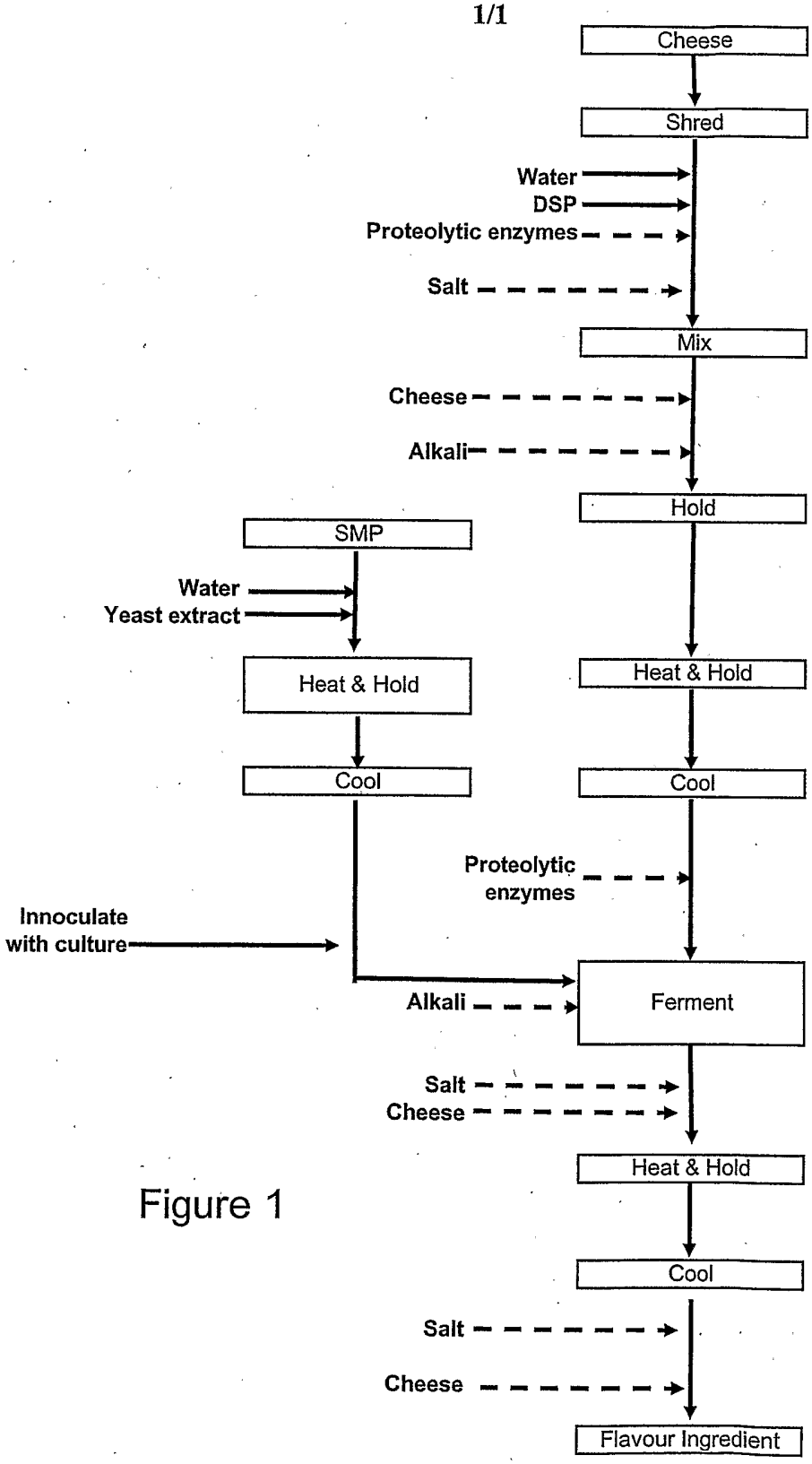
Cheese
Shred
Water
DSP
Proteolytic enzymes
Salt
Mix
Cheese
Alkali
Hold
SMP
Water
Yeast extract
Heat & Hold
Heat & Hold
Cool
Cool
Proteolytic enzymes
Innoculate with culture
Alkali
Ferment
Salt
Cheese
Heat & Hold
Cool
Salt
Cheese
Flavour Ingredient

Figure 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ02/00266

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A23C 20/00, A23L 1/23, A23L 1/30, C12N 1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IC7: A23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, CA, FSTA: enterococcus, staphylococcus, psuedomonas, protein, protease proteolyitc, ferment, flavor, flavour, cheese, dairy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 675 193 A (Bourdeaux) 23 June 1987 whole of document	1-4, 6-23, 26-42
X	EP 137 536 A1 (Stauffer Chemical Company) 17 April 1985 whole of document	1-4, 6-13, 19-23, 26-42
X	US 4 708 876 A (Yokoyama *et al*) 24 November 1987 whole of document	1-4, 6-13, 19-23, 26-42
X	GB 1 449 279 A (L. Givausan & CIE S.A.) 15 September 1976 whole of document	1-4, 6-17, 19-23, 26-42

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2003	26 FEB 2003
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **GARETH COOK** Telephone No : (02) 6283 2541

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ02/00266

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6 054 151 A (Kwon *et al*) 25 April 2000 whole of document	
A	US 4 172 900 A (Dooley) 30 October 1979	
X	EP 508 701 A2 (Quest International B.V.) 14 October 1992 whole of docuement	43, 44, 49
X	GB 930 107 A (Sigurta Farmaceutici) 3 July 1963 whole of document	45, 49
X	WO 99/35240 A1 (Erber Aktiengesellschaft) 15 July 1999 whole of document	46, 49
X	ChemAbs Accession 106:83290, JP 61274663 (Kureha Chemical Industry) A, 1986 whole of document	47, 49
X	Derwent Abstract Accession 77-87396Y, JP 52128286 (Tanabe Seiyaku KK) A, 1977 whole of document	48, 49

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ02/00266

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No II: Observations where unity of invention is lacking

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. In coming to this conclusion the International Searching Authority has found that there are different inventions as follows:

1. Claims 1 to 42 are to a method of manufacturing a cheese flavour ingredient, and food products comprising the cheese flavour ingredient. The method of manufacturing a cheese flavouring ingredient is considered to be the first "special technical feature".

2. Claims 43, 44 and 49 are to a biologically pure culture of *Enterococcus faecium* and a food product comprising it. *Enterococcus faectum* is considered to comprise the second "special technical feature".

3. Claims 45 and 49 are to a biologically pure culture of *Enterococcus faecalis* and a food product comprising it. *Enterococcus faecalis* is considered to comprise the third "special technical feature".

4. Claims 46 and 49 are to a biologically pure culture of *Enterococcus casselifavus* and a food product comprising it. *Enterococcus casselifavus* is considered to comprise the fourth "special technical feature".

5. Claims 47 and 49 are to a biologically pure culture of *Staphylococcus simulans* and a food product comprising it. *Staphylococcus simulans* is considered to comprise the fifth "special technical feature".

6. Claims48 and 49 are to a biologically pure culture of *Pseudomonas putida* and a food product comprising it. *Pseudomonas putida* is considered to comprise the sixth "special technical feature".

Since the abovementioned groups of claims do not share any of the technical features identified, a "technical relationship" between the inventions, as defined in PCT rule 13.2 does not exist. While the bacterial strains of inventions two to six may be used in the first invention, inventions two to six do not have any features of the first invention. Accordingly the international application does not relate to one invention or to a single inventive concept, a priori.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/NZ02/00266

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	4 675 193	CA	1 220 075				
EP	137 536	AU	32452/84	DK	4034/84	JP	60078582
		CA	1 220 074				
US	4 708 876	AU	56415/86	JP	61242542		
GB	1 449 279	CA	1 009 498	FR	2 210 359	NL	7 317 015
		CH	576 239	JP	49094875	US	4 001 437
		DE	2 362 998				
US	6 054 151	EP	1 085 817	AU	45091/99	WO	99/63834
		EP	1 219 176				
US	4 172 900	AU	50048/72	DE	2 300 490	FR	2 167 561
		BE	793 362	ES	410 397	GB	1 377 120
		CA	1 032 401				
EP	508 701	AU	14738/92	JP	5 252 934	US	5 589 168
		CA	2 064 954	NZ	242 195	US	5 728 380
		IE	921 092				
WO	99/35240	AT	2204/97	CA	2 317 065	HU	200100696
		AU	16465/99	EP	1 042 449	ZA	9811816
		BR	9814521				

END OF ANNEX