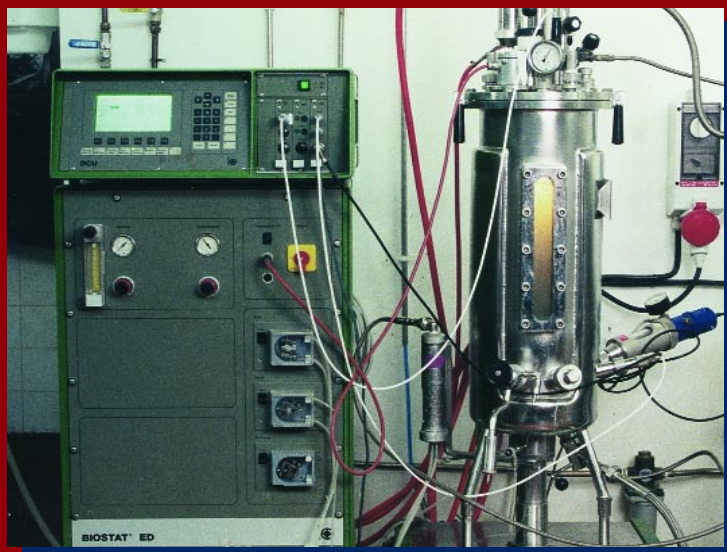


Enzyme Modified Cheese Flavour Ingredients

Dr. M. Wilkinson and Mr. K. Kilcawley

These studies provided an in-depth understanding of the key process parameters and methods of flavour production used in the manufacture of enzyme modified cheese (EMC) products, thereby facilitating the successful production of these cheese flavour ingredients.



This report is based on two complementary projects:

* Development of flavour ingredients for processed consumer foods through the use of enzyme technology (Armis No. 4338)

* Process optimisation for the generation of a range of hydrolysed dairy fractions for use in the flavouring of processed consumer foods (Armis No. 4540)

(Enzyme Modified Cheese Flavour Ingredients)

Project Team:

M. Wilkinson (Leader)

K. Kilcawley

E. Mulholland

The Dairy Products Research Centre
Moorepark, Fermoy, Co. Cork.

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Teagasc 19 Sandymount Avenue
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Summary and Conclusions

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Enzyme-modified cheeses (EMCs) are defined as concentrated cheese flavours produced enzymatically from cheeses of various ages and are

principally used as an ingredient in processed foods, where they provide a cost-effective alternative to natural cheese. They can be used as the sole source of cheese flavour to intensify an existing cheese taste, or to impart a specific cheese character to a more bland product. Their main applications are in processed cheese, analogue cheese, cheese spreads, snack foods, soups, sauces, biscuits, dips and pet foods. Their main advantages over other cheese flavour ingredients are: low production costs, consistency, high flavour intensity, diverse flavour range, extended shelf-life, low storage costs and increased functionality.

EMCs are generated utilising the same flavour pathways that occur in natural cheese ripening i.e. proteolysis, lipolysis and glycolysis. They are not as easy to differentiate as natural cheeses, as they are characterised by flavour and aroma alone as texture is not a factor in EMC production. The relationship of the flavour of EMCs to the flavour of the corresponding natural cheese remains unclear. This is especially true for Cheddar EMC which is commercially available in a range of "Cheddar flavours". Despite the fact that a wide range of commercial EMCs are available, there is very little detailed information available regarding their properties or the specific production processes used.

The main objective of this research was to build a knowledge base on EMC products and to utilise this to develop a biotechnological process for the production of improved enzyme modified cheeses for use as flavour ingredients. The strategy was to establish quantitative relationships between the compositional, proteolytic and lipolytic parameters and the sensory characteristics of EMCs. This data would then be used to develop a predictive model for flavour development in EMC production and the subsequent generation of an optimised EMC process enabling the generation of a range of cheese flavours from single or multiple substrates.

Main Conclusions and Achievements

High levels of proteolysis and lipolysis are an integral feature of Cheddar EMC products. However, most commercial products tested were not perceived as having a Cheddar cheese flavour profile. Manipulation of proteolytic, lipolytic and compositional parameters, as well as the addition of flavour potentiators, organic acids and bulking agents were identified as being used to produce Cheddar EMC products of different characteristics.

A number of commercial enzyme preparations were identified for their flavour generating potential in EMC systems. Commercial proteinase, peptidase and lipase preparations from microbial and animal sources were found to contain large differences in active enzymes and protein content.

A laboratory scale EMC system was developed and successfully used to assess the de-bittering potential of a combination of purified peptidase enzymes (aminopeptidases).

A prototype pilot plant EMC process has been developed and used to generate Cheddar flavoured EMC products.

Research and Results

Basis of EMC production

The basis of EMC production is the utilisation of specific enzymes, under optimum conditions, to produce intense cheese flavours rapidly from dairy substrates (usually cheese curd). The technology was developed in the late 1960s where fresh curd and a NaCl solution were mixed to make an emulsion of approximately 40% solids. Enzymes and preservatives were added and the mixture was incubated under controlled conditions, after which characteristic cheese flavours developed. This work demonstrated the potential of generating a range of intense cheese flavours in a short time from cheese substrates by modification of process parameters.

Many commercial procedures are based on similar principles and in general, production involves incubating mature or immature cheese with specific exogenous enzymes and/or micro-organisms, terminating the process by heat treatment, and standardising the final product to a desired flavour intensity and composition. EMCs are generally produced either by a one step process (Fig 1 - Page 10), in which hydrolysis of fat and protein occur simultaneously, or by a component approach (Fig 2 - Page 11), where several different flavour components are created separately and then blended together. The component approach allows greater flexibility, facilitating greater diversity of products.

The actual processing conditions used depend upon many factors and directly relate to the enzymes being used. In general, EMCs are produced between 12 to 72h in a temperature range of 30 - 45°C, and between pH of 5 - 7 (Kilcawley *et al.*, 1998). Once a product has been produced it may be formulated to a desired composition depending upon its final application. The pH may be altered to improve storage stability, or flavour, while bulking agents or carriers may be added to increase yield, obtain a desired flavour intensity or to facilitate spray drying.

Experimental Approach

The major objective of this research was to develop a biotechnological process for the production of EMCs for use as flavour ingredients.

The steps to achieving this objective involved:

- *Compilation of a database of the compositional, proteolytic, lipolytic indices and sensory evaluation of a range of commercial EMC products*

- *Compilation of a database of the enzyme activities (proteinase, peptidase, lipase and esterases) present in commercial enzyme preparations used in the manufacture of EMCs*

- *Development of a laboratory-scale EMC process to investigate the flavour generating and/or de-bittering potential of various enzyme combinations*

- *Development of a prototype pilot biotechnological process for the manufacture of EMC*

Database of Cheddar EMC products

Initially, a wide range of EMC flavours were investigated, such as Blue, Swiss, Gouda, Mozzarella and Parmesan. However, because of its commercial importance in Ireland it was decided to focus on Cheddar EMC. Cheddar EMC was also of interest because it is available as a range of purported Cheddar flavours and intensities from different manufactures.

Fifteen commercial Cheddar EMC products were obtained from eight suppliers in paste and powdered formats. The compositional parameters analysed were moisture, protein, fat, NaCl, pH, ash, phosphate and calcium. Proteolysis was measured by urea-PAGE, the percentage of total N soluble in water at pH 4.6 (pH 4.6 WSN) and 5% phosphotungstic acid (PTA-N), the molecular mass distribution of soluble peptides in the pH 4.6 water soluble extract (pH 4.6 WSE), the free amino acid content (FAA) of the pH 4.6 WSE, and peptide profiles by Reverse Phase -HPLC of pH 4.6 WSE.

Results shown in Table 1 are the averages of the compositional parameters analysed for paste Cheddar EMC products and for typical natural Cheddar cheeses. Table 2 shows the average proteolytic data from these same products. The compositional and proteolytic indices varied widely, with most products having high levels of proteolysis. *It was evident from these results that manipulation of both compositional and proteolytic parameters is a feature of Cheddar EMCs. The use of emulsifying salts, flavour potentiators, bulking agents, cheeses of varying fat and/or the addition of exogenous protein or fat was also indicated in these commercial products. This work is presented in full in Kilcawley *et al.* (2000).*

Table 1: Compositional data for commercial Cheddar EMC pastes and typical natural Cheddar cheese

	n	Moisture (%)		Protein (mg/g*)		Fat (mg/g*)		NaCl (mg/g*)		pH	
		Av	sd	Av	sd	Av	sd	Av	Sd	Av	sd
Cheddar EMC	13	54.4	7.9	35.1	7.8	52.2	10.9	7.4	4.6	4.8	0.7
Cheddar cheese * dry wt basis	4	36.5	1.0	40.6	1.1	54.0	2.3	2.8	0.2	5.0	0.1

Table 2: Proteolytic data for commercial Cheddar EMC pastes and typical natural Cheddar cheeses

	n	WSN (%)		PTA (%)		FAA (mg/g*)	
		Mean	sd	Mean	sd	Mean	sd
Cheddar EMC	13	49.7	18.5	26.7	21.9	60.7	40.7
Cheddar cheese * dry wt basis	4	22.2	6.9	5.6	2.8	22.3	13.9

WSN = pH 4.6 water soluble nitrogen. PTA = 5% Phosphotungstic acid soluble nitrogen. FAA = Free amino acid.

The free fatty acid (FFA) and lactic acid contents of these same Cheddar EMC products were also quantified. The lactic acid content was determined using a UV test kit method. Short chain volatile FFA (C_{2:0}, C_{3:0}, C_{4:0}) were extracted by steam distillation and quantified using ion - partition HPLC, medium and long chain FFAs (C_{6:0}, C_{8:0}, C_{10:0}, C_{12:0}, C_{14:0}, C_{16:0}, C_{18:0}, C_{18:1}, C_{18:2}, C_{18:3}) were isolated, esterified and quantified by RP-HPLC.

Results for FFA and lactic acid contents of the paste Cheddar EMCs and natural commercial Cheddar cheeses are summarised in *Table 3*. *These results confirm that levels of lipolysis were higher in commercial Cheddar EMCs than in natural Cheddar cheese. Levels of lipolysis varied widely between individual EMCs indicating that manipulation of lipolysis is used to generate a range of Cheddar EMC products (in addition to manipulation of composition and proteolysis). Addition of exogenous acetic acid, butyric acid and lactic acid was evident in certain EMCs and indicates their possible uses as flavour potentiators and/or to reduce pH. (Kilcawley et al. [In Press]).*

Table 3: Levels of different acids in commercial Cheddar EMC pastes and typical natural Cheddar cheese

	n	Acetic Acid (mg/g*)		Propionic Acid (mg/g*)		Butyric Acid (mg/g*)		Lactic Acid (mg/g*)		Total FFA (C _{6:0} -C _{18:3})* (mg/g*)	
		Av	Sd	Av	Sd	Av	Sd	Av	Sd	Av	Sd
Cheddar EMC	13	36.1	97.1	3.4	5.9	36.0	34.4	50.6	72.5	24.4	9.5
Cheddar cheese	4	0.7	0.4	nd		0.6	0.1	19.7	2.2	3.5	1.7
*dry wt basis											

The sensory characteristics of EMCs were determined by quantitative descriptive analysis by a trained sensory panel. Due to the high flavour intensity, the commercial EMCs were assessed in a bland cheese slurry at 10% (w/w) of its original concentration. *The sensory profiles of the Cheddar EMCs were found to be significantly different to the sensory characteristic used to describe natural Cheddar cheese. Positive correlation was found between flavour intensity and certain compositional parameters. This study is described in detail in Hulin-Bertaud et al. (In Press).*

Database on commercial and novel enzymes

Fifty commercially available enzyme preparations were obtained from 10 different enzyme suppliers. These preparations consisted of proteinases, peptidases, lipases or mixtures thereof. All of the proteinases were derived from microbial sources (*Bacillus*, *Aspergillus* or *Rhizomucor* spp.), as were the peptidases (*Aspergillus*, *Rhizomucor* or *Lactococcus lactis*). The lipases were derived from both animal (calf, kid and lamb) and microbial sources (*Aspergillus*, *Rhizopus*, *Mucor*, *Candida* or *Penicillium* spp.).

All enzyme preparations were analysed for protein content, semi-quantitative tests for the presence of lipase, proteinase, peptidase, phosphatase and carbohydrase activity, using the

API-ZYM test method. Each preparation was also analysed for specific proteinase activity at pH 5.5 and pH 7.0 at 37°C using azocasein.

Proteinases of interest were further evaluated in a sodium caseinate system, monitoring hydrolysis by urea-PAGE and RP-HPLC and free amino acid analysis and assessed for specific peptidase, lipase and esterase content at pH 7.0 at 37°C. Peptidase preparations were assessed for a number of specific peptidase activities; at both pH 7.0 and pH 5.5 at 37°C (general amino peptidase activity; leucine aminopeptidase, Post-proline dipeptidyl aminopeptidase; PepX, proline imino-peptidase; PepI, aminopeptidase A; PepA, aminopeptidase P; PepP, aminopeptidase M; PepM, endoproteinase O; PepO, carboxypeptidase and endopeptidase activity).

Lipase preparations were assessed for both esterase and lipase activity at pH 7.0 at 37°C.

Brief summary of evaluations

Proteinases

The protein content of the proteinase preparations was found to vary as was their specific proteinase activities. All preparations were found to contain other contaminating side activities. The preparations derived from *Aspergillus* and *Rhizomucor* species contained more peptidase side activities than the preparations derived from *Bacillus* species. Only two products contained lipase activity, but most contained esterase activity.

Bacillus species had higher proteinase activity at pH7.0 and *Aspergillus* and *Rhizomucor* species had higher proteinase activity at pH5.5.

Peptidases

The protein content and proteinase activities of these preparations were also found to vary. All preparations contained varying amounts of different specific peptidase activities, with higher activities at pH 7.0 than at pH 5.5. Most preparations contained contaminating enzyme side activities.

In general those derived from Aspergillus had the highest levels of general aminopeptidase activity, with those derived from Lactococcus lactis having the highest levels of PepX activity.

Lipases

The protein content of the lipase preparations was found to vary. Little or no peptidase activity was determined in these preparations. *Large differences were determined in the esterase and lipase activities of each preparation.*

Novel purified enzymes

The de-bittering potential of a combination of purified aminopeptidases was evaluated in a laboratory-scale EMC system. A curd slurry substrate was inoculated with a high dosage

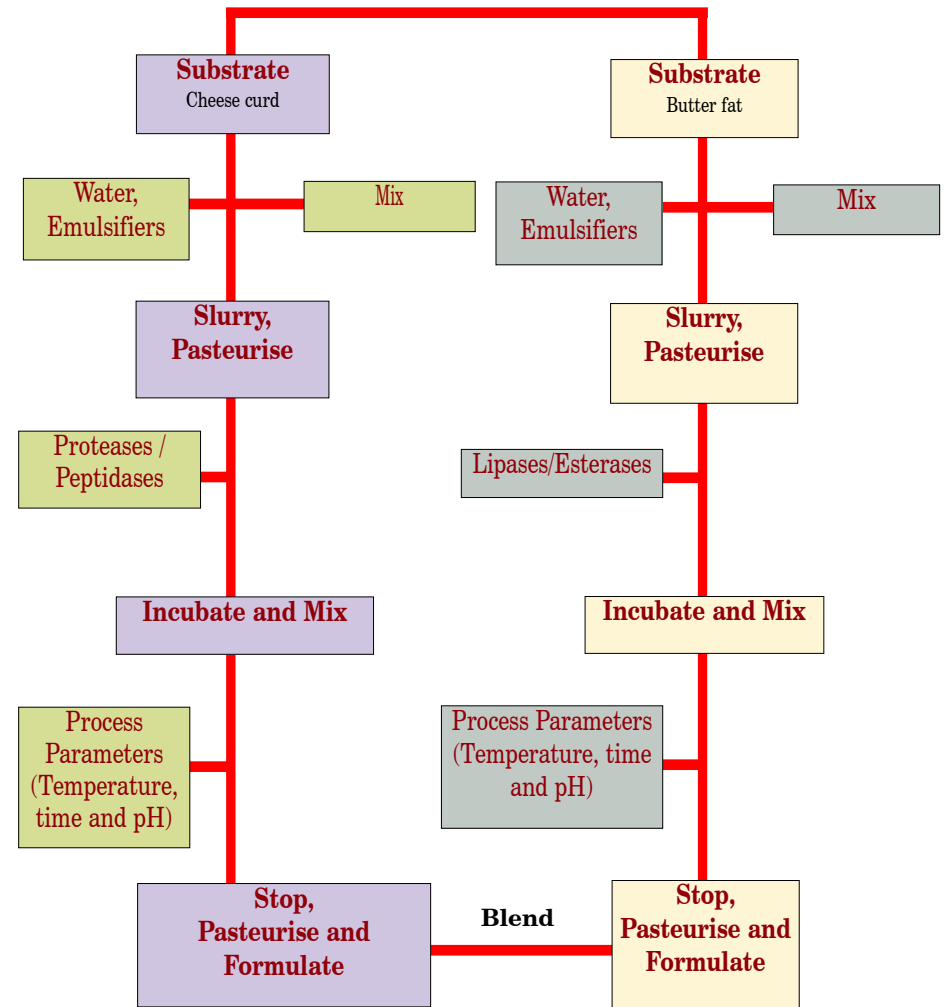
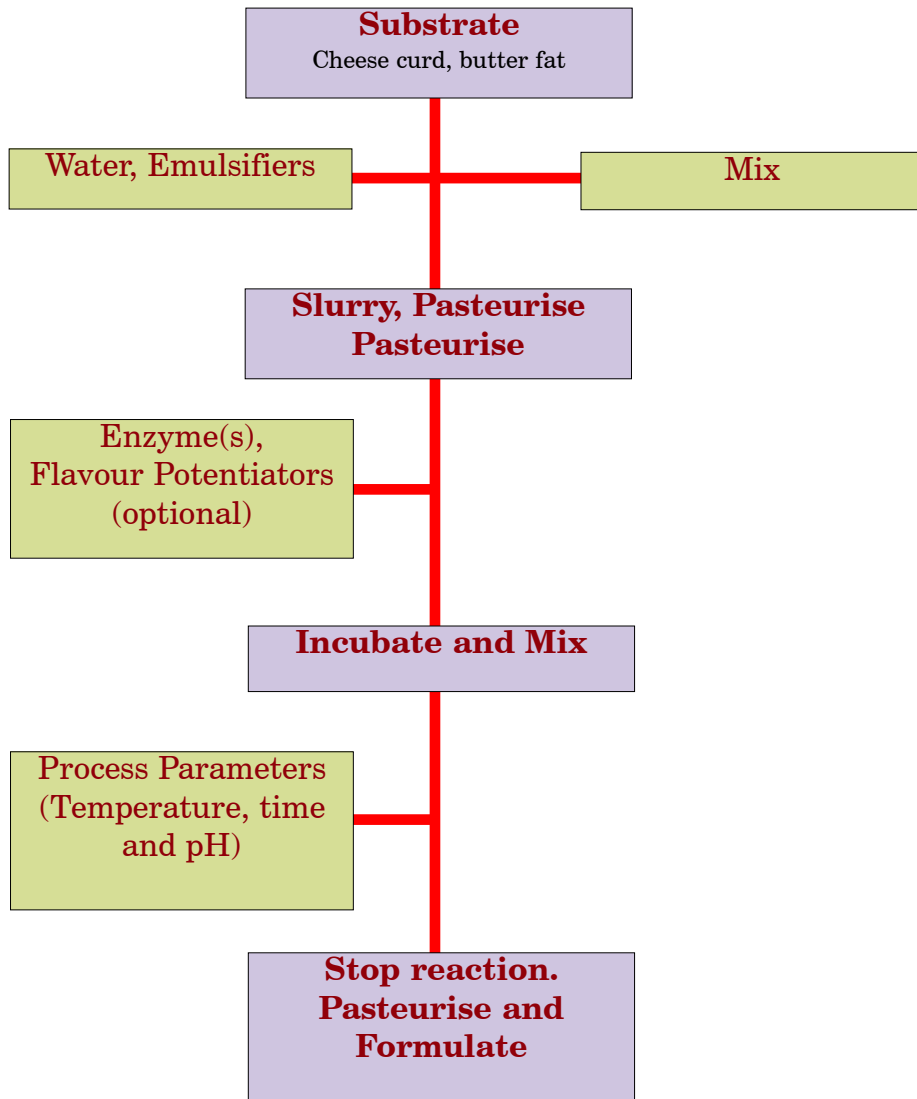
of the commercial proteinase, Neutrase 0.5L (Novo Nordisk A/S) derived from *Bacillus subtilis* which had previously been shown to accelerate Cheddar cheese flavour development, but accumulate bitterness. Neutrase 0.5L was used in this study to generate an excessively bitter curd slurry which was then treated with a combination of three purified peptidases isolated from *Lactococcus lactis* subsp. *cremoris* AM2; aminopeptidase KpNA-H (lysyl-nitrilide hydrolyase), PepX and PepP. KpNA-H cleaves all amino acids from the N-terminal of peptides up to the imido bond (the peptide bond preceding the proline residue). The proline present in the second position was then removed by the action of PepX, which also enables the general aminopeptidase to continue to hydrolyse the peptide. However if the imido bond is followed by consecutive proline residues (e.g. in positions 2 and 3) PepX cannot remove the Pro-X dipeptide due to the presence of this second proline. PepP can however, remove the amino acid preceding proline (position 1) which then enables PepX to remove the Pro-Pro dipeptide, thus allowing the general aminopeptidase to continue to hydrolyse the peptide and reduce bitterness. Addition of these purified peptidases were shown to significantly reduce bitterness (~ 40%) in the Neutrase-treated cheese slurry. *This work has demonstrated the potential of utilising purified peptidases to alleviate the problem of bitterness in specific applications, such as in EMC manufacture.*

Development of a prototype EMC process

Preliminary work to develop a prototype pilot plant EMC process has utilised the knowledge gained of commercial Cheddar EMCs, commercial enzymes and the novel laboratory scale de-bittering process. *Preliminary trials of this process have enabled the successful production of Cheddar and Blue EMC flavours at laboratory scale and Cheddar EMC flavour at pilot scale.*

Fig 1: EMC Process - One Step Approach for the Production of Enzyme Modified Cheeses

Fig 2: EMC Process - Component Approach for the Production of Enzyme Modified Cheeses





Dr. M. Wilkinson



Mr. K. Kilcawley

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For further information, please contact Dr. Martin Wilkinson or Mr. Kieran Kilcawley



DAIRY PRODUCTS RESEARCH CENTRE

Moorepark, Fermoy, Co. Cork, Ireland

Tel: +353 (0) 25 42222 - Fax: +353 (0) 42340

E-mail: reception@moorepark.teagasc.ie