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HETEROLOGOUS EXPRESSION OF *BACILLUS LICHENIFORMIS* α-AMYLASE IN *PICHIA PASTORIS*

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ABSTRACT. The *Pichia pastoris* expression system has the potential for a very high production level of foreign proteins. The α-amylase enzyme (1,4-α-D-glucan-glucanohydrolase) catalyze the endohydrolysis of 1,4-α-D-glucosidic linkages of starch and related poly- and oligosaccharides producing maltose and larger oligosaccharides. *Bacillus licheniformis* α-amylase (BLA) is a highly thermostable enzyme which is widely used in biotechnological processes. In this study, the gene encoding the α-amylase from *Bacillus licheniformis* was amplified by PCR and cloned into *P. pastoris* X33 host strain using the vector pPICZαA allowing methanol induced expression and secretion of the protein, using the methanol-controlled alcohol oxidase *(AOX1)* expression system promoter. The ultimate goal of our research is the development of a new technology for amylolytic enzyme production, applicable in fermentation processes of starch-containing materials, technology which can be applied in case of different industrial enzymes as well.

Keywords: *Bacillus licheniformis, α-amylase, heterologous expression, Pichia pastoris, AOX1 promoter*

INTRODUCTION

The methylotrophic yeast *Pichia pastoris* has been developed as a commercial expression system for recombinant proteins [1,2]. The tightly controlled and methanol inducible *AOX1* gene [3], encoding the enzyme responsible for a vast majority of the alcohol oxidase activity in the cell has proven to be a valuable tool in the expression of recombinant proteins in this host organism [4]. Vectors are designed for carrying the *AOX1* promoter region, selectable marker which endows antibiotic resistance and the α-mating factor from *Saccharomyces cerevisiae* which enables signaling of extracellular secretion of the cloned protein [5].

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Recombinant protein production in the yeast strain has several advantages like very high levels of secretion, posttranslational modifications glycosylation, methylation, a promoter derived from the alcohol oxidase I gene *(AOX1)* of *P. pastoris* that is uniquely suited for the controlled expression of foreign genes that integrate into the *Pichia* genome [6] and the ability to engineer secreted proteins that can be purified from growth medium without harvesting the yeast cells themselves [7]. The used expression vectors have an expression cassette, promoter region, multiple cloning site (for foreign coding sequence), alcohol oxidase open reading frame, α-mating factor and selectable markers [6-8].

Because of its remarkably high thermal resistance *Bacillus licheniformis* α-amylase has become the most widely used in starch liquefaction processes [9]. α-Amylase's official name is 1,4-α-D-Glucan glucanohydrolase; EC 3.2.1.1 [9-11], acts upon large polymers of starch by randomly cleaving α-1,4-glucosidic linkages, to give diverse products including dextrins and progressively smaller polymers composed of glucose units [12-14]. α-Amylases have potential application in wide number of industrial processes such as food, fermentation, textile to paper industries [12,14-17]. However, the cost of producing this enzyme is high [18] due to the extreme living conditions of the *Bacillus* species (high temperature, pH, complex medium) [19].

One possibility to resolve these problems is the heterologous expression of the thermostable enzyme. The expressed α-amylase used in starch industry must be active and stable at low pH and high temperature.

Expression of a foreign gene in *P. pastoris* requires three basic steps: (a) the insertion of the gene into an expression vector; (b) integration of the expression vector into the *P. pastoris* genome; and (c) selection of potentially expressing strains for the foreign gene [7]. In this article, we report a simple, efficient, economical and industrially applicable and reproducible method for heterologous expression. The α-amylase encoding gene was isolated from the thermophilic bacteria *B. licheniformis* and after transformation into *Escherichia coli* the amylase coding gene was cloned and expressed in *P. pastoris*. The expressed proteins can be concentrated and purified by subjecting the supernatant to ultrafiltration, precipitation, and/or adsorption/elution chromatography [9].

RESULTS AND DISCUSSION

The SamyL gene of *B. licheniformis* was amplified with forward and reverse primers that introduced *Eco*RI and *Xba*I sites respectively.

The small fragment containing the coding sequence for the α-amylase was obtained essentially as described in Experimental section. **Figure 1** shows the PCR products where the reaction was carried out in seven samples and the molecular weight is 1500 bp.

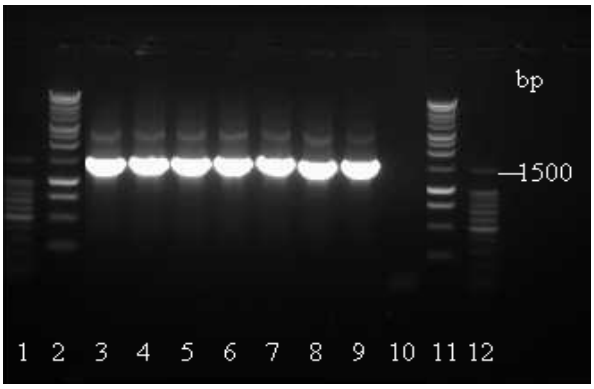

Figure 1. Image of the electrophoresis gel illustrating the PCR reaction results Lanes 1,12: 100 bp DNA ladders; lanes 2,11: 1 kb DNA ladders; lanes 3-9: PCR reaction products; lane10: negative control

Construction of the pPICZαA-SamyL vector

Construction of the recombinant pPICZαA-SamyL vector was performed as described in Experimental section; the total ligation reaction and the control reaction being transformed into chemically competent *Escherichia coli* TOP10F' cells provided in the Invitrogen EasySelect Pichia Expression Kit. 100 µL of each of the heat shocked cell suspensions were placed on solid low salt LB medium with 25 µg/mL Zeocin. On figure 2 the result of the ligation are shown.

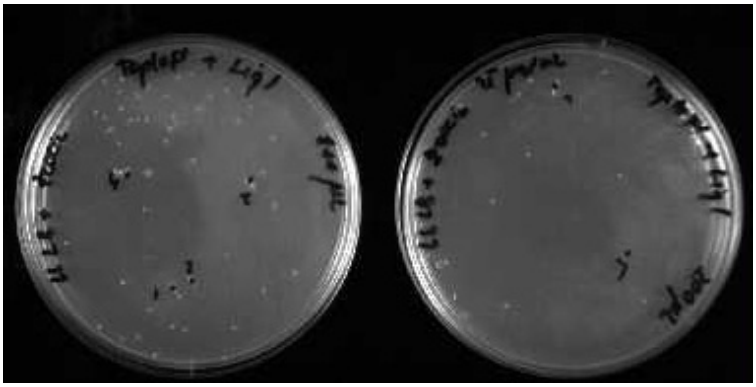

Figure 2. Results of ligation

Minipreps were prepared from 6 colonies on plate as described in Experimental section, and analyzed for the presence of the insert by double digestion with *Xba*I and *Eco*RI, respectively in order to check for the correct

conformation of the obtained recombinant plasmid and for the presence of the insert. Minipreps were obtained in 50 µL of TE buffer, from which 4 µL were loaded on an 1% TAE agarose gel and separated by electrophoresis at 7 V/cm^2 voltage. The **figure 3** (system GelDoc, BioRad) illustrates the fragments that were used to construct the vector and the results from the digestion reactions.

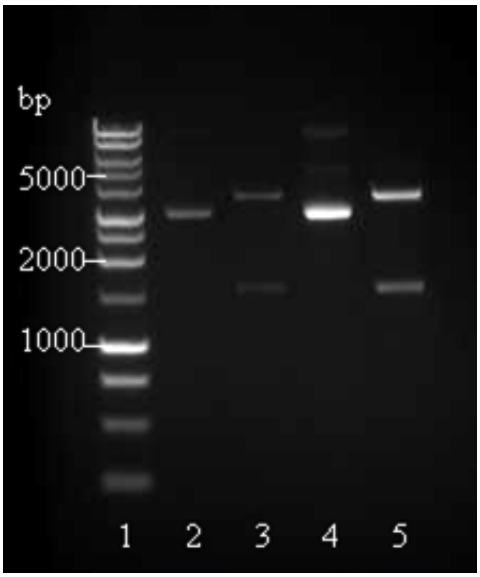

Figure 3. Illustration and verification of the obtained pPICZαA-SamyL expression vector

Lane 1: 1 kb DNA ladder (Promega);
lanes 2,4: undigested pPICZαA-SamyL;
lanes 3,4: digested with *Xba*I and *Eco*RI

We can observe that the coding sequence of the α-amylase is integrated into the vector and the double digested fragment size is 1500 bp.

Expression of α-amylase in Pichia pastoris

The obtained pPICZαA-SamyL vector was linearized with the restriction endonuclease *Sac*I, which cleaves the *AOX1* site. Digestion reaction with *Sac*I was performed as described in Experimental section and verified on a 1% agarose gel by electrophoresis. The **figure 4** presents the linearized pPICZαA-SamyL vector.

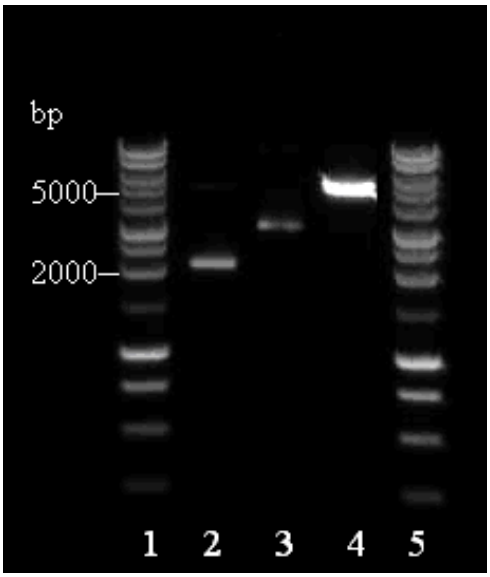

Figure 4. Image of the electrophoresis gel illustrating linearization of the pPICZαA-SamyL vector with *Sac*I

Lanes 1,5: 1 kb DNA ladders (Fermentas);
lane 2: pPICZαB 50 ng;
lane 3: pPICZαA-SamyL;
lane 4: pPICZαA-SamyL *Sac*I digest

Transformation of the *Sac*I digested plasmid pPICZα-SamyL into electrocompetent host cells of *P. pastoris* resulted in over one hundred colonies on YPD (Zeocin 100 μg/mL). Ten of these colonies were randomly chosen for screening by PCR.

Total genomic DNA was isolated in order to screen by PCR reaction for those transformants, which have successfully integrated the α-amylase coding sequence into their genome. The PCR reaction was set up including the recombinant pPICZαA-SamyL vector as a template for the positive control reaction. On **figure 5** is presented the electrophoresis gel with the PCR reaction products.

As shown on figure 5, two (colonies nr. 6,7) of the ten transformed colonies resulted the expected PCR products, representing the integrated coding sequence of the amylase. In consequence, production cultures were prepared starting from colony nr. 6 in order to achieve extracellular expression of the target protein.

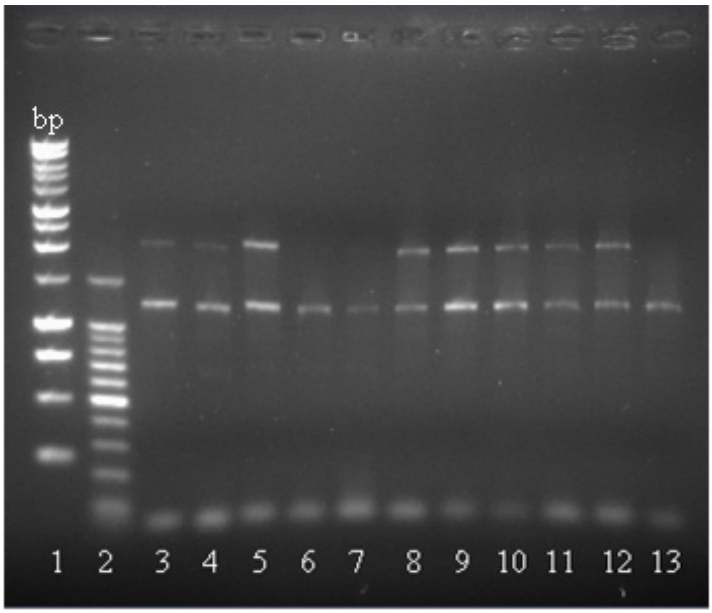

Figure 5. Illustration of the PCR screening results for genomic integration of the target sequence

Lane 1: 1 kb DNA ladder (Promega);
lane 2: 100 bp DNA ladder (Promega);
lanes 3-12: PCR products of genomic DNA from 10 transformed colonies;
lane 13: positive control

Expression was carried out as described in Experimental section, in a final volume of 100 mL in BMMY inducer medium. As temperature has a major effect on induction of the *AOX1* promoter, the cultures were grown at 30ºC; continuous induction was achieved by supplementing the growth medium with 0.5% of methanol every 24 hours. Presence of the protein of interest in the culture supernatant was verified by SDS-PAGE; 1 mL of the culture have been sampled before induction and every 24 hours from the induced culture, samples from the supernatant containing secreted proteins were analyzed on 12.5% acrylamide gels after TCA precipitation, adding equal volume of SDS-sample buffer to the samples. According to the data obtained with the ProtParam Tool (Expasy Proteomics Server), the molecular weight of the expressed amylase is in the range of 58 kDa. In figure nr. 6, results obtained after electrophoretic separation of the extracellularry-produced proteins are presented.

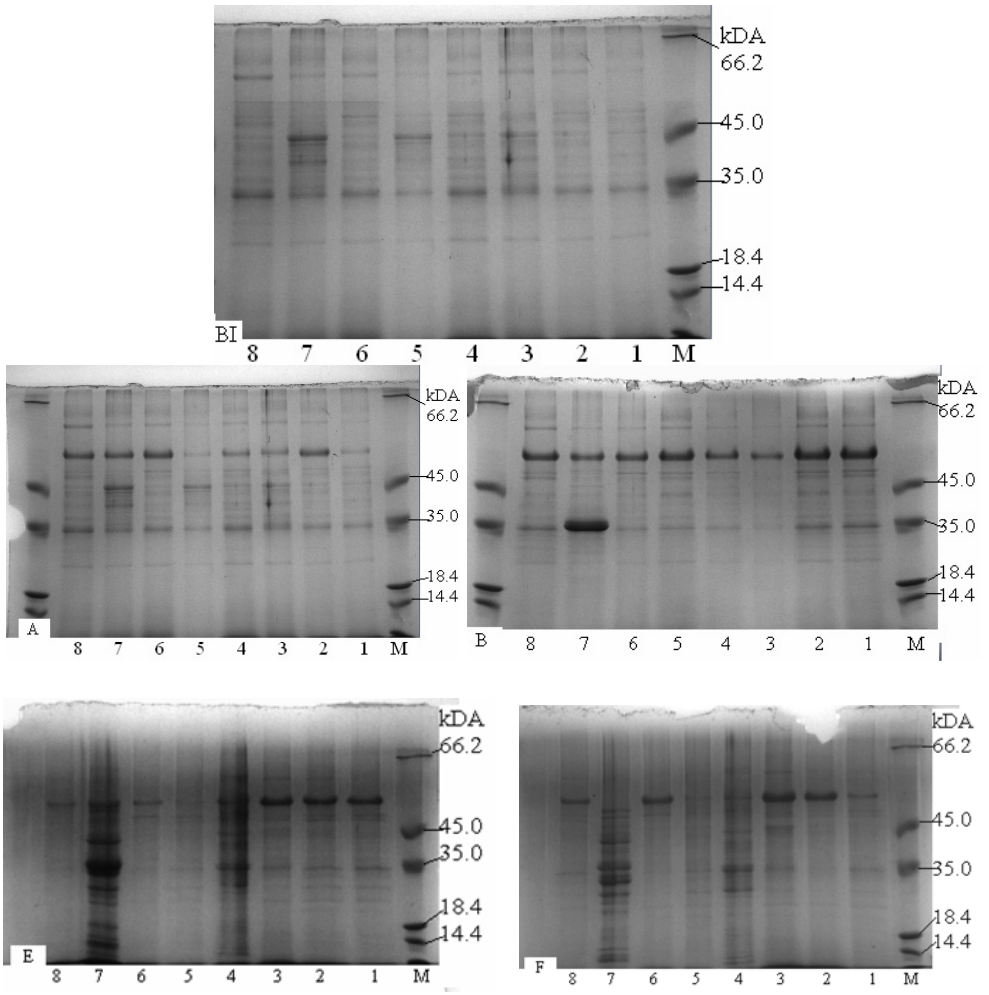

Figure 6. Illustration of the obtained proteins from culture supernatants of *Pichia* X33 transformants Part BI: before induction; Part A: secreted proteins of X33/6 after 1 day of induction; lane M: molecular weight marker; lanes 1-8: different colonies secreted proteins; Part B: secreted proteins of X33/6 after 2 days of induction; Part E: secreted proteins of X33/6 after 5 days of induction; Part F: secreted proteins of X33/6 after 6 days of induction

Analyzing the obtained proteins we can observe that after adding 0.5% methanol the expression has started. The amount of the expressed amylase was decreasing after the 5'th and 6'th days, but we can observe that in case of the 2,3,6 and 8 colonies the protein quantity remain constant.

CONCLUSIONS

In the present study we demonstrate a practical procedure for high-level production of full-length recombinant proteins from *Pichia pastoris*. The α-amylase coding gene of *Bacillus licheniformis* was cloned and expressed successfully in *P. pastoris* by using the pPICZαA vector. The α-amylase genes encoding the plasmids were integrated into the yeast genome as a result of genetic recombination and were replicated autonomously.

The *Pichia* expression system was considered to provide high-level extracellular expression of a heterologous protein offering simple purification possibilities. The data presented in the Results chapter show that construction of the recombinant pPICZαA-SamyL vector was completed successfully. The restriction analysis of the isolated vector gave results that confirm the calculated lengths of the restriction fragments. All digestion reactions yielded fragments with the expected length, the restrictase *Sac*I was chosen to linearize the vector.

Our expression experiments revealed that single transformant colony showed large variations in expression levels after few days. Transformation of electrocompetent *Pichia* X33 cells was completed successfully; expression of the α-amylase protein proved to be successful in *Pichia* X33 strain.

Future application of this enzyme could be considered as a saccharifying enzyme in the starch syrup production [1,6]. Further study will be required to determine the structure, the activity and optimum pH range of the expressed amylase.

EXPERIMENTAL SECTION

Plasmids are the most frequently used cloning and expression vectors, due to their reliability. In the past three decades there have been a number of straightforward methods described for cloning into plasmid vectors.

The *Bacillus licheniformis* amylase gene was a generous gift from University of Szeged. *P. pastoris* X33 and pPICZαA purchased from Invitrogen were used as host and vector for heterologous expression of the amylase. Chemically competent *E. coli* TOP10F' was used for plasmid construction. The easiest and most successful cloning strategy is based on use of two different restrictases on both the carrier and the passenger DNA molecule, which create noncomplementary protruding termini, making the further ligation reaction more effective.

Construction of the pPICZαA-SamyL vector

Amplification of the SamyL gene was performed using (Palm Cycler, Corbett Research) thermocycler with the oligonucleotids (primers)

Samyl_R1 R, (5' CCC T_CT AGA GAT CTT TGA ACA TAA ATT GAA ACC 3') and Samyl_F2 F, 5' GC G_AAT TCA ACA TCA AAA AGT TTG CAA AAC AAG C 3' designed to incorporate *Xba*I and *Eco*RI restriction sites to the PCR product. The PCR reaction was realised in a total volume of 50 µL (0.1 µg template, 0.2 mM dNTP mix, 2.5 mM $MgCl_2$, 0.5 µM oligonucleotides, 5U/µL aTaq polimerase, Promega).

For the construction of the pPICZαA-SamyL vector, the restriction endonucleases *Eco*RI and *Xba*I were used; in the first step the α-amylase vector, containing the target gene was digested with the two enzymes, resulting in the small fragment. 10 µg of plasmid DNA was digested with 10 U of *Xba*I (Fermentas) in 100 µL of 1x Red buffer (Fermentas), at 37°C for 4 hours, and verified by an 1% agarose gel electrophoresis; the digest was precipitated with ethanol, redissolved in 50 µL 1xY Tango buffer (Fermentas) and further digested with 10 U of *Xba*I for 4 hours at 37°C, and 2 µL were analysed by 1% agarose gel electrophoresis in TAE (Tris-acetate-EDTA) buffer with ethidium-bromide staining and visualized by GelDoc System from BioRad.

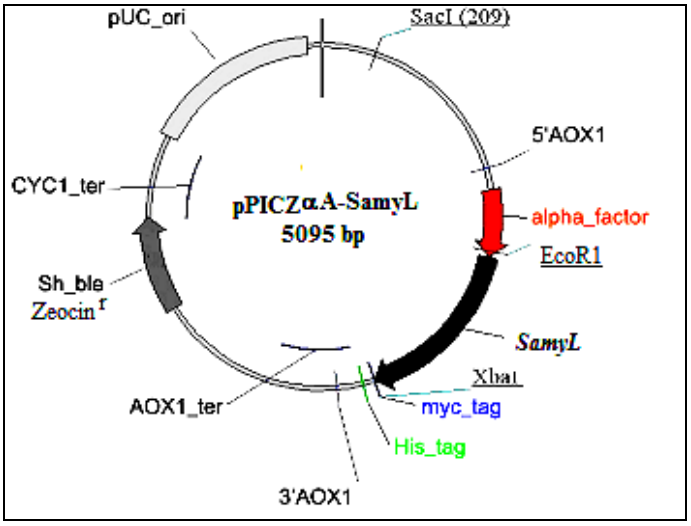

Figure 7. Map of pPICZαA-SamyL

The figure 7 summarizes the features of the vector. The vector map was designed with the VNTI software on the basis of data provided by Invitrogen in the users manual EasySelect *Pichia* Expression Kit.

The total amount of the digestion mixture was separated on a preparative 1% agarose gel and the 1500 bp small fragment containing the coding sequence was excised and isolated by the MiniElute Gel Extraction Kit (Qiagen) according to the instructions of the manufacturer. The isolated fragment

was obtained in 50 µL of TE buffer (Tris-EDTA, Fluka). For the preparation of the large fragment, 100 µg of pPICZαA (Invitrogen) plasmid DNA were digested with 10 U of *Eco*RI (Fermentas) and *Xba*I (Fermentas) in 1x Y Tango Buffer (Fermentas) for 4 hours at 37°C; the large fragment was obtained as described above in 30 µL of TE buffer. The purity of both isolates (large and small fragment) was verified by gel electrophoresis. The ligation reaction consists in formation of new phospohodiesther bonds between the 5' termini and 3' hydroxyl groups of the double stranded DNA molecules, which is carried out by the enzyme ligase, among which the T4 phage ligase is considered highly effective, thus frequently used in molecular biology studies. The ligation reaction was set up in a total volume of 20 µL, containing 14 µL of small fragment, 2 µL of pPICZαA large fragment and 1 µL T4 ligase (Invitrogen) in 2 µL 10x Ligation Buffer (Invitrogen) at 25°C for 1 hour, together with one control ligation without the small fragment. The ligation and control mixtures were transformed into 100 µL of chemically competent *E. coli* Top10F' cells (Invitrogen) and plated on low salt LB medium (Bactotryptone 1%, Yeast extract 0.5%, NaCl 0.5%, pH 7, Fluka) with 25 µg/mL Zeocin (Invitrogen). To isolate the recombinant plasmid, 6 transformed colonies were picked up with sterile toothpicks and grown overnight (12 hours) in 5 mL of low salt LB with Zeocin (25 µg/mL), at 37°C and 150 rpm. Cells were harvested by centrifugation at 5000 rpm for 2', plasmid DNA was isolated and purified by the Plasmid Miniprep Kit (Qiuagen), according to the instructions of the manufacturer. The plasmid preparates were obtained in 50 µL of TE buffer and verified by agarose gel electrophoresis. Insertion of the fragment of interest and the correct assembling of the recombinant plasmid were checked by double digestion with *Xba*I and *Eco*RI for the presence of the insert, and loaded for verification on a 1% agarose gel together with the obtained minipreps for separation by electrophoresis.

Extracellular expression in Pichia pastoris

For high-level extracellular secretion of the α-amylase, *Pichia pastoris* strain X33 with *his4* genotype, His^+, Mut^+ phenotype was used (Invitrogen). As *Pichia* strains express only a low number of native proteins and also at low levels extacellularry, obtention of the target protein in a secreted form assures simple and effective isolation and purification of the product. Transformation into *Pichia* strains requires linearization of the recombinant vector, as it integrates the coding sequence of interest via homologous recombination between the transforming DNA and regions of homology within the genome. Linearization of the recombinant pPICZαA-SamyL vector was carried out by digestion with the restriction endonuclease *Sac*I*,* which has a unique restriction site at the *AOX1* locus of the vector, thus permits efficient integration into

the *Pichia* genome. The digestion reaction was set up as follows: 40 µL of isolated plasmid preparate was digested with 5 µL of 15 U of *Sac*I (Sigma) in 6 µL of 10x *Sac*I Buffer (Sigma) in a total volume of 61 µL for 2 hours at 37ºC, and checked by agarose gel electrophoresis. The resulted linearized plasmid DNA was further used for transformation of the chosen *Pichia* strain X33. Electroporation of the *Pichia* strain was carried out using a BioRad GenePulser eletroporation apparatus, electrocompetent *Pichia* X33 cells being obtained by a modified protocol published in the *Pichia* EasySelect Expression Kit from Invitrogen.

Preparation of electrocompetent cells was carried out by diluting a fresh overnight culture of X33 cells to OD_{600} = 0.1 in a total volume of 100 mL of YPD media, incubating overnight again at 30°C, until an OD_{600} of 1.5. Cells were harvested by centrifugation at 1500xg for 5 minutes at +4°C, then resuspended in 100 mL of ice-cold sterile water. Cells were centrifuged again as in the previous step and the pellet resuspended in 25 mL of sterile water; followed by centrifugation as above and resuspension in 4 mL of ice-cold 1 M sorbitol (Sigma). After a final centrifugation at 1500xg for 5 minutes at +4°C, the cell pellet was resuspended in 200 µL of 1 M sorbitol, and aliquots of 80 µL were used for transformation by electroporation. For every transformation 80 µL of electrocompetent *Pichia* cells were used by adding 10 µL (~5 µg) of linearized pPICZαA-SamyL recombinant vector into chilled 2 cm electroporation cuvettes (BioRad) with linearized plasmids using a Bio-Rad GenePulser, at C = 25 µF; PC = and kept on ice for 5 minutes. The cells were transformed 200 Ω, V = 2.0 kV with a time constant of 5.2 ms.

They were allowed to recover in 1 mL of ice-cold 1 M sorbitol, the cell suspension was transferred into a sterile tube and incubated at 30°C for 1,5 hours without shaking.

Transformants were selected on YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% agar-agar, reagents from Merck) agarized plates containing 100 µg/mL Zeocin. Control transformation reactions were also set up, containing no plasmid DNA; incubation at 30°C was prolonged until colonies were visible (on average 3-4 days).

PCR analysis of *Pichia* integrants was performed in order to scan for transformed colonies, which have integrated the amylase coding sequence into their genome. The 5' *AOX1* primer 5'- respectively the 3' *AOX1* primer were used. Genomic DNA was isolated from 10 transformed X33 colonies by the Wizard Genomic DNA Purification Kit (Promega), according to the instructions of the manufacturer; purified genomic DNA was obtained in a volume of 50 µL. The PCR reaction was set up as follows: 5 µL 10x Taq buffer, 5 µL 25 mM $MgCl_2$, 1 µL 25 mM dNTP, 1 µL of 5', respectively 3' *AOX1* primer (10 pmol/µL), 5 U Taq Polymerase and 1 µg of template genomic DNA for a reaction volume of 50 µL. For amplification controls 100 ng of recombinant

plasmid pPICZαA-SamyL (positive control). The PCR reaction was performed in Corbett Research Thermal Cycler, with the following program: 1 cycle of heat soak at 94°C 2 min, 25 cycles of denaturation at 94°C 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and 1 cycle of final extension at 72°C for 7 min. PCR products were loaded onto an 1% TAE agarose gel for electrophoresis.

Controlled expression of the recombinant protein was achieved by use of the methanol-inducible promoter *AOX1,* which is known to yield highest expression levels upon induction with 0.1-5% methanol. To ensure derepression, the recombinant colonies were firstly grown on complex media supplemented with glycerol.

Small-scale expression experiments of the recombinant *Pichia* strains were carried out in glass reaction tubes. The inoculums was prepared by transfer of a single recombinant colony to 10 mL BMGY (Buffered Glycerol-Complex Medium: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% Yeast Nitrogen Base, 4 x 10^{-5}% biotin, 1% glycerol, all reagents from Merck); to ensure good aeration, baffled flasks were used in which the culture volume did not exceed 20% of the flask volume. Cultures were grown at 30°C with shaking at 150 rpm (CERTOMAT BS-T, SARTORIUS) until OD_{600} reached 3-4 (16-20 hours), corresponding to log-phase growth.

Cells were harvested by centrifugation at 3000xg for 5', and the supernatant was treated with trichloracetic acid (TCA) to extract total protein. The expression experiments was prepared by cell resuspension in 100 mL inducer medium BMMY (Buffered Methanol-Complex Medium: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% Yeast Nitrogen Base, 4 x 10^{-5}% biotin, 0.5% glycerol, all reagents from Merck) to an OD_{600} of 1 to induce expression. *Pichia* cultures were further incubated at 30°C with shaking at 150 rpm and grown in shake flasks with baffles for expression of the α-amylase up to 6 days. To maintain induction, the media was supplemented every 24 hours with 0.5% methanol; 1 mL samples for further analysis by SDS-PAGE were taken before induction and every 24 hours post-induction, centrifuged at maximum speed in a micro centrifuge (Hettich), and both the pellets and the separated supernatant were stored at -80°C until assaying. After 6 days of expression, culture supernatants were separated by centrifugation and stored at -80°C until purification procedures.

SDS-PAGE

Denaturation of the proteins and a linear structure, as well as negative surface charge is assured by the β-mercaptoethanol and SDS loading buffer contained in the loading buffer, as well as by keeping the samples for 10' at 95°C.

Separation of the proteins was carried out in 12.5% polyacrylamide running gel (acrylamide-bis-methylene-acrylamide monomer (Sigma Aldrich), SDS 10%, ammonium persulphate, TEMED), in a 2 phase-2 electrolyte system (electrophoresis apparatus – BioRad Tetra cell). 15 µL of each sample was run at one time, together with a molecular weight marker (Promega), with the parameters of the electromagnetic field of 30 mA constant amperage and 150 W power.

Comassie Blue G250 (Sigma Aldrich) dyeing was used for visualization of the electrophoretic bands, destaining with 50% ethanol and 10% acetic acid solution.

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