PROTEOLYTIC ACTIVITY OF *Penicillium nalgiovense*

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Background

Degradation of proteins, peptides and amino acids are important factors in fermentation of meat products. Endogenic enzymes are responsible for degradation of proteins and large peptides , while microbial enzymes are important for degradation of smaller peptides (Trigueros *et al* 1995). Andersen (1995) showed that moulds were increasingly important during storage in natural fermented sausages, and **Penicillium nalgiovense** often constituted a major proportion of the flora. The mould *P.nalgiovense* is also used as a commercial strain.
A large number of substrates are used for detecting peptidase activity, often substrates conjugated with chromogenic groups (Cholette,H. and McKellar,R.C. (1990).

Objective

The objective of the investigation was to study the productivity of extracellular peptide degradative enzymes in *P. nalgiovense* using two para-nitroanilide-leu and p-nitroanilide-lys together with azocasin at relevant values of pH, temperature, and salt during growth of the mould on agar plates.

Methods

P. nalgiovense (Edelschimmel Kulmbach, supplied by Chr.Hansen A/S, Denmark) was heavily inoculated onto plates of Czapek dox broth+1,2 % agar (Oxoid) and incubated for specified times and temperatures. 0.012 g of freeze dried culture was mixed with 18.7 ml Millipore water and 0.1 ml plated onto each plate.
Enzyme extract: agar from 4 plates was mixed with 100 Millipore water in a Stomacher bag for 2 x 30s. After filtering the mixture through a filter covered with cellulose filteraid, the extract was filtered sterilized through a 0,45µ filter, and stored at -18°C until used.

Test for protease activity was done using a mixture of 0.5 ml 1% azocasein in 0.1 M K_2HPO_4, (pH 6.73) 0.2 ml extract, incubated for 3 h at 25°C. Reaction was stopped by adding 0.7 ml 10% trichloracetic acid, a blank was included and absorbance measured on a spectrophotometer at 336 nm. Leu-p-nitroanilid and Lys-p-nitroanilid was used as substrates for aminopeptidase activity test. Test system was: 0.1 ml 5mM Leu-p-nitroanilid + 0.2 ml concentrated enzyme extract + 0.3 ml 0.1 M Na_2HPO_4 buffer solution (pH 7.05). After incubation at 35°C for 2h absorbance was measured at 410nm in a spectrophotometer, blanks were included in the test (modified after Cholette and McKeller 1990).
After several test at different temperature/time combination a 3^2 factor experiment was performed with factor levels pH 5.0, 6.6 and 8.0; temperature 18°C, 24°C and 30°C; and NaCl concentration 0%, 5% and 10%. 8 agar plates were incubated at each level of parameters, 4 plates were harvested after 1 week and the other 4 plates after 2 weeks. Results were examined using Modde (Umetri). Activities were quite low especially after one week making it necessary to concentrate the extracts. This was done pouring a certain amount into a dialysis tube membrane, closing the ends and concentrating the liquid against normally 3-5 g Aqualic CA W-3 ( ) storing at 2°C. Activities given as abs units/(mg substrate* ml enzymes extract*h* concentration factor).

Results and Discussion

As the activity generally was small a concentration step was necessary. This resulted in the enzyme extract being concentrated up to 78 times although the general level was 20-50 times.
The results were examined using Modde and showed the effect of salt, pH and temperature during growth of the mould. The growth was influenced by the factors, thus the growth was slowest at 18°C and 10 % NaCl. No correction of this was done, but the results were comparable as the full content of the agar plates were used in all instances.
The results showed that after one weeks incubation productivities were significantly higher at higher temperature and pH (not azocasein activity) values (Fig. 1 and 2). For lys activities, a significant interaction of salt*temperature and significant effect of salt was found, with higher activities af lower salt concentrations.
Contrary to this, after 2 weeks incubation there was a significantly increased enzyme production as measured by the tests of p-N-Leu and p-N-Lys and azocasein degrading activities with increasing salt concentration (Fig.2 and 3)) and a slightly higher productivity at lower pH levels, although this effect was much smaller. Temperature only had a significant positive effect on p-N-Leu and the effect was small (results not shown). There was a marked effect of incubation conditions on growth of the culture, and this of course influences the results. The productivity is not corrected for cell mass, but a mirror of the amount of enzyme pr agar plate. Thus the high salt concentration markedly reduced the growth. Zapelena *et al* (1999) found that added fungal protease was active during fermentation when a_w fell fram 0.94 to 0.86. The amount of free fatty acid was higher throughout fermentation, but amino acids from peptides was higher only after 15 days. The production of the tested degradating enzymes was markedly influenced by incubation temperature. However, the results showed that enzyme production is not inhibited by high salt concentration or low pH values. Further the effect of temperature although significant was quite small. This means that the culture is capable of producing enzymes that could have an importance for the metabolic changes in the product leading to enhanced flavour. It stands to be tested what the actual activity of the enzymes are at the processing conditions.

Conclusions

In the study it was shown that it was possible to test *P.nalgiovense* for different enzyme activities related to protein/peptide degradation. The productivity was strongly influenced by salt concentration with a higher productivity at higher salt concentrations after 2weeks. The temperature effect was after 2 weeks incubation of minor importance. Th eresults indicate that *P. nalgiovense* is capable of taking part in the degradation of peptides/proteins at the processing parameters used.

References

Andersen,S. (1995) Compostitional changes in surface mycoflora during ripening of naturally fermented sausages. J.Food Prot, 58, 426.
Cholette,H. and McKellar,R.C. (1990) Influence of pH on the properties of *Lactobacillus helveticus* aminopeptidase J.Dairy Sci.73, 2278.

Trigueros,M.L.,Garcia,M.L., Casas,J.A. Ordonez,J.A., and Selgas,M.D. (1995) Proteolytic and lipolytic activities of mould strains isolated from spanish dry fermented sausages. Z.Lebensm.Unters.Forsh. 201:298.
Zapelena,M.J.,Astiasarán,I., and Bello,J. (1999). Dry fermented sausages made with a protease from *Aspergillus oryzae* and/or a starter culture. Meat Sci. 52; 403.

Fig.1. Degradation of leu-p-nitroanilide,1 week, 5 %NaCl
(Abs units/mg substrate*ml enzyme extract*h)

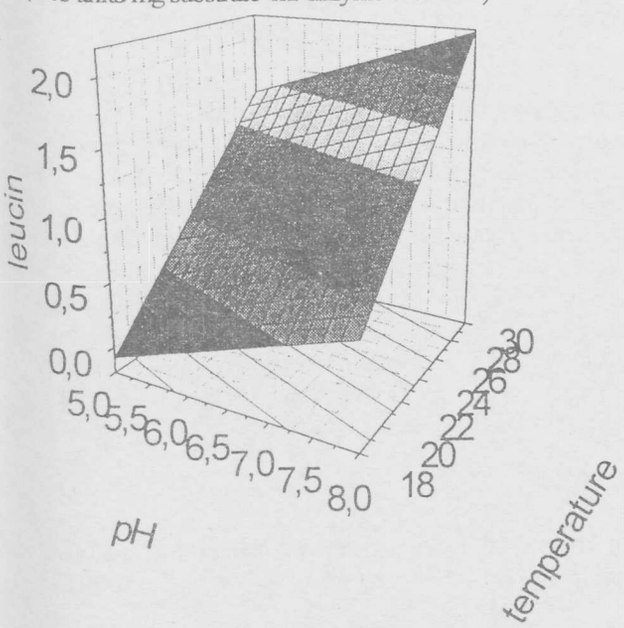

Fig.2. Degradation of Leu-p-nitroanilide 2 weeks, 24C
(Abs units/mg substrate*ml enzyme extract*h)

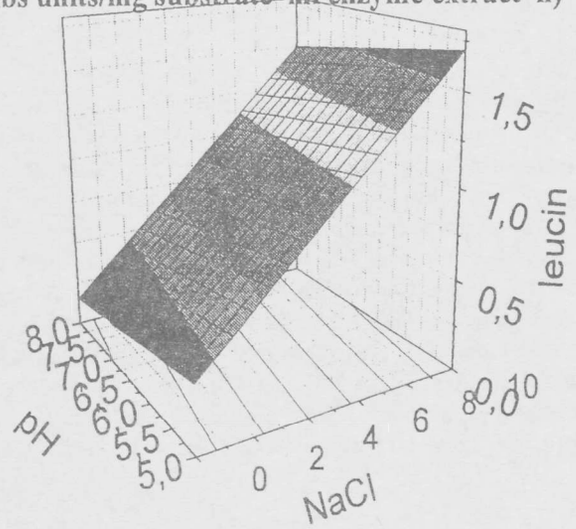

Fig.3. Degradation of azocasein, 2 weeks, 24C
(Abs units/mg substrate*ml enzyme extract*h)

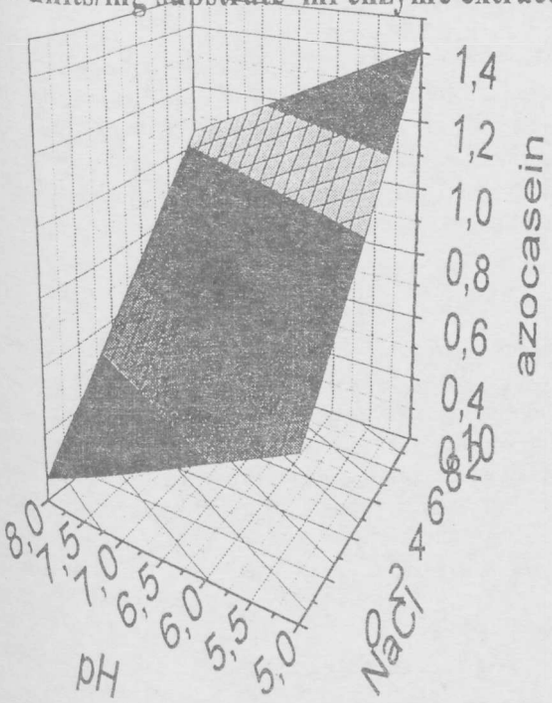

Fig.4 Degradation of lys-p-nitroanilid, 2weeks, 18C
(Abs units/mg substrate*ml enzyme extract*h)