DEPARTMENT OF MICROBIOLOGY

UNIVERSITY OF STELLENBOSCH

PROTEIN RESEARCH PROJECT

FUNCTIONAL YEAST PROTEIN SUPPLEMENT TO MONOGASTRIC ANIMAL FEED

EXECUTIVE SUMMARY
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LABORATORIES OF:

Prof. W.H. van Zyl
Tel.: 021-8083160
Fax: 021-8083611
E-Mail: whvz@maties.sun.ac.za

Prof. H.J.J. van Vuuren
Tel.: 021-8084866
Fax: 021-8083611
E-Mail: hjjvv@maties.sun.ac.za

BACKGROUND AND RATIONALE

The employment of microbial enzymes (hydrolases) in the animal-feed industries to enhance the utilisation of wheat- and barley-based animal feeds by monogastric animals (poultry and pigs) is gaining considerable support world-wide. The aim of this Protein Research Trust project is the cloning and overexpression of genes encoding microbial ß-xylanase, ß-glucanase and phytase enzymes, as well as a synthetic gene high in the essential amino acids lysine and methionine, in bakers' yeast (*Saccharomyces cerevisiae*). The resulting recombinant yeast culture (in a dried form) can be added as a functional protein supplement to wheat- and barley-based animal feeds to not only provide a more balanced protein profile, but to also assist in the digestibility and assimilation of the animal feeds, hence enhancing growth performance and alleviating the negative impact of the antinutritional activities present in wheat and barley.

The Western Cape is not a maize-producing region and therefore has to "import" maize from the Western Tranvaal region (Lichtenburg) for animal feed rations. However, the Western Cape region is at the same time "exporting" wheat to the rest of South Africa. The estimated transport costs are about R 150 per tonne grain both ways. The Elsenburg Agricultural Development Institute, Stellenbosch commissioned a taskgroup in 1992 to investigate the potential production of alternative animal feed in the Western Cape region. The taskgroup found that the Western Cape produces an excess of 36 800 tonne barley per year and that only 40,6% of the wheat produced are used in the Western Cape, meaning that 59,4% (435 000 tonne per year) have to be exported. However, the annual animal feed demands for monogastric animals (egg-laying hens, broilers and pigs) are about 580 000 tonnes. They also concluded that Alpha wheat (for animal feed) can be produced at an average costs of about R 400 per tonne, compared to a calculated break-even price of about R 500 per tonne for animal feeders to replace maize with wheat. If the non-starch polysaccharides (NSPs) fraction of wheat can be removed or reduced by microbial enzymes produced by *S. cerevisiae*, the excess wheat production can thus be considered as a cost-effective, alternative animal feed in the Western Cape.

The objectives for the first year of this project (commenced in January 1995) were completed. It involved the cloning of suitable ß-xylanase (*XYN5* from *Aspergillus niger*) and ß-endoglucanase (*BEG1* from a *Bacillus* species) genes, as well as the construction of an synthetic gene high in essential amino acids lysine and methionine (*HLM* gene). The specific objectives for the second year of the project (1996) were the fusion of the three different genes to yeast expression control elements (promoter / secretion / terminator sequences) and expression of the genes in genetically engineered laboratory strains of *S. cerevisiae*. A suitable *PHY* phytase gene will also be cloned, characterised and fused to yeast expression control elements. If the cloning and expression of these genes in *S. cerevisiae* proved to be successful, a stable industrial baker's strain of *S. cerevisiae* will be genetically engineered in

the third year (1997) to co-express the four genes, *END*, *XYN*, *HLM*, and *PHY* for testing and application in pilot plant fermentation studies.

RESULTS AND DISCUSSION

Sequencing of the ß-xylanase gene (XYN5), its expression in yeast and the characterization of the recombinant ß-xylanase produced by yeast: The nucleotide sequence of the *A. niger XYN5* gene was determined and found to contain an open reading frame (ORF) of 636 bp which encodes for a protein of 211 amino acids. When the protein sequence of the Xyn5 ß-xylanase was compared to the protein sequences from the EMBL/GenBank databases revealed that it is between 90% and 92% homologous to ß-xylanase isolated from other *Aspergillus* species.

An autoselective *S. cerevisiae* strain was constructed that successfully expressed the *XYN5* gene, under transcriptional control of the inducible *ADH2* promoter, from a multicopy yeast vector. The recombinant Xyn5 ß-xylanase was effectively secreted from the yeast cells and the maximum ß-xylanase activity obtained after 55 h in YPD medium was 56 nkat ml^{-1}. The Xyn5 ß-xylanase exhibited a temperature and pH optimum of 60°C and pH 4, respectively. The recombinant ß-xylanase has a molecular mass of ca. 30 kDa that differs significantly from the theoretical molecular mass of 20 kDa, which suggested that Xyn5 was probably hyperglycosylated. The hyperglycosylation appeared not to affect its secretion or enzymatic activity.

Sequencing of the bacterial endo-1,3-1,4-ß-glucanase gene (beg1) and its expression in yeast: The nucleotide sequence of the *B. subtilis beg1* gene was determined and found to contain a single ORF of 717 bp, encoding 239 amino acid residues. When the protein sequence of the Beg1 endo-1,3-1,4-ß-glucanase was compared to the protein sequences from the EMBL/GenBank databases it revealed that it is 97% identical to BglA from *Bacillus amyloliquefaciens* but only 92% to BglS from *B. subtilis*, suggesting that the *beg1* gene and its product corresponds more to that of *B. amyloliquefaciens* than that of *B. subtilis.*

An autoselective *S. cerevisiae* strain was constructed that successfully expressed the *beg1*gene, also under transcriptional control of the inducible *ADH2* promoter, from a multicopy yeast vector. A plate and liquid assay for endo-β-1,3-1,4-glucanase activity, using barley β-glucan/Congo-red complex, was developed. The plate assay with Congo-red showed the formation of a large clearing zone, clearly indicating that the endo-β-1,3-1,4-glucanase dramatically alter the polysaccharide structure of the β-glucan/Congo-red complex. When the autoselective *S. cerevisiae* strain expressing the *beg1* gene was cultivated for 72 h on YPGE medium, it produced endo-β-1,3-1,4-glucanase activity that allowed the breakdown of the glucan/Congo-red complex in liquid, as measured by a 16% reduction in the red color absorbancy at 540 nm. These results underlined the ability of the Beg1 endo-β-1,3-1,4-

glucanase to attack barley β-glucan and break this complex molecule in smaller oligosaccharides.

Cloning of the phytase gene from A. niger. The fungus *A. niger* will be cultivated on minimal medium containing corn meal as carbon and organic phosphate source and total cellular RNA will be extracted. The messenger RNA (mRNA) will be purified from the total RNA and first strand complementary DNA (cDNA) prepared from the poly-A^+ mRNA. Two synthetic oligonucleotides have been prepared for the amplification of the *PHY* phytase gene from first strand cDNA by the polymerase chain reaction (PCR), similarly as was done for the *XYN5* gene of *A. niger*. Initial attempts to amplify the *PHY* gene have not been successful, however, we will continue trying to clone the *PHY* gene through PCR technology by altering the PCR conditions, as well as using alternative *A. niger* strains.

Construction of the HLM gene and cloning of this gene into expression vectors pVS2 and pVS5. Two complementary oligonucleotide strands *(see previous report)* were synthesized by a local company. The oligonucleotides were purified, annealed and kinased. Numerous attempts were made to clone the 63 bp blunt ended DNA fragment into expression vectors pVS2 and pVS5. After an extensive study we found that the locally produced oligonucleotides were defective. New oligonucleotides were ordered from an overseas supplier and the oligonucleotides were again purified, annealed and phosphorylated. The *HLM* gene has now successfully been cloned into both expression vectors. The clones are currently being sequenced to establish orientation. Once an in-frame clone has been found, expression of the *HLM* gene and secretion of the lysine-methione rich peptide will be determined by standard methods.