Methanol as a fermentation substrate for the production of phenylalanine, tyrosine and tryptophan by the facultative methylotroph Nocardia sp. 239

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The proteinases of the strains SK11 and AM1 show very specific caseinolytic activities. In contrast to the other strains, AM1 and SK11 also attack α-casein which might account for the observed low level of bitter off-flavour production. In order to explain the characteristic caseinolytic properties of the SK11/AM1 proteinases and to provide the tools for the over-production of these 'non-bitter' proteinases in dairy starter cultures, a genetic investigation of the proteolytic enzymes of the cheese-making strains SK11 and AM1 was initiated.

As it had been observed that the proteinases present in bitter-producing strains are coded for by plasmid DNA, the genetic location of the proteinases of strain SK11 and AM1 was determined by curing and genetic transfer experiments (De Vos et al., 1984). It appeared that the proteinase genes of strain SK11 as well as AM1 were located on a 77 kb plasmid. Moreover, restriction enzyme mapping showed that the 77 kb plasmids in AM1 and SK11 were identical.

In order to isolate the proteinase gene(s) restriction enzyme fragments of the large 77 kb plasmid were cloned in λ phage EMBL3. Using antibodies against the proteinase(s) (Hugenholtz et al., 1984) of strain Wg2 several positive clones were isolated. More detailed mapping of the phage λ insert (~ 11 kb) will enable us to determine the exact size of the proteinase gene(s) and to make a more detailed study of the synthesis and its regulation of these important proteins in dairy industry.

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Glucose and molasses are substrates which are often used as feedstocks in industrial fermentations but compared with another potential substrate, methanol, they are relatively expensive. We are interested in the possible use of methanol as a carbon- and energy source in the microbial production of fine chemicals, for instance aromatic amino acids.

Studies on microbial growth on C_1-compounds have shown that carbon assimilation via the ribulose monophosphate cycle of formaldehyde fixation is energetically most favourable. In these studies it also became clear that facultative methylotrophs are generally more amenable to environmental and genetic manipulation than obligate methylotrophs. On the basis of these considerations *Nocardia* sp. 239, a facultative methylotroph employing the RuMP cycle, was selected for further investigation.

In any biotechnological process it is of prime importance that utilization of desired end-products, for instance in catabolic reactions, does not occur. *Nocardia* sp. 239, however, is able to grow on tyrosine and phenylalanine (but not on tryptophan) as the sole carbon and energy source. This organism even uses methanol and phenylalanine or tyrosine, when present in a mixture, simultane-

ously. Therefore, methods for the isolation of mutants of *Nocardia* sp. 239 have been developed, and mutants blocked in phenylalanine and tyrosine catabolism are currently being isolated.

Before over-production of these aromatic amino acids can be induced, a clear understanding of the regulatory mechanisms involved must be obtained. In general, regulation occurs via feed-back inhibition and/or repression mechanisms, affecting the activities and levels of a number of key enzymes, in response to the intracellular concentrations of intermediates or end-products in the pathway. In *Nocardia* sp. 239 two of these control points, DAHP synthase and chorismate mutase, have been identified sofar.

Initial studies showed that growth of *Nocardia* sp. 239 is strongly inhibited by analogues of tryptophan and tyrosine. Analogue-resistant mutants are therefore being isolated followed by selection of strains in which enzyme regulation has been abolished to a sufficient degree to result in over-production of the aromatic amino acids.

Energetics of peptide hydrolysis in *Streptococcus cremoris*

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Dutch starter cultures used in cheese-manufacture consist mainly of *Streptococcus* spp. These organisms are nutritionally very fastidious and require an exogenous source of amino acids for growth. In milk the free amino acid concentration is very low, therefore these organisms are dependent on the milk protein casein which is hydrolysed by cell-wall-bound proteases to peptides and amino acids. These peptides are hydrolysed by peptidases to amino acids. We studied some characteristics of peptidase activity in *S. cremoris* Wg2 with the dipeptide leucyl-leucine as substrate.

In order to detect how many peptidases in *S. cremoris* Wg2 are responsible for leu-leu hydrolysis, immunological methods were used. Rabbit antibodies against a cell-free extract of *S. cremoris* Wg2 were obtained and used in crossed-immunoelectrophoresis experiments to detect the leu-leu dipeptidases. With a zymogram-staining technique in which the released leucine could be detected upon leu-leu hydrolysis only one leu-leu hydrolysing peptidase was found in *S. cremoris* Wg2.

A proton motive force (Δp) in *Streptococcus* spp. can be generated by hydrolysis of ATP (generated by substrate-level phosphorylation) by membrane-bound ATPase. Peptide hydrolysis in whole cells of *S. cremoris* Wg2 appeared to be dependent on the presence of an energy source (lactose). Addition of an uncoupler (which dissipates the Δp) to a cell suspension with lactose inhibited the hydrolysis of leucyl-leucine. These experiments indicate that the generation of a Δp is necessary for peptide hydrolysis. To establish which component of the Δp could be responsible for hydrolysis different ionophores were used. Valinomycin (dissipating the $\Delta\psi$ component) stimulated the hydrolysis, and nigericin (dissipating the ΔpH component) inhibited leu-leu hydrolysis. By measuring the Δp components it could be shown that after addition of valinomycin the $\Delta\psi$ collapsed and the ΔpH increased, while addition of nigericin reduced the ΔpH and increased the $\Delta\psi$. Also addition of the ATPase inhibitor DCCD inhibited the leu-leu hydrolysis.

These experiments indicate that the ΔpH component of the Δp plays an important role in controlling the activity of the leu-leu peptidase in *S. cremoris* Wg2.