The aim of the work is to develop a protein expression system which can be used to overexpress proteins. The system would be based on a methylotrophic Pichia strain which is capable to utilise methanol as a sole carbon and energy source. The strain was isolated from nature by the co-workers of the Institute, so the system – if developed – could be used without any legal restriction.

Known alcohol oxidases (AO) were compared at the nucleic acid and amino acid levels, evolutionary conserved motifs were identified, degenerate oligonucleotides were synthesised accordingly and used in PCR reactions to amplify segments of the AO coding regions. The templates in these reactions were genomic DNAs isolated from the Pichia sp. and from two other methylotrophic Candida boidinii strains for comparison. The amplified products were cloned and sequenced. The partial sequences revealed the presence of two genes (aoxA and aoxB) in the Pichia sp. and one in each Candida boidinii strain. Gene specific PCR primers were synthesised on the bases of the sequence information gained, and the complete AO ORFs were subsequently isolated in several steps.

The expression of aoxA and aoxB was analysed by RT-PCR. RNAs for templates in these reactions were isolated from yeast grown on dextrose or methanol. In the presence of dextrose a constitutive, low-level expression of aoxA but not aoxB was detected. Both genes were induced in the presence of methanol, although to different extent.

The DNA segments that contain the promoter of the genes were isolated in several successive steps. The isolated genomic segment is 1920 bp long in the case of aoxB and 1661 bp long in the case of the aoxA. The 3’ non-translated regions which probably contain the transcriptional terminators were also isolated as a 469 bp (aoxA) and a 430 bp (aoxB) long PCR fragments, respectively.

For the localisation of the functional elements of the promoter and for the identification of the minimal functional promoter, a number of transcriptional fusions were constructed. For a reporter in these fusions we used the fluorescent protein DsRed2.

For a functional expression system, some kind of a selection is essential. Plasmids, therefore, were constructed that indicate the successful transformation through the manifestation of a resistance gene (zeocin). Zeocin, as an antibiotics, though works at a laboratory level, might not be used for commercial productions. For future usage we also isolated the entire his4 gene from the Pichia sp. which could be used for selection, provided a strain carrying a mutant version of this gene was isolated, too.

The effective transformation requires the stable maintenance of the transformed DNA, either through integration into the genome, or by replication. For integrative stabilisation the his4 sequence can be used. The replicative stabilisation requires genomic regions the presence of which confers replication to the transformed DNA by the cellular functions. Such autonomously replicating sequences were isolated from the Pichia sp. and their ARS function were confirmed.