UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Luka KRANJC

HETEROLOGOUS EXPRESSION OF VINYLPHENOL REDUCTASE OF YEAST *Brettanomyces bruxellensis* IN YEAST *Saccharomyces cerevisiae*

DOCTORAL DISSERTATION

Ljubljana, 2016

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HETEROLOGNA EKSPRESIJA ENCIMA VINILFENOL REDUKTAZE KVASOVKE *Brettanomyces bruxellensis* V KVASOVKI *Saccharmyces cerevisiae*

DOKTORSKA DISERTACIJA

Ljubljana, 2016

On the basis of the Statute of the University of Ljubljana and by decisions of the Senate of the Biotechnical Faculty and the decision of University Senate, dated from November, 13th, 2013, the continuation to Interdisciplinary Doctoral Programme in Biosciences, field: Biotechnology, was approved. Prof. Dr. Peter Raspor, as the supervisor, and Assist. Prof. Dr. Krešimir Gjuračić, as the co-advisor, were confirmed.

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Senata Univerze z dne 13.11.2013 je bilo potrjeno, da kandidat izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študijskem programu Bioznanosti, znanstveno področje biotehnologija. Za mentorja je bil imenovan prof. dr. Peter Raspor ter za somentorja doc. dr. Krešimir Gjuračić.

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Date of defense (datum zagovora):

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Luka Kranjc

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AB Yeast *Brettanomyces bruxellensis* is one of the most common wine spoilage causing microorganisms due to the formation of high levels of volatile phenols, namely 4-ethylphenol which contributes to huge economic losses in wine industry worldwide. However, in low concentrations 4-ethylphenol imparts smoky and leathery aromas in wine, which contribute to the complexity and can be characteristic for certain types of red wines. The conversion of 4-vinylphenol to 4-ethylphenol is catalyzed by vinylphenol reductase enzyme. Nevertheless, use of the *B. bruxellensis* in wine industry is not desired, mainly because these yeasts also form the opacity of wine, high levels of acetic acid and tetrahydropiridines. In addition, they are very difficult to control. A genetically engineered wine strain of *Saccharomyces cerevisiae* that would be able to produce low levels of 4-ethylphenol might present the solution for winemakers who want to achieve desired complexity without use of *B. bruxellensis*. In our study we evaluated selected *B. bruxellensis* strains for their ability to convert 4-vinylphenol to 4-ethylphenol through relative conversion rates. We measured a number of viable cells and changes in 4-vinylphenol and 4-ethylphenol concentrations in static fermentation cultures under microaerobic conditions in defined synthetic medium supplemented with 4-vinylphenol. The results obtained showed that there were differences in the conversion rates among different strains, indicating differences in the vinylphenol reductase activity. The comparison of the proposed vinylphenol reductase gene sequences of different *B. bruxellensis* strains revealed differences in the nucleotide composition, which resulted in changes in the amino acid composition and, in some cases, in the length of the protein. This indicated that differences in the specific conversion rates might have emerged due to polymorphisms. We used that knowledge to construct plasmid vectors for high inducible expression of vinylphenol reductase in *S. cerevisiae*. The *in-vivo* activity of the expressed enzymes was assessed by fermentation experiments in the defined synthetic medium and their expression was confirmed by the Western blot analysis; however, no *in-vivo* activity was detected. We repeated the experiments, first with vectors harbouring Cu/Zn superoxide dismutase of *B. bruxellensis*, which was also reported to possess the vinylphenol reductase activity. Since no *in-vivo* enzyme activity was observed, the same experiments were repeated with our own vinylphenol reductase candidates, namely triosephosphate isomerase, putative cytochrome p450 monooxigenase and cytochrome p450, which we obtained through literature search. Although we confirmed the expression of these enzymes, we failed to confirm the *in-vivo* activity of the chosen candidates. In conclusion, our experiments showed that vinylphenol reductase of *B. bruxellensis* remains an elusive and interesting research topic and that additional investigations are needed to discover the potential of this intriguing enzyme.

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AI Kvasovka *Brettanomyces bruxellensis* je zaradi sposobnosti tvorbe visokih koncentracij hlapnih fenolov, predvsem 4-etilfenola, v svetovnem merilu eden izmed najpogostejših vzrokov za kvar vina. Čeprav je visoka koncentracija 4-etilfenola pojmovana kot napaka vina, imajo lahko nizke koncentracije 4-etilfenola pozitiven doprinos h kompleksnosti arome. Nizko koncentracijo 4-etilfenola v vinu zaznamo kot aromo po dimljenem in usnju in je celo značilna za nekatere vrste rdečih vin. Za pretvorbo 4-vinilfenola v 4-etilfenol je v kvasoki *B. bruxellensis* odgovoren encim vinilfenol reduktaza. Kjub potencialno pozitivnem doprinosu k aromi vina je uporaba kvasovke *B. bruxellensis* nezaželena, saj je poleg visokih koncentracij hlapnih fenolov sposobna proizvesti visoke koncentracije drugih neželenih metabolitov, hkrati pa jo je težko vzdrževati v nizkem številu tekom fermentacije vine. Konstrukcija gensko spremenjene kvasovke *Saccharomyces cerevisiae*, ki je sposobna proizvesti majhne količine 4-etilfenola tekom fermentacije vina, bi tako predstavljala rešitev za vinarje, ki si želijo doseči večjo kompleksnost vina, hkrati pa ne želijo prisotnosti kvasovke *B. bruxellensis*. Preverili smo, kakšne so razlike v hitrosti pretvorbe 4-vinilfenola v 4-etilfenol med izbranimi sevi kvasovke *B. bruxellensis*. To smo dosegli s spremljanjem koncentracije živih celic ter sprememb v koncentraciji 4-vinilfenola in 4-etilfenola v statičnih testnih fermentacijah v kemično definiranim gojišču z dodanim 4-vinilfenolom in iz pridobljenih rezultatov izračunali relativno hitrost pretvorbe 4-vinilfenola v 4-etilfenol. Rezultati so pokazali, da obstajajo razlike v hitrosti pretvorbe 4-vinilfenola v 4-etilfenol med različnimi sevi kvasovke *B. bruxellensis*. Sklepali smo, da je to posledica različne encimske aktivnosti encima vinilfenol reduktaza. Primerjava nukleotidnega zapisa za encim vinilfenol reduktaza različnih sevov je pokazala, da se sevi med seboj razlikujejo v zapisu, ki se posledično odraža tudi v aminokislinskem zaporedju ter celo v dolžini samega encima, kar bi lahko vplivalo tudi na njegovo katalitično aktivnost. Sledila je priprava vektorjev za visoko inducibilno izražanje vinilfenol reduktaze v kvasovki *S. cerevisiae*. Fermentacijski poskusi so bili izvedeni v kemično definiranem sintetičnem gojišču, izražanje encima pa potrjeno z analizo po Westernu. Izražanje encima je bilo uspešno, vendar njegove *in-vivo* aktivnosti nismo mogli potrditi. Poskuse smo ponovili s transformantami, z zapisom za Cu/Zn superoksid dismutazo kvasovke *B. bruxellensis*, ki naj bi prav tako vsebovala vinilfenol reduktazno aktivnost. Ker *in-vivo* aktivnosti zopet nismo uspeli potrditi, smo sami skušali najti gene, katerih produkti bi lahko posedovali vinilfenol reduktazno aktivnost. S ponovnim pregledom literature smo prišli do treh kandidatov – trioza fosfat izomeraza, predvidena citokrom p450 oksidoreduktaza in citokrom p450 – eksperimente smo ponovili, vendar *in-vivo* vinilfenol reduktazne aktivnosti nismo mogli potrditi. Rezultati našega eksperimentalnega dela so pokazali, da vinilfenol reduktaza kvasovke *B. bruxellensis* ostaja zanimiva raziskovalna tema ter da bodo potrebne še nadaljne raziskave, preden bomo lahko izkoristili celoten potencial tega izmuzljivega encima.

TABLE OF CONTENTS

KEY WORDS DOCUMENTATION III
KLJUČNA DOKUMENTACIJSKA INFORMACIJA IV
TABLE OF CONTENTS V
LIST OF TABLES VIII
LIST OF FIGURES X
LIST OF ANNEXES XIII
ABBREVIATIONS AND SYMBOLS XIV

1 INTRODUCTION 1
1.1 RESEARCH GOALS AND HYPOTHESES 2

2 LITERATURE OVERVIEW 3
2.1 YEASTS IN WINE FERMENTATION 3
2.2 YEAST *Brettanomyces bruxellensis* 4
2.2.1 *Brettanomyces bruxellensis* in wine 6
2.3 PRODUCTION OF VOLATILE PHENOLS BY MICROORGANISMS 7
2.3.1 Formation of vinylphenols by microorganisms 9
2.3.2 Formation of vinylphenols by *B. bruxellensis* 9
2.3.3 Formation of ethylphenols by microorganisms 10
2.3.4 Vinylphenol reductase of *B. bruxellensis* 10
2.4 GENETIC ENGINEERING OF *Saccharomyces cerevisiae* 11
2.4.1 Vectors 11
2.4.2 Construction of vectors and expression cassettes 13
2.4.3 Integration of the expression cassettes 13
2.4.4 Transformation of *S. cerevisiae* 15
2.4.5 Regulation of protein abundance 16
2.4.6 Use of genetically modified strains in wine industry 17

3 MATERIALS AND METHODS 20
3.1 MATERIALS 20
3.1.1 Microbial strains 20
3.1.1.1 *Brettanomyces bruxellensis* and *S. cerevisiae* strains used for fermentation experiments 20
3.1.1.2 *Escherichia coli* strain used for molecular cloning 21
3.1.1.3 *Saccharomyces cerevisiae* strains used for plasmid construction and heterologous gene expression 21
3.1.2 Culture media 21
3.1.2.1 Standard media 21
3.1.2.2 Differential media 22
3.1.2.3 Selection media 23
3.1.2.4 Fermentation medium 25
3.1.2.5 Media for heterologous expression 25
3.1.2.6 Storage media 27
3.1.3 PCR, RFLP and molecular cloning reagents 28
3.1.3.1 PCR reagents 29

3.1.3.2 Reagents for RFLP analysis 29
3.1.3.3 Plasmids used for heterologous expression 30
3.1.3.4 Control plasmids 30
3.1.3.5 Plasmid isolation 30
3.1.3.6 Reagents for ligation of pRS426-based plasmids 30
3.1.3.7 Chemical transformation reagents 30
3.1.4 Oligonucleotide primers 31
3.1.5 HPLC reagents 33
3.1.5.1 Volatile phenols mobile phase 33
3.1.5.2 Standard solutions for preparation of calibration curves 33
3.1.5.2.1 4-vinylphenol standard curve 33
3.1.5.2.2 4-ethylphenol standard curve 34
3.1.6 Western Blot reagents 34
3.1.6.1 Protein isolation reagents 34
3.1.6.2 SDS-PAGE and protein transfer buffers 35
3.1.7 Other reagents 36
3.1.8 Laboratory materials and equipment 37
3.2 METHODS 39
3.2.1 Isolation and identification of novel *B. bruxellensis* strain 41
3.2.1.1 Isolation of novel *B. bruxellensis* strain 41
3.2.1.2 Isolation of genomic DNA from *B. bruxellensis* 41
3.2.1.3 Identification of *B. bruxellensis* with RFLP method 41
3.2.2 Determination of specific conversion rate of 4-vinylphenol to 4-ethylphenol of *B. bruxellensis* strains 42
3.2.2.1 Inoculum preparation 42
3.2.2.2 Fermentations in defined synthetic medium 42
3.2.2.3 Determination of metabolically active cells with microscope 43
3.2.2.4 Determination of 4-vinylphenol and 4-ethylphenol concentration with HPLC 43
3.2.2.5 Calculation of specific conversion rates 44
3.2.3 Search for additional candidates for vinylphenol reductase genes 44
3.2.3.1 Search for additional candidate genes 44
3.2.3.2 Design of oligonucleotide primers used for sequencing 45
3.2.3.3 Amplification of selected candidate genes 45
3.2.3.4 Sequencing of vinylphenol reductase candidate genes 45
3.2.3.5 Comparison of vinylphenol reductase candidate genes 46
3.2.4 *In-silico* vector construction 46
3.2.5 Plasmid construction and transformation of *E. coli* and *S. cerevisiae* 46
3.2.6 Isolation of genomic DNA from *B. bruxellensis* 46
3.2.6.1 Oligonucleotide primer design for the PCR amplification of candidate genes for plasmid construction 46
3.2.6.2 PCR amplification of plasmid inserts for pYES2.1-based plasmids 47
3.2.6.3 Cloning reaction of pYES2.1-based plasmids and transformation into *E. coli* 47
3.2.6.4 Cloning reaction of pRS426-based plasmids and transformation into *E. coli* 47

3.2.6.5 Amplification, isolation and verification of pYES2.1 based plasmid DNA 49
3.2.6.6 Transformation of *S. cerevisiae* 50
3.2.7 Heterologous expression of vinylphenol reductase in *S. cerevisiae* and confirmation of *in-vivo* enzyme activity through fermentation experiments 51
3.2.7.1 Induction of heterologous expression 51
3.2.7.2 Assessment of conversion of 4-vinylphenol to 4-ethylphenol with HPLC analysis 51
3.2.8 Confirmation of gene expression by Western blot 52
3.2.8.1 Protein isolation 52
3.2.8.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) 52
3.2.8.3 Transfer of protein to nitrocellulose membrane 53
3.2.8.4 Detection of target protein tagged with V5 epitope 53

4 RESULTS AND DISCUSSION 54
4.1 ISOLATION OF A NOVEL *Brettanomyces bruxellensis* STRAIN 54
4.2 DETERMINATION OF VINYLPHENOL REDUCTASE ACTIVITY OF THE SELECTED *B. bruxellensis* STRAINS 54
4.3 NUCLEOTIDE SEQUENCE COMPARISON OF VINYLPHENOL REDUCTASE OF THE SELECTED *B. bruxellensis* STRAINS 59
4.4 *IN-SILICO* PLASMID DESIGN 60
4.5 FERMENTATION EXPERIMENTS 70
4.6 SEARCH FOR ADDITIONAL VINYLPHENOL REDUCTASE CANDIDATES 77
4.6.1 Comparison of *SOD* sequences 77
4.6.2 Search for other candidates with possible vinylphenol reductase activity 78
4.7 DESIGN OF ADDITIONAL VECTORS 79
4.8 FERMENTATION EXPERIMENTS WITH NEW VINYLPHENOL REDUCTASE CANDIDATES 83
4.9 GENERAL DISCUSSION 88

5 CONCLUSIONS 91

6 SUMMARY (POVZETEK) 92
6.1 SUMMARY 92
6.2 POVZETEK 93

7 REFERENCES 99

ACKNOWLEDGEMENTS

ANNEXES

LIST OF TABLES

Table 1: List of *B. bruxellensis* strains with whole genome sequences 6

Table 2: Strains of *B. bruxellensis* and *S. cerevisiae* used for fermentation experiments with their designation and origin 20

Table 3: Genotype and references of *S. cerevisiae* T334 and YPH250 strains 21

Table 4: Composition of solid YPD medium (Kurtzman et al., 2011) 22

Table 5: Composition of liquid YPD medium (Raspor and Smole Možina, 1993) 22

Table 6: Composition of DBDM (Rodrigues et al., 2001) 23

Table 7: Composition of oxytetracycline glucose yeast extract agar 23

Table 8: Composition of solid SC-URA medium 24

Table 9: Composition of LB solid medium supplemented with ampicillin (Sambrook et al., 1989) 24

Table 10: Composition of LB liquid medium supplemented with ampicillin (Sambrook et al., 1989) 25

Table 11: Composition of YNB (Yeast Nitrogen Base) medium supplemented with 4-vinylphenol 25

Table 12: Composition of liquid SC-URA medium 26

Table 13: Composition of liquid SC-URA medium supplemented with 2 % galactose and 10 mg/L 4-vinylphenol 26

Table 14: Composition of liquid SC-URA medium supplemented with 2 % galactose, 2 % glucose and 10 mg/L 4-vinylphenol 27

Table 15: Liquid storage media for wild-type yeast cultures 27

Table 16: Composition of *E. coli* storage medium 28

Table 17: Composition of liquid SC-URA medium for storage of yeast transformants 28

Table 18: Polymerases used in PCR amplifications 29

Table 19: 50x Tris Acetic acid EDTA buffer (TAE) 29

Table 20: Agarose gel preparation 29

Table 21: Oligonucleotide primers for amplification of *B. bruxellensis* vinylphenol reductase gene as proposed by Tchobanov et al. (2008) 31

Table 22: Oligonucleotide primers for amplification of internal transcribed spacer sequence of fungal rDNA (Guillamón et al., 1998) 31

Table 23: Oligonucleotide primers supplied by Invitrogen for sequencing of pYES2.1 insert 31

Table 24: Oligonucleotide primers for amplification of alternative vinylphenol reductase candidate genes 32

Table 25: Oligonucleotide primers for pRS426 based expression plasmid construction 32

Table 26: Oligonucleotide primers for verification of correct assembly of pRS426 based expression plasmid 33
Table 27: Composition of 0.25 M NaOH solution with 1 % 2-mercaptoethanol 34
Table 28: Composition of protein resuspension buffer 34
Table 29: Composition of 10x SDS-PAGE buffer (CSH Protocols, 2006) 35
Table 30: Composition of 10x protein transfer buffer stock 35
Table 31: Composition of 10x Tris base saline (TBS) buffer 35
Table 32: Composition of protein transfer buffer 35
Table 33: Composition of Ponceau S solution for detection of membrane bound proteins 36
Table 34: Composition of 1x TBS buffer supplemented with 3 % powdered milk (TBSM) 36
Table 35: 6x SDS sample buffer 36
Table 36: Table of frequently used materials 37
Table 37: Table of equipment used for experimental work 38
Table 38: Preparation of acrylamide gel for SDS-PAGE 53
Table 39: Table of pYES2.1 and pRS426-based plasmids created in this study, listing their respective plasmid backbones and gene insert sources 61
Table 40: Table of pRS416-based plasmids designed in this study 66
Table 41: Table of pYES2.1-based plasmids created in this study, listing their respective plasmid backbones and gene insert sources 79

LIST OF FIGURES

Figure 1: Formation of 4-ethylphenol through conversion of *p*-coumaric acid (Suárez et al., 2007) 9

Figure 2: Flow-chart representing course of the experiments (*VPR* – vinylphenol reductase) 40

Figure 3: Growth curves during 6 day fermentation in static 250 mL Erlenmeyer flasks in 200 mL of YNB medium supplemented with 20 g/L glucose and 6 mg/L 4-vinylphenol under microaerobic conditions. Each growth curve represents either one of the *B. bruxellensis* strains or *S. cerevisiae* control strain. 55

Figure 4: Consumption curves of 4-vinylphenol (A) and production curves of 4-ethylphenol (B) during 6 day fermentation in static 250 mL Erlenmeyer flasks in 200 mL of YNB medium supplemented with 20 g/L glucose and 6 mg/L 4-vinylphenol under microaerobic conditions. Each curve represents either one of the *B. bruxellensis* strains or *S. cerevisiae* control strain or control flask without inoculum. 56

Figure 5: Consumption rates of 4-vinylphenol (A) and specific production rates of 4-ethylphenol (B) during 6 day fermentation in static 250 mL Erlenmeyer flasks in 200 mL of YNB medium supplemented with 20 g/L glucose and 6 mg/L 4-vinylphenol under microaerobic conditions. Each curve represents one of the *B. bruxellensis* strains. 57

Figure 6: Dendrogram representing distances among vinylphenol reductase longest ORF sequences of the selected *B. bruxellensis* strains performed in Unipro UGENE software with PHYLIP Neighbour Joining method. 60

Figure 7: Plasmid maps, constructed with Unipro UGENE software, representing plasmids pYES2.1_VPR_ORF1_V5 and pYES2.1_VPR_ORF1 for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae*. 62

Figure 8: Plasmid maps, constructed with Unipro UGENE software, representing plasmids pYES2.1_VPR_ORF2_V5 and pYES2.1_VPR_ORF2 for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae*. 63

Figure 9: Plasmid maps, constructed with Unipro UGENE software, representing plasmids pYES2.1_VPR_ORF3_V5 and pYES2.1_VPR_ORF3 for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae*. 64

Figure 10: Plasmid map, constructed with Unipro UGENE software, representing pRS426_VPR plasmid for high constitutive expression of vinylphenol reductase gene of *B. bruxellensis* in *S. cerevisiae.* 65

Figure 11: Plasmid maps, constructed with Unipro UGENE software, representing pRS416_P(GPD)_VPR and pRS416_P(CYC1)_VPR plasmids for high (GPD) and low (CYC1) constitutive expression of vinylphenol reductase gene of *B. bruxellensis* in *S. cerevisiae.* 67

Figure 12: Plasmid maps, constructed with Unipro UGENE software, representing pRS416_P(CIT2)_VPR and pRS416_P(ADH1)_VPR plasmids for low constitutive expression of vinylphenol reductase gene of *B. bruxellensis* in *S. cerevisiae.* 68

Figure 13: Plasmid maps, constructed with Unipro UGENE software, representing pRS416_ura3Δ::KanMX4 plasmid for low constitutive expression of vinylphenol reductase from *B. bruxellensis* in *S. cerevisiae.* 69

Figure 14: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES_VPR_ORF1_V5_701 (A), pYES_VPR_ORF1_V5_2499 (B) and pYES_VPR_ORF1_V5_1762 (C). 71

Figure 15: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_VPR_ORF2_V5_701 (A), pYES2.1_VPR_ORF2_V5_2499 (B) and pYES2.1_VPR_ORF2_V5_1762 (C). 72

Figure 16: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_VPR_ORF3_V5_701 (A), pYES2.1_VPR_ORF3_V5_2499 (B) and pYES2.1_VPR_ORF3_V5_1762 (C). 73

Figure 17: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_VPR_ORF1_2499 (A), pYES2.1_VPR_ORF2_2499 (B) and pYES2.1_VPR_ORF3_2499 (C). 74

Figure 18: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* YPH250 transformants, containing plasmids pYES2.1_VPR_ORF1_V5_2499 (A), pYES2.1_VPR_ORF2_V5_2499 (B), pYES2.1_VPR_ORF3_V5_2499 (C), pYES2.1_VPR_ORF1_2499 (D), pYES2.1_VPR_ORF2_2499 (E) and pYES2.1_VPR_ORF3_2499 (F). 75

Figure 19: Dendrogram presenting distances among Cu/Zn superoxide dismutase longest ORF sequences of different *B. bruxellensis* strains performed in Unipro UGENE software with PHYLIP Neighbour Joining method. 78

Figure 20: Plasmid maps, constructed with Unipro UGENE software, representing pYES2.1_SOD_V5 and pYES2.1_SOD plasmids for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae.* 81

Figure 21: Plasmid maps, constructed with Unipro UGENE software, representing pYES2.1_TPI_V5 and pYES2.1_PCp450M_V5 plasmids for high inducible expression of vinylphenol reductase candidate genes of *B. bruxellensis* in *S. cerevisiae.* 82

Figure 22: Plasmid map, constructed with Unipro UGENE software, representing pYES2.1_Cp450_V5 plasmid for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae.* 83

Figure 23: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_SOD_V5 (A) and pYES2.1_SOD (B). 84

Figure 24: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_TPI_V5 (A), pYES2.1_PCp450M_V5 (B) and pYES2.1_Cp450_V5 (C). 85

Figure 25: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* YPH250 transformants, containing plasmids pYES2.1_SOD_V5 (A), pYES2.1_SOD (B). 86

Figure 26: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* YPH250 transformants, containing plasmids pYES2.1_TPI_V5 (A), pYES2.1_PCp450M_V5 (B) and pYES2.1_Cp450_V5 (C). 87

LIST OF ANNEXES

ANNEX A: Sample standard curves for determination of 4-vinylphenol and 4-ethylphenol concentration

ANNEX B: Sample picture of viable *B. bruxellensis* cells, stained with methylene blue solution that was used for determination of viable cell concentration with ImageJ software

ANNEX C: Picture of agarose gel with RFLP profiles for species determination of potential *B. bruxellensis* candidate colonies

ANNEX D: Pictures of agarose gels with PCR amplicons of the vinylphenol reductase candidate genes of *B. bruxellensis*

ANNEX E: Sample pictures of agarose gels with PCR amplicons that were used for construction and verification of pRS426-based plasmid

ANNEX F: Sample alignment region of vinylphenol reductase sequences from different *B. bruxellensis* strains aligned in Unipro UGENE program

ANNEX G: Alignment of amino acid sequences of vinylphenol reductase enzyme from different *B. bruxellensis* strains performed with EMBL-EBI MUSCLE software

ANNEX H: Graphical representation of codon used between *S. cerevisiae* (red) and *B. bruxellensis* (black) as determined with GCUA – Graphical Codon Usage Analyzer software

ANNEX I: Sample alignment region of Cu/Zn superoxide dismutase sequences from different *B. bruxellensis* strains aligned in Unipro UGENE program

ANNEX J: Alignment of amino acid sequences of Cu/Zn superoxide dismutase enzyme from different *B. bruxellensis* strains performed with EMBL-EBI MUSCLE software

ANNEX K: Plasmid maps of plasmids for positive (pYES2.1_RAD59) and negative (pYES2.1_neg._control) of gene expression constructed with Unipro UGENE software

ANNEX L: Fermentation experiments in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L glucose and 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C at 160 rpm. Fermentations were performed with ZIM 2512 positive control (A) and *S. cerevisiae* T334 transformed with negative control plasmid pYES2.1_neg._control (B)

ANNEX M: Sample pictures of nitrocellulose membrane with heterologously expressed proteins hybridized with anti-V5-HRP antibodies and visualized with chromogenic substrate

ABBREVIATIONS AND SYMBOLS

4EP	4-ethylphenol
4VP	4-vinylphenol
ADH1	Gene encoding alcohol dehydrogenase in *Saccharomyces cerevisiae*
AmpR	Gene encoding ampicillin resistance; β-lactamase
CIT1	Gene encoding citrate synthase in *Saccharomyces cerevisiae*
Cp450	Cytochrome p450 from *Brettanomyces bruxellensis*
CYC1	Gene encoding cytochrome c in *Saccharomyces cerevisiae*
DBDM	*Dekkra Bruxellensis* Differential Medium
GAL1	Gene encoding galactokinase from *Saccharomyces cerevisiae*
dH_2O	Distilled water
HPLC	High-performance liquid chromatography
GPD	Gene encoding glyceraldehyde-3-phosphate dehydrogenase in *Saccharomyces cerevisiae*; also known as *TDH3*
ITS	Internal transcribed spacer
kanMX	Hybrid gene encoding G418 and kanamycin resistance; aminoglycoside phosphotransferase under the control of *TEF* promoter from *Ashbya gossypii*
ORF	Open reading frame
PAD	Gene encoding phenolic acid decarboxylase in various microorganisms
PCp450M	Gene encoding putative cytochrome p450 monooxigenase in *Brettanomyces bruxellensis*
SC-URA	Synthetic Complete drop-out medium without uracil
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Gene encoding Cu/Zn superoxide dismutase in *Brettanomyces bruxellensis*
TBS	Tris buffered saline
TPI	Gene encoding triosephosphate isomerase in *Brettanomyces bruxellensis*
URA3	Gene encoding orotidine-5'-phosphate decarboxylase in *Saccharomyces cerevisiae*
V5	Epitope derived from paramyxovirus of simian virus 5
VPR	Gene encoding vinylphenol reductase from *Brettanomyces bruxellensis*

1 INTRODUCTION

Yeast *Brettanomyces bruxellensis* has long been considered as one of the major spoilage microorganisms that cause taint in wine, resulting in huge economic and material losses in the wine industry. *B. bruxellensis* causes wine spoilage through the formation of cloudiness in wine, acetic acid formation, formation of tetrahydropyridines, biogenic amines and volatile phenols (Suárez et al., 2007). *B. bruxellensis* can tolerate high alcohol content (Childs et al., 2015) and grow in environments with low residual sugars (Wedral et al., 2010), which made this yeast one of the most researched non-*Saccahromyces* wine yeasts in the past decades. Nevertheless, the presence of non-*Saccharomyces* yeast during wine fermentation proved to be beneficial in terms of the formation of desirable aromatic compounds in wine (Childs et al., 2015, Comitini et al., 2011; Dashko et al., 2015; Renault et al., 2015) and many wineries throughout the world have started using spontaneous and mixed-culture fermentations in past few years (Jolly et al., 2014).

The ability of *B. bruxellensis* to produce volatile phenols, namely 4-ethylphenol, which is the most abundant volatile phenol in wine infected with *B. bruxellensis*, is possessed only by a handful of other microorganisms, such as a few species from the genus *Candida*, *Kluyveromyces lactis*, *Debaromyces hansenii* and *Pichia guilliermondii*, although none of these is able to form 4-ethylphenol in the wine medium (Dias et al., 2003). Some species of *Lactobacillus* sp. have also been reported to form 4-ethylphenol; however, these species are rarely present in wine and there are no reports of phenolic taint caused by *Lactobacillus* spp. in wine (Barthelmebs et al., 2000; Couto et al., 2006). Conversion of 4-vinylphenol to 4-ethylphenol in *B. bruxellensis* is performed by enzyme vinylphenol reductase (Godoy et al., 2008; Tchobanov et al., 2008).

High concentrations of 4-ethylphenol are considered as wine flaw, since the aromas can be reminiscent of that of horse sweat, burnt plastic and band-aid®. However, low concentrations of 4-ethylphenol can be regarded as positive in certain types of red wines and are part of "Bordeaux" character (Jolly et al., 2006). When 4-ethylphenol is present in wine in concentrations between 0.23 and 0.68 mg/L, its contribution is considered as positive and can give wine smoky and leather aromas (Suárez et al., 2007).

B. bruxellensis started to gain attention a couple of decades ago, first as lambic beer production organism, then wine spoilage organism and, more recently, as an alternative organism for bioethanol production. All of these resulted in the publication of the first complete genome sequence in 2012 (Curtin et al., 2012). The complex metabolism remains to be connected to genetic information deposited in *B. bruxellensis* genome. Our attempt is just one of the many in the recent years trying to connect physiological and genetic information of this interesting organism.

1.1 RESEARCH GOALS AND HYPOTHESES

B. bruxellensis has been until recently intensively researched mainly because of the ability to form volatile phenols under winemaking conditions (Steensels et al., 2015). There were many research articles in which researchers tried to assess the ability of *B. bruxellensis* to convert 4-vinylphenol to 4-ethylphenol. Some researchers already tried to assess the ability of *B. bruxellensis* to produce volatile phenols (Harris et al., 2008), while others tried to elucidate under which circumstances the formation of 4-ethylphenol could be prevented (Benito et al., 2009). There was also a lot of interest in how the mixed-culture fermentation of *S. cerevisiae* and *B. bruxellensis* affects the formation of volatile phenols (Kosel et al., 2014). The wealth of research on this topic shows the significance of *B. bruxellensis* in winemaking.

Although the high concentration of 4-ethylphenol in wine is considered a wine fault, low concentrations of 4-ethylphenol can make positive contribution to wine aroma (Jolly et al., 2006). Since *B. bruxellensis* produces high concentrations of acetic acid, tetrahydropyridines, biogenic amines and also causes the opacity of wine (Suárez et al., 2007; Oelofse and Pretorius, 2008), its use in winemaking has not been considered yet.

Fortunately, modern molecular techniques allow us to tailor the wine yeast *S. cerevisiae* to enhance the organoleptic, nutritional or technological aspects of wine (Pretorius, 2000; Schuller and Casal, 2005; Donalies et al., 2008). Vinylphenol reductase, the enzyme that is responsible for formation of 4-ethylphenol, has been described by Tchobanov et al. (2008), who also published the nucleotide sequence coding for this enzyme. Since there is a wealth of information on promoters of *S. cerevisiae* (Partow et al., 2010) as well as on promoter engineering (Blazeck et al., 2012), we are able express the desired product in concentrations of our choosing. Through the regulation of expression of vinylphenol reductase enzyme of *B. bruxellensis* in *S. cerevisiae*, we were planning to create a *S. cerevisiae* strain that would be able to produce low enough concentrations of 4-ethylphenol, which would enhance the aroma of wine and would have the potential to be used in the winemaking industry.

We have made the following hypotheses:

- Various strains of *B. bruxellensis* differ both in the activity of the enzyme as well as nucleotide sequence for vinylphenol reductase enzyme
- Active vinylphenol reductase enzyme from *B. bruxellensis* can be expressed in the laboratory strain of *S. cerevisiae*
- Expression of vinylphenol reductase enzymes can be regulated to such an extent that the concentration of 4-ethylphenol is within the limits of acceptability for wine
- Vinylphenol reductase enzyme can also be expressed in the wine strain of *S. cerevisiae*

2 LITERATURE OVERVIEW

2.1 YEASTS IN WINE FERMENTATION

Yeasts are unicellular eukaryotic microorganisms, which are members of fungus kingdom are usually unicellular although some species can also exist in multicellular form (Kurtzman and Fell, 2006). Wine is the result of complex biochemical processes, that starts with grape harvesting, continues with the alcoholic and malolactic fermentations, wine aging and bottling (Romano et al., 2003). This definition of wine clearly states the importance of yeasts in wine fermentations due to their ability to perform alcoholic fermentation with biochemical transformation of grape sugar to ethanol and carbon dioxide, as well as other chemical compounds, which can have significant impact on the sensory properties of wine (Heard and Fleet, 1985; Fleet, 1993, 2003; Fugelsang, 1997). The beginnings of wine production can be traced to as far 7000 years into the history (Jolly et al., 2006) and wine has now become of one the most popular and massively produced alcoholic beverages in the world (Bisson et al., 2002; Chambers and Pretorius, 2010).

Until few decades ago, wine has been made with spontaneous fermentation, which often resulted in poor quality of the end product. Introduction of starter cultures composed of selected strains of *Saccharmoyces cerevisiae* into winemaking industry in 1980's allowed producers to control the process and minimize the impact of other microbial species on wine quality (Reed and Nagodawithana, 1988). Although the introduction of starter cultures allowed producers to reach results that are more constant, it also reduced the complexity of the produced wines. Fortunately, with the development of modern molecular techniques as well as the expanded knowledge of ecology, physiology and biochemistry of yeasts involved in wine production, the use of non-*Saccharomyces* in wine making has regained its interest in the past decade (Fleet, 2008). At the same time, wines produced with spontaneous fermentation as well as biodynamic and "natural" wines gained popularity among the consumers, resulting in the interest of the industry to offer the consumers such products (Jolly et al., 2014).

Although *S. cerevsiae* quickly starts to dominate wine fermentations due to the superior adaptation to grape must and wine environment (Martini, 1993; Cray et al., 2013), up to this date, more than 40 different yeast species have been isolated from grape must, some of which can have significant impact on the final product (Jolly et al., 2006; Ciani et al., 2010). These non-*Saccharomyces* yeasts can be divided into three groups: yeasts, which are predominantly aerobic such as *Pichia* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Candida* spp. and *Cryptococcus albidus*; apiculate yeasts with low fermentative activity such as *Hanseniaspora uvarum*, *Hanseaniaspora guilliermondii*, *Hanseniaspora occidentalis* and yeasts with fermentative metabolism such as *Kluyveromyces marxianus*, *Torulaspora delbruckeii*, *Metchnikowia pulcherrima*, *Brettanomyces bruxellensis* and *Zygosaccharomyces bailii* (Jolly et al., 2014). Microbiological analysis of yeast biota associated with natural fermentation of grape juice revealed that the fermentation of grape juice is conducted in stages – in the first stage, the apiculate yeasts are most abundant; they are replaced in three to four days by *S. cerevisiae* (Pretorius, 2000). Yeasts from other yeast genera, such as *Candida, Pichia, Zygosaccharomyces, Torulaspora, Kluyveromyces* and *Metchnikowia* can be isolated in various stages of fermentation (Ciani et al., 2010).

The presence of these yeasts during wine fermentation can improve the reduction of acetic acid (*T. delbruckeii*; Bely et al., 2008), enhance glycerol content (*C. stellata*; Ferraro et al., 2000), improve the aroma complexity (*H. uvarum*, *P. fermentans*; Mendoza et al., 2007; Clemente-Jimenez et al., 2005), reduce the content of malic acid through malolactic fermentation (*Shizosaccharomyces pombe*; Ciani, 1995). However, some of these yeasts, such as *B. bruxellensis* can also have negative impact on the quality of wine due to formation of high concentrations of volatile phenols (Chatonnet et al., 1995), acetic acid (Uscanga et al., 2007), biogenic amines (Caruso et al., 2002) and tetrahydropyridines (Heresztyn, 1986a) and some species from genus *Zygosaccharomyces*, which can cause the refermentation and CO_2 formation in sweet wines (Zuehlke et al., 2013).

Although a lot of research has been done on the non-*Saccharomyces* yeasts that contribute are present in grape must and during fermentation of wine, most of the research has been done on physiology of these yeasts. Up to this that date, only handful of the these 40 non-*Saccharomyces* yeasts full genome sequences were published, namely *T. delbrukeii*, *H. uvarum*, *H. vinae*, *Lachancea thermotolerans*, *Lachancea kluyveri*, *D. hansenii*, *P. kurdriavzevii*, *P. membranifaciens*, *P. anomala*, *Z. bailii* (Masneuf-Pomarede et al., 2015) and *B. bruxellensis* (Curtin et al., 2012; Piškur et al., 2012; Valdes et al., 2014). Since only a handful of these species have been completely sequenced – in most cases only a single strain – linking of physiological with genetic data currently presents one of the main challenges in this field of research.

Though only yeasts are mentioned in this chapter as contributors to wine sensory properties, lactic acid bacteria, namely bacteria from genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* also have significant impact on sensory properties of wine due to their ability to perform malolactic fermentation (Davis et al., 1988). On the other hand, the presence of acetic acid bacteria is not desired in wine since acetic acid bacteria can produce high levels of acetaldehyde and acetic acid in the presence of oxygen (Bartowsky and Henschke, 2008).

2.2 YEAST *Brettanomyces bruxellensis*

Yeast *Brettanomyces bruxellensis* was first isolated from beer by N. H. Claussen in New Carlsberg brewery in 1904 and he named *Brettanomyces* which translates as "British sugar fungus" and it was thought that this yeast was responsible for characteristic aroma of the finest English stock ales (Gilliland, 1961). When more isolates were obtained from lambic beers in 1921, "*Brettanomyces*" was first proposed as genus name and "*bruxellensis*" as name of the species as a tribute to region known for production of lambic-style beers (Kufferath and Van Laer, 1921). First observation of *B. bruxellensis* in wine happened later (Custers, 1940; Peynaud and Domercq, 1956) and was since then regarded mainly as wine spoilage organism (Loureiro and Malfeito-Ferreira, 2003). Research of the physiology of *B. bruxellensis* regained interest in this yeast in 1960's, when the decrease in glucose consumption in the absence of oxygen was observed in *B. bruxellensis* yeast. When cells were again exposed to low levels of oxygen, the anaerobic fermentation proceeded, with the formation of the acetic acid as a side product. The observed phenomenon was designated as Custers effect (Scheffers, 1961; Scheffers and Wikén, 1969). In recent years, however, *B. bruxellensis* regained its interest as an interesting alternative to standard organisms, that are used in the production of bioethanol (Passoth et

al., 2007), which regained the interest of scientific community, which can be clearly seen in the high number of recent review articles by Zuehlke et al. (2013), Curtin and Pretorius (2014), Schifferdecker et al. (2014), Blomqvist and Passoth (2015) and Steensels et al. (2015).

B. bruxellensis is predominantly found in industrial environments that include alcoholic fermentation, such as cider (Morrissey et al., 2004), kefir (Laureys and De Vuyst, 2014), kombucha (Teoh et al., 2004), bioethanol (de Souza Liberal et al., 2007) and wine (Peynaud and Domercq, 1956). The only other niche that from which *B. bruxellensis* was isolated are grape berries (Renouf and Lonvaud-Funel, 2007). Although *B. bruxellensis* can have positive contribution and is even characteristic for certain types of red wines (Jolly et al., 2006) it has been until recently associated only with wine spoilage (Chatonnet et al., 1992; Suárez et al., 2007).

The taxonomy of genus *Brettanomyces* has seen many changes through the years. Early classification was based on species that reproduced asexually (anamorph form) through multipolar budding (Custers, 1940). When the ascospores were observed in genus *Dekkera*, which meant that it can reproduce sexually (teleomorph form), that was fact was introduced into taxonomy (Van der Walt, 1984). Although *Brettanomyces* genus was composed by many species, current taxonomy includes five species in genera *Brettanomyces*. These are anamorphs *Brettanomyces bruxellensis*, *Brettanomyces anomalus*, *Brettanomyces custercianus*, *Brettanomyces naardenesis* and *Brettanomyces nanus*. There are two existing teleomorphs for the first two species, *Dekkera bruxellensis* and *Dekkera anomala* (Oelofse and Pretorius, 2008). The distinction between *Dekkera* and *Brettanomyces* is not clear as molecular techniques uncovered no variance between anamorph and teleomorph states (Oelofse and Pretorius, 2008). Currently, only *B. bruxellensis* is associated with grape and wine contamination (Egli and Henick-Kling, 2001). Although some of the *Brettanomyces* species have also been classified under teleomorphic genus *Dekkera*, it is likely that the original name will be used in the future according to Melbourne Convention (Daniel et al., 2014). Recent investigation into genome confirmed that *B. bruxellensis* was separate from the Saccharomycetaceae or CTG group defined by Dujon (2010). Comparison of its genome with twenty other fungi by Curtin et al. (2012) revealed that *B. bruxellensis* is most similar to several *Pichia* species while Ravin et al. (2013) discovered that *B. bruxellensis* and *Ogataea polymorpha* exhibit greatest conservation of synteny and likely share the most common ancestor.

Large differences in the physiological behavior were observed among different *B. bruxellensis* strains. Research revealed that there is wide variation in their growth rates (Abbott et al., 2005), the extent of substrate inhibition (Aguilar-Uscanga et al., 2000), their ability to metabolize different carbon sources (Uscanga et al., 2007), ethanol production efficiency (Blomqvist et al., 2010), acetic acid tolerance (Moktaduzzaman et al., 2015) and production rates (Castro-Martinez et al., 2005) and their volatile phenols conversion rates (Agnolucci et al., 2009) to list a few. Large differences in the physiological characteristics among different *B. bruxellensis* strains were attributed to large differences in the genetic makeup of different *B. bruxellensis* strains (Conterno et al., 2006; Curtin et al., 2007; Agnolucci et al., 2009). Hellborg and Piškur (2009) demonstrated, that number of chromosomes can vary among different strains, as the strains can possess from four and up to nine chromosomes, which is rare occurrence among eukaryotic species.

As interest for *B. bruxellensis* started to shift from that of a wine spoilage organism towards a potential bioethanol producing organism due to demonstration of more efficient substrate utilization for ethanol production compared to *S. cerevisiae* (Passoth et al., 2007; Tiukova et al., 2013), first efforts to understand this organism on the genomic level were made. Woolfit et al. (2007) published first partial genomic sequence of the *B. bruxellensis* CBS 2499, covering 40 percent of the genome. In 2012, first whole genome sequences of two *B. bruxellensis* were published by Curtin et al. (2012) and Piškur et al. (2012), which was soon followed by publication of the first transcriptome of *B. bruxellensis* (Tiukova et al., 2013). Since then, whole genome sequences of few other strains were published and are listed in Table 1.

Table 1: List of *B. bruxellensis* strains with whole genome sequences
Preglednica 1: Preglednica sevov *B. bruxellensis* s popolnimi sekvencami genoma

Strain	Genome size (Mb)	Predicted ORFs	Reference
AWRI 1499	12.7	4969	Curtin et al., 2012
CBS 2499	13.4	5600	Piškur et al., 2012
ST05.12/22	13.0	5255	Crauwels et al., 2014
AWRI 1608	-	-	Borneman et al., 2014
AWRI 1613	-	-	Borneman et al., 2014
LAMAP 2480	26.9	12225	Valdes et al., 2014

Allele comparison revealed that all of the sequenced strains are heterozygous. Strains AWRI 1499 and AWRI 1608 are predicted to be triploids (Curtin et al., 2012; Borneman et al., 2014), while CBS 2499 and ST05.12/22 exhibited diploid characteristics (Piškur et al., 2012; Crauwels et al., 2014). Third set of chromosomes of triploid strains AWRI 1499 and AWRI 1608 revealed divergent characteristics (Curtin et al., 2012; Borneman et al., 2014), which are reminiscent of interspecies hybrids in genus *Saccharomyeces* that likely occurred as rare mating events (Libkind et al., 2011). Although the whole genome sequences provided new insights into physiological behaviour of *B. bruxellensis*, there are still a few challenges that scientific community needs to overcome to be able to link physiological with the genetic data, among the most important being more efficient tools for the genetic manipulation of *B. bruxellensis* (Curtin and Pretorius, 2014).

2.2.1 *Brettanomyces bruxellensis* in wine

Brettanomyces genus currently contains five species, however *B. bruxellensis* is most commonly associated with wine (Cocolin et al., 2004). Although there have also been few occurrences of *B. anomalus* and *B. custerscianus* in wine (Esteve-Zarzoso et al., 2001) reports that indicate their presence are rare. *B. bruxellensis* is usually isolated from wineries – more frequently from red wines and rarely from white wines, which might be due to the higher concentrations of sulphur dioxide that is usually present in white wine and is more efficient at lower pH (Loureiro and Malfeito-Ferreira, 2006). Although their presence on grape barriers has been reported by Renouf and Lonvaud-Funel (2007), the isolation of these yeasts is usually associated with winery equipment and due to its tolerance to high concentrations of ethanol in late stages of fermentation (Passoth et al., 2007; Childs et al., 2015). In the past it has been difficult to prove the presence of *B. bruxellensis* with the isolation techniques in wine, due to low cell concentrations (Fugelsang, 1997). However with development of selective media (Rodrigues et al., 2001)

and molecular techniques the task of detecting its detection became much easier (Cocolin et al., 2004; Martorell et al., 2006).

The main problems of wine contaminated with *B. bruxellensis* are high concentrations of volatile phenols, namely ethylphenols (Chatonnet et al., 1992; Suárez et al., 2007; Oelofse and Pretorius, 2008). Although few other microbial species are able to synthesize these compounds, *B. bruxellensis* remains the only known species that is able to produce them in wine-like environments (Dias et al., 2003). Low levels of volatile phenols can impart pleasant aromas, reminiscent of cloves, leather and smoke, while high levels resemble aroma of horse sweat, barnyard, band-aid® and medicinal aromas, which are considered major wine defects (Chatonnet et al., 1992, 1995; Edlin et al., 1995). However, exactly these aromas are characteristic for certain types of red wines and are described as wines with "Bordeaux" character (Jolly et al., 2006; Romano et al., 2009).

Although volatile phenols are considered as main problem of wine contaminated with *B. bruxellensis*, there are also other factors that make this yeast undesired in winemaking environment. It has been reported that presence of *B. bruxellensis* causes discoloration of wine due to presence of β-glucosidase (Fugelsang et al., 1993), high levels of acetic acid (Licker and Henick-Kling, 1998), "mousiness" or mouse urine aroma, caused by the high levels of tetrahydropyridines (Snowdon et al., 2006) and biogenic amines (Caruso et al., 2002). Nevertheless, these facts make presence of *B. bruxellensis* even less desired and inappropriate for use during fermentation and aging of wine.

Research indicated that late stages of wine fermentation and wine ageing present more favorable conditions for growth of *B. bruxellensis* due to low concentrations of residual sugars and sulfur dioxide as well as the availability of low concentration of oxygen, which is usually available in wooden wine barrels and corked bottles due to porous nature of the material (Chatonnet et al., 1992; Fugelsang et al., 1993; Suárez et al., 2007). Such wines are usually of higher quality and spoilage of such wines represents even bigger financial loss for the wine maker. Although the sanitation of stainless steel equipment contaminated with *B. bruxellensis* does not present a challenge for the winemaker, contaminated wooden barrels do. It has been demonstrated that *B. bruxellensis* can resist most of the classical as well as modern sanitation techniques (Chatonnet et al., 1999) and can even form biofilms, which makes this species even more resistant to sanitation and possess additional financial burden on the wine maker (Joseph et al., 2007). However, not all of the strains present the same threat for the winemaker due to large physiological differences that have been reported in this species and are in most cases depend on the geographical region, from which the strain has been isolated (Conterno et al., 2006; Curtin et al., 2007; Agnolucci et al., 2009).

2.3 PRODUCTION OF VOLATILE PHENOLS BY MICROORGANISMS

The presence of high concentrations of volatile phenols in wine is one of the indicators of microbial spoilage of wine. Main volatile phenols in wine are 4-vinylphenol, 4-ethylphenol, 4-vinylguaiacol and 4-ethylguaicol, among which 4-vinylphenol and 4-ethylphenol are usually more abundant in wine. In low concentrations, these chemical compounds are regarded as beneficial to wine aroma, since they can impart the aromas that are reminiscent of cloves, smoke, leather and spices, however in high concentrations these

aromas are perceived as barnyard, horse sweat and medicinal aromas and are considered as a wine flaws (Heresztyn, 1986; Chatonnet et al., 1992). Representation of the formation of volatile phenols from hydroxycinnamic acids is presented below (Oelofse and Pretorius, 2008).

Hydroxycinnamic acid		**Hydroxystyrenes**		**Ethyl derivatives**
p-coumaric acid	→	4-vinylphenol	→	4-ethylphenol
Ferulic acid	→	4-vinylguaiacol	→	4-ethyguaiacol
Caffeic acid	→	4-vinylcatechol	→	4-ethylcatechol

Sensorial threshold for 4-ethylphenol is 230 μg/L (Chatonnet et al., 1990). In red wines, the range between where presence of 4-ethylphenol has positive contribution to the aroma is between 230 μg/L and 680 μg/L, while wines with phenolic taint can have up to 1.74 mg/L or even over 3 mg/L of 4-ethylphenol (Joseph and Bisson, 2004). It has been reported that the variety of grape also affects the perception of ethylphenols, as well as the wine style and consumers perceptive abilities (Curtin et al., 2005). More recent evaluation performed by Romano et al. (2009) showed that some wines, which were considered without phenolic taint contained from 5 to 1370 μg/L of ethylphenols, while other wines, which were considered as heavily tainted, contained from 196 to 746 μg/L of ethylphenols which shows the importance of all of the factors mentioned before for the perception of volatile phenols in wine.

Volatile phenols in wine originate from grapes, where they are esterified with tartaric acid or anthocyanin esters (Dugelay et al., 1993). Presence of enzymes with cinnamoyl-esteraze activity releases these weak (hydroxycinnamic) acids to their respective free forms which are inhibitory to many microorganisms (Barthelmebs et al., 2000). However, some microbial genera possess an enzyme, which is able to decarboxylate hydroxycinnamic acids into respective vinylphenols. This ability has been described in many microorganisms, such as *S. cerevisiae* (Clausen et al., 1994), *Lactobacillus plantarum* (Cavin et al., 1997), species of the genus *Bacillus* (Degrassi et al., 1995; Cavin et al., 1998), species of the genus *Clostridium* (Chamkha et al., 2001), *Candida cantarelli*, *C. wickerhamii*, *D. hansenii*, *K. lactis*, *P. guilliermondii*, as well as yeast from *Torulaspora* spp. and *Zygosaccharomyces* spp. (Dias et al., 2003) and genus *Brettanomyces* (Heresztyn, 1986) to name just a few. On the other hand, the ability to perform the second step of conversion – to convert vinylphenol into respective ethyl derivatives – has been to this date observed only in handful of microbial species. Up to this date, the reduction of vinylphenols into ethyl derivatives has been described only in handful of microbial species, namely *B. bruxellensis* and *B. anomalus* (Heresztyn, 1986; Chatonnet et al., 1992; Dias et al., 2003), few species from genus *Lactobacillus* (Chatonnet et al., 1995; Fras et al., 2013; de Castro et al., 2015) and genus *Candida* (Suezawa and Suzuki, 2007) and *P. guilliermondii* (Barata et al., 2006). Although all of these microorganisms possess the ability to produce ethylphenols, only *B. bruxellensis* has been reported to be able to produce them under wine like conditions and therefore presents the biggest problem for winemaking industry (Couto et al., 2006; Suárez et al., 2007) which has been attributed to the enzymatic activity of vinylphenol reductase (Dias et al., 2003). Picture representing formation of 4-ethylphenol from *p*-coumaric acid is presented in Figure 1 (Suárez et al., 2007).

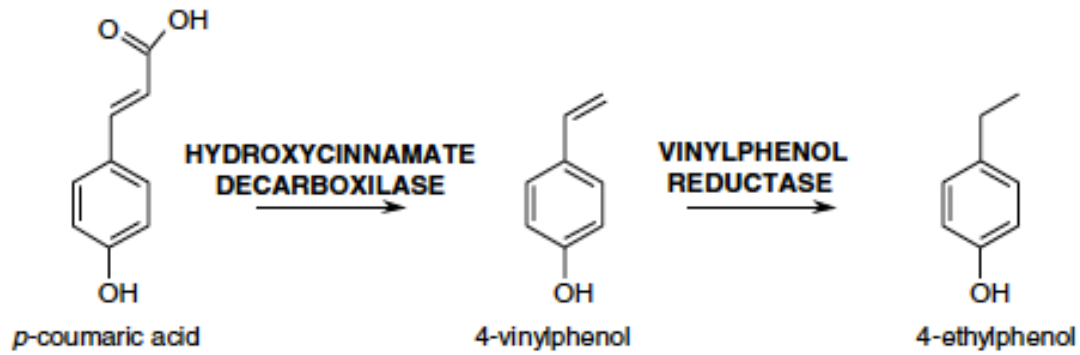

Figure 1: Formation of 4-ethylphenol through conversion of *p*-coumaric acid (Suárez et al., 2007)
Slika 1: Nastanek 4-etilfenola s konverzijo p-kumarne kisline (Suárez in sod., 2007)

2.3.1 Formation of vinylphenols by microorganisms

Many microbial species possess the hydrocinnamate decarboxylase activity (including *B. bruxellensis*; Godoy et al., 2008, 2014), which is the first step in the formation of ethylphenols, however, most of the research on metabolism of hydroxycinnamic acids has been done on yeast *S. cerevisiae* and on bacteria from *Lactobacillus* spp. (Gury et al., 2004; Licandro-Seraut et al., 2008). On the other hand, the reduction of vinylphenols into their respective ethyl derivatives have been studied mostly in *B. bruxellensis* (Suárez et al., 2007; Schifferdecker et al., 2014) and *Lactobacillus* spp. (Rodríguez et al., 2009).

It has been shown that the main function of the enzymes with hydrocinnamate decarboxylase activity in bacterial species is to reduce the toxicity of hydroxycinnamic acids, which explains its strict regulation (Cavin et al., 1997; Gury et al., 2004). In *S. cerevisiae*, the enzymes with hydrocinnamate decarboxylase activity, such as phenylacrylic and ferulic acid decarboxylase, which are products of *PAD1* and *FDC1* genes, are also involved in the process of ubiquinone synthesis, which is used for electron transport in mitochondria and is the essential component of the respiratory metabolism in yeast, which can explain their constitutive expression (Mukai et al., 2010). Different functions of these enzymes can therefore explain why homologous enzymes with hydroxycinnamate decarboxylase of bacterial origin can share up to 84 % identity in their amino acid sequences (Cavin et al., 1998; Barthelmebs et al., 2000) and no homology with *PAD1* of *S. cerevisiae*.

2.3.2 Formation of vinylphenols by *B. bruxellensis*

When the first whole genome sequence of *B. bruxellensis* was published by Curtin et al. (2012), no gene exhibited homology with the *PAD1* of *S. cerevisiae*. The authors suggested that nucleotide sequence for the enzyme with hydroxycinnamate decarboxylase activity might be one of the sequences that had homology in bacterial species. Godoy et al. (2014) later characterized phenolic acid decarboxylase of *B. bruxellensis* and showed that *B. bruxellensis PAD* (*BbPAD*) gene indeed shares 34 % identity with bacterial homologues and shares homology in yeast only with *Meyerozyma guilliermondii*. However, cloning of *B. bruxellensis PAD* gene into *S. cerevisiae* and subsequent biochemical analyses revealed that *BbPAD* exhibited lower conversion rates compared to *PAD1* of *S. cerevisiae*, which might prove the hypothesis of Sáez et al. (2010) that *B. bruxellensis* exhibits higher

production of ethylphenols in mixed fermentations with *S. cerevisiae*, where *S. cerevisiae* converts *p*-coumaric acids into vinylphenols, which are then utilized by *B. bruxellensis*.

2.3.3 Formation of ethylphenols by microorganisms

Unlike with enzymes with hydroxycinnamic acid decarboxylase activity, where the function of enzyme in the physiology of the organisms has been identified, and the physiological function vinylphenol reductase has yet to be discovered. Research done by Barthelmebs et al. (2000) gave convincing indication that there is at least one more pathway for the formation of ethylphenols from vinylphenol precursors in *Lactobacillus* spp. – even though the researches knocked-out the gene encoding phenolic acid decarboxylase (*PadA*), ethylphenols were still formed from hydroxycinnamic acids. Despite all the research on the formation of volatile phenols by *Lactobacillus* spp., enzyme responsible for reduction of vinylphenols into ethylphenols has yet to be characterized.

2.3.4 Vinylphenol reductase of *B. bruxellensis*

Until recent discovery that *B. bruxellensis* is efficient ethanol producer, its ability to form ethylphenols in wine-like conditions, made this yeast one of the most researched non-*Saccharomyces* wine yeast (Steensels et al., 2015). The ability to convert vinylphenols into ethylphenols has been attributed to the presence of vinylphenol reductase enzyme (Dias et al., 2003).

It has been demonstrated, that the efficiency of production of ethylphenols varies widely among different *B. bruxellensis* strains, which is attributed to the large genetic variability in this species (Sturm et al., 2015). It has been also shown, that high presence of SO_2 in wine inhibits the production of volatile phenols by *B. bruxellensis* although the inhibitory concentration of SO_2 was showed to be strain dependent (Zuehlke and Edwards, 2013).

One of the proposed functions of vinylphenol reductase was to further reduce the toxicity of the vinylphenols through the reduction to ethylphenols. Harris et al. (2008) provided evidence, that this hypothesis might be wrong, since the ethylphenols exhibited higher toxicity then vinylphenols. Joseph et al. (2013) observed that significantly higher concentrations of acetic acid were formed in the presence of coumaric acid, suggesting that formation of ethylphenols may aid in the recycling of oxidized cofactor NAD^+.

The first to characterize vinylphenol reductase of *B. bruxellensis* were Tchobanov et al. (2008). Vinylphenol reductase enzyme was isolated through protein isolation, purification of HPLC fractions that exhibited vinylphenol reductase activity and separated them with SDS-PAGE. Monomeric protein with the molecular mass of 26 kDa was obtained and peptide fragments were then analyzed with mass spectrometry. Obtained amino acid sequences were then used to identify the nucleotide sequence through use of BLASTp algorithm in NCBCI database. One matching ORF was found in the genomic sequence of *B. bruxellensis* CBS 2499, which had at that time 40 % of its genome sequenced (Woolfit et al., 2007) and there were no other significant matches in any of the other species sequenced at the time of publishing.

Later that year, Godoy et al. (2008) published another characterization of the vinylphenol reductase enzyme, which exhibited different size (37 kDa) and kinetic properties then the enzyme published by Tchobanov et al. (2008). Although another study was published by the same group (Godoy et al., 2009), no amino acid or nucleotide sequences were provided.

In 2014, Granato et al. (2014) published characterization of another vinylphenol reductase of *B. bruxellensis* which has been shown to be Cu/Zn superoxide dismutase with vinylphenol reductase activity being its 'moonlight' function. Characterization of vinylphenol reductase enzymes from different strains showed, that different strains indeed had different conversion kinetics. Their results indicated that the enzyme is homodimer with molecular mass of single protein being 16 kDa (20 kDa in its denaturated form). Amino acid sequence comparison showed highest similarity with Cu/Zn superoxide dismutase of *Komagataella pastoris*. Although enzyme exhibited different kinetic properties from that of the enzymes characterized by other authors, peptide fragments of the enzyme, characterized by Tchobanov et al. (2008) were also detected by Granato et al. (2014) and vice versa.

2.4 GENETIC ENGINEERING OF *Saccharomyces cerevisiae*

Yeast *Saccharomyces cerevisiae* is one of the first domesticated organisms and has been used for the production of food and beverages for millennia and for the greater part of the past century, research on *S. cerevisiae* was focused on its role in the traditional biotechnology (Barnett, 2003). In the past few decades, it has gained more attention as model eukaryotic organism and was the first eukaryote to have its genome completely sequenced (Goffeau et al., 1996). Since then, *S. cerevisiae* was quickly established as one of the most important models for basic research of eukaryotic organisms (Winzeler et al., 1999), model for human disease (Spradling et al., 2006), bioethanol (Bothast and Schlicher, 2005), as well as production of drugs (Ro et al., 2006), vaccines (Wansley et al., 2008) and bulk chemicals (Borodina and Nielsen, 2014) to list just a few. This has become possible with the development of efficient transformation methods, which enabled genetic engineering of *S. cerevisiae*. Classical techniques such as selection, mutagenesis, mating, hybridization and directed evolution present some possibilities of strain improvement allow us to improve desired characteristics of industrial strains to some extent (Attfield and Bell, 2003) – however, molecular techniques have two major advantages; the modification is directed without the accumulation of undesired mutations and molecular techniques allow us to introduce genes of foreign organisms to achieve the desired effect. This topic however is too vast to be summed up on a few pages, so the following chapters present only select topics from this field.

2.4.1 Vectors

One of the first things to consider when we want to introduce exogenous DNA into *S. cerevisiae* is the vector, with which we will introduce the DNA into the cell. Yeast vectors can be roughly divided into two categories – linear and circular vectors. Linear vectors such as yeast integrative plasmid lack the yeast origin of replication and must be integrated into genome to ensure the propagation of the desired DNA element. The positive side of use of such vectors is that they are stable even in the absence of selective

pressure; however they are usually integrated into genome in single or low copy numbers. Three different circular vectors have been most commonly used in the studies of *S. cerevisiae* – yeast replicating plasmid, which contains autonomously replicating sequence (ARS) derived from the yeast chromosome but tends to be unstable and is frequently lost during budding; yeast episomal plasmid and yeast centromere plasmid. Among these yeast integrative, episomal and centromere plasmids are usually used as vectors for heterologous expression (Stearns et al., 1990).

Yeast episomal plasmids are based on the natural *S. cerevisiae* 2 micron (2μ) plasmid from which the 2μ origin of replication is derived. It replicates in the nucleus at an average copy number of 40-60 plasmids per cell (Ludwig and Bruschi, 1991). If these plasmids are present in the cell in too high copy numbers, they can be deleterious to the cell due to cell cycle misregulation (Chen et al., 2005). However, the copy number of these vectors can be regulated if the specific regulatable elements are included (Romanos et al., 1992). Yeast episomal vectors are usually vectors of choice for the overexpression of the desired products in the laboratory strains of *S. cerevisiae* (Mumberg et al., 1995).

Yeast centromere vectors are plasmids that incorporate part of an autonomous replicating sequence as well as a part of centromere sequence, which allows them to replicate and behave as a chromosome. These vectors are usually present in the cell in single copies and are very stable due to the presence of centromere sequence (Clarke and Carbon, 1980; Polumienko et al., 1986).

Plasmids have to possess the following features in order to be used as vectors for gene expression: origin of replication, which ensures the propagation of plasmid through cell divisions; selectable marker or reporter gene, which ensures that plasmid is maintained in the cell population under selectable pressure; multiple unique restriction sites for integration of the DNA construct; and DNA insert with associated promoter and terminator region (Cohen et al., 1973; Cohen, 2013). Since the propagation and isolation of plasmid DNA from yeast is difficult, most of the plasmids used for the transformation also include origin of replication from *E. coli* as well as a selectable marker – vectors that are constructed and propagated in one organism for its use in another are called "shuttle-vectors" (Hill et al., 1986).

One of the important features to consider when constructing a plasmid is the choice of the selectable markers, which will insure the maintenance of plasmid in the population of cells. In laboratory strains of *S. cerevisiae* auxotrophic markers are most commonly used, as many laboratory strains of *S. cerevisiae* are auxotrophic due to mutations in certain genes. Most commonly used auxotrophic markers are *URA3*, *HIS3*, *LEU2*, *TRP1* and *LYS2*, which complement the mutations in these genes and the reversion rate of these mutations is very low (Jones and Prakash, 1990). However, most of these markers cannot be used as reporter genes in industrial strains of *S. cerevisiae*, which are usually prototropic. For these strains, dominant markers must be used, with the most common being kanMX4, hphMX4, natMX4, patMX3 and NEO, which confer the resistances to Geneticin, Hygromicin B, Nourseothricin, bialaphos and Neomycin (Goldstein and McCusker, 1999; Sadowski et al., 2008).

2.4.2 Construction of vectors and expression cassettes

For the most part of past few decades, cloning of desired DNA product into vector of choice was performed through restriction-ligation reactions (Cohen et al., 1973; Sambrook and Russell, 2001). In recent years new techniques that do not require restriction reactions have emerged, which allow faster and more specific construction of plasmid vectors. Although efforts to eliminate restriction and/or ligation enzymes from cloning reactions have been made before, one example being TA cloning, which allowed simple cloning of PCR cassette with 3' end adenine overhangs into vector with 3' end thymine overhangs (Trower and Elgar, 1994; Zhou and Gomez-Sanchez, 2000). This method was very efficient for assembly of plasmids with single inserts but lacked the potential to include multiple inserts. Sequence- and ligation-independent cloning (SLIC), which mimics in vivo homologous recombination by relying on exonuclease-generated ssDNA overhangs in insert and vector fragments, and the assembly of these fragments by recombination in vitro (Li and Elledge, 2007) was one of the first methods that showed the potential of assembling multiple fragments in one reaction. Quan and Tian (2009) have developed circular polymerase extension cloning method (CPEC), which can be used for joining of multiple DNA fragments for the assembly of vectors, libraries and multi-component pathways. The method is based on polymerase overlap extension and is therefore free of restriction digestion, ligation or single-stranded homologous recombination. One of the most popular methods was published by Merryman and Gibson (2012), which allows joining of multiple DNA fragments in a single, isothermal reaction. The method is based on the exonuclease that removes nucleotides from 5' end of each of the PCR amplicons of homologous flanking regions, which reanneal after the digestion. The gaps are then filled by DNA polymerase and covalently joined by DNA ligase in single reaction. Ligase chain reaction published by Kok et al. (2014) is also based in the thermocycling reaction to join DNA fragments – assembly is performed with single-stranded bridging oligonucleotides, complementary to the ends of neighbouring DNA parts, a thermostable ligase to join DNA backbones and multiple denaturation-annealing-ligation temperature cycles to assemble complex DNA constructs. When optimized for specific reaction, this method can be used for one-step assembly of up to 20 DNA parts into up to 20 kb DNA constructs. Method for vector construction that takes into account the specific trait of *S. cerevisiae*, namely very efficient homologous recombination – one of the first applications of homologous recombination in yeast for the construction of plasmids from DNA fragments was described by Ma et al. (1987). The real power of this technique was demonstrated by Gibson et al. (2008), when authors assembled the complete *Mycoplasma genitalium* genome from 25 overlapping DNA fragments through transformation of these fragments into *S. cerevisiae*, where they were assembled by the cell's recombination machinery. This technique was further improved for plasmid assembly by Kuijpers et al. (2013), which demonstrated that transformation of *S. cerevisiae* with PCR based cassettes with 60 bp homologous flanking regions very efficiently produces circular plasmids. These so called transformation associated recombination tools made *S. cerevisiae* one of the most interesting tools for assembly of large DNA constructs (Mizutani, 2015).

2.4.3 Integration of the expression cassettes

When the construction of more stable strains is needed, the integration of gene constructs into the genome is needed. One of the first techniques was use of the yeast integrative

plasmids as already mentioned. Fact that integration of gene constructs into *S. cerevisiae* through homologous recombination was discovered early, with several hundred base pair homologous flanking regions – it soon became clear that 30 to 50 bp homology regions are sufficient to induce recombination, which allowed use of the PCR-mediated strategies, where homology regions were introduced with primers (Baudin et al., 1993). Another efficient method to integrate heterologous DNA into the genome of *S. cerevisiae* is the introduction of the double-stranded breaks at the desired locus – these methods are more labor intensive but on the other hand result in higher transformation efficiencies and facilitate the screening process because unrepaired double-stranded brakes are usually deleterious to the cell. One of such methods was published by Kuijpers et al. (2013), where the authors first created the platform with the integrated expression cassette containing selectable marker and the open reading frame of I-*Sce*I meganuclease with inducible promoter and the whole cassette was flanked on both ends with I-*Sce*I restriction sites. After the platform strain was transformed with linear DNA fragments, which had homologous regions with the genome, the expression of the I-*Sce*I meganuclease was induced, which introduced the double stranded breaks at the specific locus – the marker was excised and the linear DNA fragments were integrated. Similar results without the need of prior platform strain construction were achieved by using zinc finger nucleases (Wang et al., 2013) and transcription activator-like effector nucleases (TALENs; Christian et al., 2010), which also introduce double stranded breaks into yeast genome at the specific locations. However, these methods are rapidly being replaced by clustered regularly-interspaced short palindromic repeats (CRISPR) due to easier manipulation methods in its effectiveness (Stovicek et al., 2015).

Integration of heterologous DNA constructs is usually targeted at rRNA genes (Lopes et al., 1989), sigma factors (Kudla and Nicolas, 1992) and delta sequences (Sakai et al., 1990; Lee and Da Silva, 1997). Integration at multiple sites is useful when multiple DNA fragments need to be integrated into genome because they tend to be more stable compared to when they are inserted in tandems (Da Silva and Srikrishnan, 2012). However, when integrating multiple DNA fragments into genome, one must also consider limited number of marker/reporter genes that can be simultaneously expressed, as well as absence of dominant markers in an organism, when considering its use in industrial applications. Example of such strategy is already described in the paragraph above by Kuijpers et al. (2013). One of the earliest strategies was based on the so-called "pop-in/pop-out" strategy, where counterselectable marker gene, such as *URA3*, was flanked with direct repeats from other microorganisms. This strategy used homologous recombination of *S. cerevisiae* to remove the marker from the integrated cassette (Alani et al., 1987) and allowed the selection of mutants on the agar plates containing 5-fluoroortic acid (Boeke et al., 1984). This method left the repeat sequences in the genome, which could interfere with efficiency if the same strategy would be deployed again as well as the genetic element introduced into genome. Similar strategies was later deployed by Tian et al. (2013), who used flanking tandem repeats that possessed the genetic elements homologous with the cassette that one wishes to integrate – this strategy allowed the introduction of a single copy of the desired element with no junk DNA left in the genome. For genetic engineering of industrial strains, strategies that applied active marker recycling proved to be the most powerful because do not need the application of counterselectable markers. In Cre-*loxP* system the selection marker is flanked by *loxP* sequences and the expression of Cre recombinase allows the excision allows the excision of simultaneous excision of multiple markers,

which significantly decreases the time of strain construction. However, the Cre recombinase has to be introduced into the cell (Storici et al., 2001; Gueldener et al., 2002; Storici and Resnick, 2006). The second system, called Flp-*FRT* recombination, is similar to the Cre-*loxP* system however the recombination involves the recombination of sequences between short flippase recognition target (*FRT*) by the recombinase (Flp) from the *S. cerevisiae* (Lloyd and Davis, 1994; Schweizer, 2003; Kopke et al., 2010).

2.4.4 Transformation of *S. cerevisiae*

Transformation is a technique with which the exogenous DNA is introduced into the cell, resulting in genetic modification. The first successful transformation of *S. cerevisae* was reported by Hinnen et al. (1978) with the transformation of spheroplasts. Since then, few other methods have been developed for the transformation of *S. cerevisiae*, which now include chemical transformation with lithium acetate, electroporation, biolistic and glass bead method. All of these methods can be also applied for the transformation of other fungi though the highest efficiencies have been obtained for *S. cerevisiae* (Kawai et al., 2010).

For the successful transformation, the exogenous DNA must pass through cell wall and plasma membrane to be delivered into cytosol and subsequently nucleus, with the exception of the spheroplast method, where the cell wall has been removed. Mechanism that underlie the transformation has still not been clarified completely although few recent studies suggest the possible mechanisms (Kawai et al., 2004; Chen et al., 2008) of which the uptake of the exogenous DNA seems the most probable as demonstrated by Kawai et al. (2004) through monitoring of transformation efficiency of *S. cerevisiae* knock-out collection.

As mentioned before, the first transformation of *S. cerevisiae* was reported by Hinnen et al. (1978) with transformation of spheroplasts. Spheroplast transformation method is composed of preparation of spheroplasts with the enzymatic digestion of the cell wall of *S. cerevisiae* and subsequent incubation of spheroplasts with exogenous DNA. It has to be stressed that the concentration of the exogenous DNA, presence of carrier DNA, concentration of cells and the amount of shear stress imposed upon the cells strongly influence the transformation efficiency (Burgers and Percival, 1987).

Use of the lithium acetate method was first published by Ito et al. (1983) and was based on the observation, that monovalent cations Na^+, K^+, Rb^+, Cs^+ and particularly, Li^+, unlike divalent cations such Ca^{2+}, which proved to be very efficient in the transformation of *Escherichia coli*, enhance the efficiency of the transformation of intact *S. cerevisiae* cells. Through shear trial-and-error experiments the authors found out, that use of LiAc gives the highest transformation efficiencies. The work that Ito et al. (1983) performed showed that the use of polyethylene glycol is essential in the transformation mixture, that use of LiAc and heat shock enhance transformation efficiency and the highest transformation efficiencies are obtained when cells are at mid-log phase. This method quickly became popular, as it was easy to perform with no special equipment required and was further improved by Gietz and Woods (2002).

Hashimoto et al. (1985) were the first to report transformation of intact *S. cerevisiae* cells by electroporation – technique in which an electrical field is applied to cells to increase the permeability of the cell membrane, which allows the introduction of DNA into the cell (Neumann et al., 1982). The original method was then further improved by introduction of sorbitol into reaction mixture (Becker and Guarente, 1991), preincubation in LiAc and DTT (Thompson et al., 1998) as well as freezing of intact cells in the solution of sorbitol and calcium before transformation (Suga and Hatakeyama, 2003).

Biolistic method is based on the principle that cells can also be transformed by DNA-coated metal particles, that are shot into the cells (Klein et al., 1987) and Armaleo et al. (1990) were the first to demonstrate the transformation of intact *S. cerevisiae* with this method. Although the efficiency of transformation with this method is low, it has been the only method, with which the mitochondrial DNA of *S. cerevisiae* could be transformed (Johnston et al., 1988).

Intact *S. cerevisiae* cells can also be transformed by agitation with glass beads (glass bead method) in the presence of carrier DNA and plasmid DNA at very low efficiency with the addition of 1.0 M sorbitol in the selective solid medium; this technique is rarely utilized due to the low transformation efficiency (Costanzo and Fox, 1988).

2.4.5 Regulation of protein abundance

The level of protein abundance in a cell can be regulated on three different levels – it can be regulated by adjusting DNA/gene content, transcription efficiency and mRNA abundance and translation efficiency (Nevoigt, 2008). The simplest way to enhance the heterologously expressed product in a cell is to increase the number of copies of the gene of choice. In *S. cerevisiae*, this can be achieved with the introduction of multicopy plasmids, carrying the gene of interest. Although 10 to 30 or even more copies of these plasmids are present in cell, the expression levels can be further amplified with the right choice of promoter regulating the expression of gene of choice (Christianson et al., 1992; Mumberg et al., 1995). Drawbacks of this method are: copy number among cells varies; selective pressure must be applied for selection of cells containing the plasmid; and high expression levels presents metabolic burden for the cells (Görgens et al., 2001). Alternatively, gene of choice can be integrated into genome in multiple copies, as already discussed in the previous chapters.

Protein expression can also be altered through replacement or modification of the upstream gene regulating sequence (promoter), which can be either constitutive or inducible. One of the first strategies employed to change the level of expression and its regulation was by putting gene of choice of under different native promoter of the host organism (Goodey et al., 1986), which is still one of the most employed strategies for the expression of the heterologous proteins (Nevoigt et al., 2006; Kaufmann and Knop, 2011). When the first genomic sequence of the *S. cerevisiae* became available, the research of the regulatory elements on global scale (Brazma et al., 1997; Zhu and Zhang, 1999) as well as the assessment of promoter libraries (Jeppsson et al., 2003) became possible. The development of the RT-PCR (Teste et al., 2009) as well as microarray and RNAseq technology (Riordan et al., 2011; Nookaew et al., 2012) further helped to elucidate the transcription and therefore regulation of different genes and consequently their promoters (Hahn and Young,

2011). Before the advent of these technologies, promoters were assessed with the expression and quantification of reporter proteins (or their activity), such as β-galactosidase (Guarente, 1983), some of which are still used today (Hammer et al., 2006). Expression studies were performed under different conditions, mostly mimicking those that yeast encounter in industrial environments (Aguilera et al., 2005) and have been subjected to many reviews (Blazeck et al., 2012; Weinhandl et al., 2014; Hubmann et al., 2014) and won't be discussed further in this section. Another strategy to change the activity of native promoter is promoter engineering through random mutagenesis with error prone DNA polymerase as demonstrated by Alper et al. (2005), who showed that mutagenesis of the well characterized *TEF1* promoter can result in a wide range of promoter activities. Insights into regulatory elements allowed the construction of hybrid (de Boer et al., 1983) and later synthetic promoters, which allowed the introduction of novel regulatory elements and pathways for precise regulation of gene expression (Hammer et al., 2006; Blazeck et al., 2012).

The third level of protein expression control is based on the control of the mRNA stability and translation efficiency. RNA interference with silencing of gene expression via antisense RNA techniques proved to be one of the most powerful tools for regulation of protein expression in eukaryotic organisms. This mechanism is conserved in diverse eukaryotic species, however *S. cerevisiae* seems to be one of the few organisms that lost this mechanism (Drinnenberg et al., 2009). Suk et al. (2011) were able to reconstitute this mechanism in *S. cerevisiae* by expressing three human proteins, Ago2, Dicer and TRB, which opened the possibilities for complex phenotype engineering through RNA interference (Si et al., 2015). Although *S. cerevisiae* does not possess all of the necessary elements for the assembly of canonical RNA silencing complex, there are indications of expression control at transcript level. Bonoli et al. (2006) showed that mRNA complementary to the 5'-untranslated region effective gene silencing in *S. cerevisiae*. Another example of such mechanisms was presented by Babiskin and Smolke (2011) – they showed that the insertion of specific sequence at 3'-untranslated region of transcript forms tetraloop by base-pairing that is recognized by RNAseIII (RntIp) enzyme, which directs cleavage at that region and in turn lowers target protein levels. On the other hand, Zhou et al. (2001) demonstrated that some of the naturally occurring nucleotide sequences at 5'-untranslated region, namely YAP1 and p150 mRNAs, function as very effective leader sequences at internal ribosomal entry sites, which enhance the translation efficiency in yeast cells. Another possibility of control of protein expression at post-transcription level is use of the riboswitches, regulatory segments of a messenger RNA molecule that bind a small molecules, resulting in a change in production of the proteins encoded by the mRNA (Bauer and Suess, 2006). Klauser et al. (2015) demonstrated, that with the use of the ribozyme-based aminoglycoside switches, gene expression in *S. cerevisiae* could be switched up to 25-fold. There are still many non-coding RNA molecules function of which still had not been characterized yet and present additional possibilities of expression control at post-transcriptional level (Harrison et al., 2009).

2.4.6 Use of genetically modified strains in wine industry

Main reasons for genetic engineering of food-related microorganisms are: improvement of propagation, fermentation and storage efficiency fermented product; enhancement of sensorial properties of the end product; enhancement of health related quality of the end

product; and improvement of microbial stability during fermentation and storage (Steensels et al., 2014). Genetic engineering of microorganisms for application in food and wine industry poses a particular challenge for many reasons. Sensory quality of foods and beverages are determined by balance among different chemical compounds. This fact makes optimization of strains to achieve the desired results very challenging (Nevoigt, 2008). Another factor to consider are genetic traits of *S. cerevisiae* industrial strains, which are genetically diverse, prototrophic, homothallic and often polyploid, aneuploidy or even alloploid and therefore hard to manipulate with tools that have been developed for the engineering of laboratory strains (Winde, 2003). However, one of the greatest obstacles for application of genetically modified yeast in food and beverage industry is that the containment of these organisms in the food-related industrial environment cannot always be guaranteed. To address this problem, such products are strictly regulated on national and international levels and must pass safety regulations and pass safety evaluations and follow labelling regulations, with European regulation being particularly strict – risk assessment procedure for genetically modified non-pathogenic organisms that are used in fermentations is the same as for genetically modified organisms from plant and animal origin (Nevoigt, 2008). Even if all of these procedures are successfully completed, this does not mean that commercialization of strain will be successful. The acceptance of consumers towards genetically modified organisms is generally negative and are influenced by the complex cultural, social, ethical, environmental and technical factors (Pretorius and Bauer, 2002; Pretorius et al., 2012).

The first commercial transgenic wine strain of *S. cerevisiae* was introduced to market in 2005. *S. cerevisiae* ML01 was genetically modified through insertion of *Schizosaccharomyces pombe* malate transporter gene (*mae1*) and *Oenococcus oeni* malolactic enzyme gene (*mleA*) at *URA3* locus, with both being regulated with native *PGK1* promoter and terminator. The insertion of heterologous genes enabled ML01 to perform malolactic fermentation, which is usually performed in wine by lactic acid bacteria (Husnik et al., 2006). After thorough analysis and risk assessment, ML01 was approved for use in winemaking in the USA, Canada and Moldova (Pretorius et al., 2012). The second genetically modified yeast that was approved for commercial use in winemaking in the USA and Canada in 2006 was ECMo01. This strain was modified with the introduction of extra copies of native *DUR1,2* genes under the control of *PGK1* regulatory sequences. *DUR1,2* gene encodes urea amidolyase, which convert urea into ammonia and carbon dioxide. In this way, substrates for ethyl carbamate production, which is a potential carcinogen, are removed (Coulon et al., 2006).

In this paragraph, one of the first efforts to enhance the production of volatile phenols by *S. cerevisiae* for its application in wine fermentations is presented. *S. cerevisiae* possesses phenylacrylic acid decarboxylase (*PAD1*) which enables it to form vinylphenols (Clausen et al., 1994). However, the activity of the *S. cerevisiae PAD1* proved to be too weak to make significant contribution to the aroma of wine. Although another enzyme, that possesses hydroxycinnamate decarboxylase function has been discovered in *S. cerevisiae* (ferulic acid decarboxylase, *FDC1*; Mukai et al., 2010) and characterized (Bhuiya et al., 2015), efforts to increase the production of vinylphenols in *S. cerevisiae* to enhance the aroma of wine had been made earlier. Smit et al. (2003) cloned various enzymes with hydrocinnamate decarboxylase activity from different microbial sources under the regulation of *S. cerevisiae PGK1* promotor. They found out, that genes cloned from

Lactobacillus plantarum (*PadA*) and *Bacillus subtilis* (*Pad*), exhibited higher activities even without codon optimization then overexpressed *S. cerevisiae PAD1* gene. Richard et al. (2015) later succeeded in overexpression of native *PAD1* and *FDC1* genes in *S. cerevisiae* that resulted in significant enhancement of the conversion rates, though this research was performed for the application in other industrial processes.

Many research articles on improving sensory, technological and health aspects of wine through genetic engineering of *S. cerevisiae* have been published. These have been subjected to many excellent reviews by Pretorius and Bauer (2002), Schuller and Casal (2005), Cebollero et al. (2006), Donalies et al. (2008), Schmidtke et al. (2012), Steensels et al. (2014), Tilloy et al. (2015) and won't be discussed in further detail.

3 MATERIALS AND METHODS

The following chapters contain all of the protocols, methods and materials that were used for experimental work. Laboratory equipment and apparatuses that were used in this study are also listed in this chapter.

3.1 MATERIALS

All of the experiments were performed with use of materials, listed in this chapter.

3.1.1 Microbial strains

All of the strains used for our experiments are listed in this chapter.

3.1.1.1 *Brettanomyces bruxellensis* and *S. cerevisiae* strains used for fermentation experiments

Fermentation experiments for determination of specific conversion rates of 4-vinylphenol were performed with *S. cerevisiae* ZIM 1927 and following *B. bruxellensis* strains: ZIM 700, 701, 702, 703, 704, 705, 2306, 2503 and CBS 72 (ZIM 1764), CBS 75 (ZIM 1762), CBS 2499. All of the listed strains except for CBS 72, 75 and 2499 were obtained from Collection of Industrial Microorganism (ZIM) from Chair of Biotechnology, Microbiology and Food Safety, Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana. Strains CBS 72, 74 (designated in following chapters as ZIM 1764 and ZIM 1762 respectively) and CBS 2499 were obtained from CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands). All of the strains used in our experiments were maintained in 20 % solution at -80°C and maintained on YPD agar plates at 28°C.

Table 2: Strains of *B. bruxellensis* and *S. cerevisiae* used for fermentation experiments with their designation and origin
Preglednica 2: Sevi kvasovk *B. bruxellensis* in *S. cerevisiae* z izvorom in oznako, ki so bili uporabljeni pri izvedbi poskusnih fermentacij

Species	**Designation**	**Origin**
B. bruxellensis	ZIM 700	Rebula wine must, Slovenia
B. bruxellensis	ZIM 701	Rebula wine must, Slovenia
B. bruxellensis	ZIM 702	Rebula wine must, Slovenia
B. bruxellensis	ZIM 703	Rebula wine must, Slovenia
B. bruxellensis	ZIM 704	Rebula wine must, Slovenia
B. bruxellensis	ZIM 705	Rebula wine must, Slovenia
B. bruxellensis	ZIM 2306	Rebula wine, Slovenia
B. bruxellensis	ZIM 2512	Wine must, Montenegro
B. bruxellensis	CBS 72 (ZIM 1764)	Lambic beer, Belgium
B. bruxellensis	CBS 75 (ZIM 1762)	Lambic beer, Belgium
B. bruxellensis	CBS 2499	Wine, France
S. cerevisiae	ZIM 1927	Malvazija wine, Slovenia

3.1.1.2 *Escherichia coli* strain used for molecular cloning

For experiments involving cloning and plasmid construction in *E. coli*, TOP10F' One Shot® (Invitrogen) chemically competent cells were used. Genotype, supplied by manufacturer is listed as follows: F′ {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

3.1.1.3 *Saccharomyces cerevisiae* strains used for plasmid construction and heterologous gene expression

Commercial *S. cerevisiae* EC1118 (Lalvin) wine strain was used as a donor strain for promoters and terminators used for the construction of pRS426-based plasmids. The strain was obtained from Collection of Industrial Microorganism (ZIM) from Chair of Biotechnology, Microbiology and Food Safety, Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana.

Two different strains of *S. cerevisiae* – T334 and YPH250 – were used in our experiments for expression of heterologous proteins. Both strains were donated by dr. Krešimir Gjuračić from Acies Bio d.o.o. (Ljubljana, Slovenia) on YPD agar plates. Upon receiving, colonies of both strains were transferred to fresh YPD plates and after 48 hour of incubation a single colony was stored in YPD medium with 20 % at -80°C till needed. Table 3 lists both strains, as well as their respective genotypes and references.

Table 3: Genotype and references of *S. cerevisiae* T334 and YPH250 strains
Preglednica 3: Genotip in reference sevov *S. cerevisiae* T334 in YPH250

***S. cerevisiae* strain**	**Genotype**	**Reference**
T334	*MATα ura3-52 leu2-3,112 Δtrp1::hisG reg1-501 gal1 pep4-3 prb1-1122*	Hovland et al., 1989
YPH250	*MAT***a** *trp1-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*	Inoue et al., 1999

3.1.2 Culture media

This chapter lists all of the media that were used for maintenance and culturing of different yeast and bacteria, used in our experiments. Media used for isolation, selection, fermentation experiments and inoculum preparation is listed in this chapter as well.

3.1.2.1 Standard media

Solid YPD (Yeast Peptone Dextrose) medium was used or cultivation and maintenance of *B. bruxellensis* and wild-type *S. cerevisiae*. Composition is listed in Table 4 (Kurtzman et al., 2011).

Table 4: Composition of solid YPD medium (Kurtzman et al., 2011)
Preglednica 4: Sestava trdnega gojišča YPD (Kurtzman in sod., 2011)

Component	**Mass/volume per 500 mL of medium**
Glucose (Sigma-Aldrich)	10 g
Peptone (Biolife)	10 g
Yeast extract (Biolife)	5 g
Agar (Biolife)	10 g
dH_2O	Up to final volume of 500 mL

All of the components were weighed into 1L laboratory flask and dissolved in 500 mL of distilled water. YPD medium was sterilized in autoclave at 121°C at 1.1 bar for 20 minutes. Flask was then transferred into water-bath and left to cool down to 55°C. When YPD medium reached desired temperature, it was spread into sterile petri dishes in laminar flow cabinet and left until it completely polymerized. Plates were stored at 4°C until needed.

YPD liquid medium (Raspor and Smole Možina, 1993) was used for preparation of inoculums of *B. bruxellensis* as well as wild-type *S. cerevisiae* strains. Composition is listed in Table 5.

Table 5: Composition of liquid YPD medium (Raspor and Smole Možina, 1993)
Preglednica 5: Sestava tekočega gojišča YPD (Raspor in Smole Možina, 1993)

Component	**Mass per 500 mL of medium**
Glucose (Sigma-Aldrich)	10 g
Peptone (Biolife)	10 g
Yeast extract (Biolife)	5 g
dH_2O	Up to final volume of 500 mL

All of the components were weighed into 1L laboratory flask and dissolved in 500 mL of distilled water. YPD medium was sterilized in autoclave at 121°C at 1.1 bar for 20 minutes. Flask was then left at room temperature until it cooled down. Flask with liquid laboratory medium was stored at 4°C until needed.

3.1.2.2 Differential media

Dekkera Brettanomyces Differential Medium (DBDM; Rodrigues et al., 2001) was used to selectively isolate *B. bruxellensis* from wine and grape must samples. Composition of DBDM is listed in Table 6.

Table 6: Composition of DBDM (Rodrigues et al., 2001)
Preglednica 6: Sestava gojišča DBDM (Rodrigues in sod., 2001)

Component	**Mass/volume per 1000 mL of medium**
YNB (Difco)	6.7 g
100 % Ethanol (Sigma-Aldrich)	6 mL
Cycloheximide (Merck)	0.01 g
p-coumaric acid (Sigma-Aldrich)	0.1 g
Bromocresol green (Kemika)	0.022 g
dH_2O	Up to final volume of 1000 mL

For preparation of DBDM, YNB, cycloheximide and *p*-coumaric acid were weighed separately and transferred to 1000 mL volumetric flask. Bromocresol green had to be dissolved in 100 % ethanol prior to addition to other chemicals. Distilled H_2O was added to 1000 mL mark and the contents were occasionally mixed until all of the components dissolved completely. DBDM was filter sterilized and stored at 4°C until needed.

Oxytetracycline Glucose Yeast Extract agar (OGYE agar) was prepared for selective isolation of yeast from mixed culture media. Composition of OGYE agar is listed in Table 7.

Table 7: Composition of oxytetracycline glucose yeast extract agar
Preglednica 7: Sestava oksitetraciklin glukoza kvasni ekstrakt agarja

Component	**Mass/volume per 500 mL of medium**
OGYE agar base (Biolife)	19 g
Oxytetracycline Antimicrobic Supplement (Biolife)	1 vial
dH_2O	Up to final volume of 500 mL

OGYE agar base was mixed with 500 mL of dH_2O until fully dissolved and sterilized in autoclave at 121°C at 1.1 bar for 20 minutes. Flask was incubated in water bath until cooled to 55°C. 1 vial of oxytetracycline antimicrobic supplement was added and spread into sterile petri dishes in laminar flow cabinet and left until it completely polymerized. Plates were stored at 4°C until needed.

3.1.2.3 Selection media

Solid SC-URA (Synthetic Complete drop-out medium without Uracil) medium was prepared for selection of positive *S. cerevisiae* transformants. Media was prepared according to pYES2.1 TOPO® TA Expression Kit's manufacturer's recommendations (Invitrogen) and modified to suit our experimental requirements. Composition of solid SC-URA medium is listed in Table 8.

Table 8: Composition of solid SC-URA medium
Preglednica 8: Sestava trdnega gojišča SC-URA

Component	**Mass per 500 mL of medium**
YNB w/o amino acids and ammonium sulfate (Sigma-Aldrich)	2.0 g
Ammonium sulfate (Sigma-Aldrich)	2.5 g
Yeast Synthetic Drop-Out Medium Supplements without uracil (Sigma)	1.5 g
Agar (Biolife)	10 g
Glucose (Sigma – Aldrich)	15 g
dH_2O	Up to final volume of 500 mL

Ammonium sulphate, agar and glucose were weighed into 1L laboratory flask and dissolved in 450 mL of distilled water. Solution was sterilized in autoclave at 121°C at 1.1 bar for 20 minutes. Flask was then transferred into water-bath and left to cool down to 55°C. YNB w/o amino acids and ammonium sulphate and Yeast Synthetic Drop-Out Medium Supplements without uracil were dissolved in 50 mL of dH_2O, filter sterilized and added to autoclaved solution. Medium was spread into sterile petri dishes in laminar flow cabinet and left until it completely polymerized. Plates were stored at 4°C until needed.

LB (Luria-Bertani) solid medium supplemented with ampicillin was prepared for selection of positive *E. coli* transformants as recommended by pYES2.1 TOPO® TA Expression Kit's manufacturer (Invitrogen). Composition is listed in Table 9 (Sambrook et al., 1989).

Table 9: Composition of LB solid medium supplemented with ampicillin (Sambrook et al., 1989)
Preglednica 9: Sestava trdnega gojišča LB z dodanim ampicilinom (Sambrook in sod., 1989)

Component	**Mass/volume per 500 mL of medium**
Tryptone (Sigma-Aldrich)	5.0 g
NaCl (Sigma-Aldrich)	5.0 g
Yeast Extract (Biolife)	2.5 g
Agar (Biolife)	10.0 g
Ampicillin stock solution (1g/L)	2.5 mL
dH_2O	Up to final volume of 500 mL

For preparation of ampicillin stock solution 100 mg of ampicillin (Roche, Switzerland) was weighed into 10 mL volumetric flask and filled up with to 10 mL mark with dH_2O. After ampicillin completely dissolved, the solution was filter sterilized and stored at -20°C until needed. Tryptone, NaCl, yeast extract and agar were weighed into 1L laboratory flask and diluted with 500 mL of dH_2O. Solution was sterilized in autoclave at 121°C at 1.1 bar for 20 minutes. Flask was then transferred into water-bath and left to cool down to 55°C. 2.5 mL of ampicillin stock solution was added to LB medium to reach recommended final ampicillin concentration of 50 μL/mL. Medium was spread into sterile petri dishes in laminar flow cabinet and left until it completely polymerized. Plates were stored at 4°C until needed.

LB (Luria-Bertani) liquid medium supplemented with ampicillin was prepared to amplify *E. coli* transformants for plasmid isolation as recommended by pYES2.1 TOPO® TA

Expression Kit's manufacturer (Invitrogen). Composition is listed in Table 10 (Sambrook et al., 1989).

Table 10: Composition of LB liquid medium supplemented with ampicillin (Sambrook et al., 1989)
Preglednica 10: Sestava tekočega gojišča LB z ampicilinom (Sambrook in sod., 1989)

Component	**Mass/volume per 500 mL of medium**
Tryptone (Sigma-Aldrich)	5.0 g
NaCl (Sigma-Aldrich)	5.0 g
Yeast Extract (Biolife)	2.5 g
Ampicillin stock solution (1g/L)	2.5 mL
dH_2O	Up to final volume of 500 mL

Tryptone, NaCl and yeast extract were weighed into 1L laboratory flask and diluted with 500 mL of dH_2O. Solution was sterilized in autoclave at 121°C at 1.1 bar for 20 minutes. Flask was then transferred into water-bath and left to cool down to 55°C. Flask was then left at room temperature until it cooled down. Flask with liquid laboratory medium was stored at 4°C until needed. 2.5 mL of ampicillin stock solution was added to LB medium to reach recommended final ampicillin concentration of 50 µL/mL prior inoculation with *E. coli*.

3.1.2.4 Fermentation medium

To measure conversion rate of 4-vinylphenol to 4-ethylphenol of different *B. bruxellensis* strains, YNB (Yeast Nitrogen Base) medium was prepared according to manufacturer's recommendations (Difco) and supplemented with 4-vinylphenol. Composition is listed in Table 11.

Table 11: Composition of YNB (Yeast Nitrogen Base) medium supplemented with 4-vinylphenol
Preglednica 11: Sestava gojišča YNB z dodanim 4-vinilfenolom

Component	**Mass/volume per 1000 mL of medium**
YNB (Difco)	6.7 g
Glucose (Sigma-Aldrich)	20 g
10 % 4-vinylphenol solution (Sigma-Aldrich)	60 µL
dH_2O	Up to final volume of 1000 mL

YNB and glucose were weighed into 1000 mL volumetric flask and filled with 900 mL of dH_2O. Solution of 4-vinylphenol was added to final concentration of 7 mg/L and mixed, till all of the constituents completely dissolved. Flask was then filled up to 1000 mL mark and filter sterilized.

3.1.2.5 Media for heterologous expression

Liquid SC-URA medium was prepared for propagation of positive *S. cerevisiae* transformants before induction of transformation. Media was prepared according to pYES2.1 TOPO® TA Expression Kit's manufacturer's recommendations (Invitrogen) and modified to suit our experimental requirements. Composition of liquid SC-URA medium is listed in Table 12.

Table 12: Composition of liquid SC-URA medium
Preglednica 12: Sestava tekočega gojišča SC-URA

Component	**Mass/volume per 1000 mL of medium**
YNB w/o amino acids and ammonium sulphate (Sigma-Aldrich)	4.0 g
Ammonium sulphate (Sigma-Aldrich)	5.0 g
Yeast Synthetic Drop-Out Medium Supplements without uracil (Sigma)	3.0 g
Glucose (Sigma-Aldrich)	20 g
dH_2O	Up to final volume of 1000 mL

All of the constituents were weighed into 1000 mL volumetric flask. Water was added up to 1000 mL mark and flask was mixed until all of the constituents completely dissolved. Medium was then filter sterilized and stored at 4°C until needed.

Liquid SC-URA medium supplemented with 2 % galactose and 10 mg/L of 4-vinylphenol was prepared for induction of expression of positive *S. cerevisiae* YPH250 transformants and detection of vinylphenol reductase activity. Media was prepared according to pYES2.1 TOPO® TA Expression Kit's manufacturer's recommendations (Invitrogen) and modified to suit our experimental requirements. Composition of medium is listed in Table 13.

Table 13: Composition of liquid SC-URA medium supplemented with 2 % galactose and 10 mg/L 4-vinylphenol
Preglednica 13: Sestava tekočega gojišča SC-URA z dodatkom 2 % galaktoze in 10 mg/L 4-vinilfenola

Component	**Mass/volume per 1000 mL of medium**
YNB w/o amino acids and ammonium sulfate (Sigma-Aldrich)	4.0 g
Ammonium sulfate (Sigma-Aldrich)	5.0 g
Yeast Synthetic Drop-Out Medium Supplements without uracil (Sigma-Aldrich)	3.0 g
Galactose (Sigma-Aldrich)	20 g
10 % (w/v) 4-vinylphenol (Sigma-Aldrich)	100 µL
dH_2O	Up to final volume of 1000 mL

All of the powdery constituents were weighed into 1000 mL volumetric flask and 900 mL of dH_2O was added. Flask was shaken until of the constituents completely dissolved. 10 % solution of 4-vinylphenol was added and filled with dH_2O up to 1000 mL mark. Medium was then filter sterilized and stored at 4°C until needed.

Liquid SC-URA medium supplemented with 2 % galactose, 2 % glucose and 10 mg/L of 4-vinylphenol prepared for induction of expression of positive *S. cerevisiae* T334 transformants and detection of vinylphenol reductase activity. Media was prepared according to pYES2.1 TOPO® TA Expression Kit's manufacturer's recommendations (Invitrogen) and modified to suit our experimental requirements. Composition of medium is listed in Table 14.

Table 14: Composition of liquid SC-URA medium supplemented with 2 % galactose, 2 % glucose and 10 mg/L 4-vinylphenol
Preglednica 14: Sestava tekočega gojišča SC-URA z dodatkom 2 % galaktoze, 2 % glukoza in 10 mg/L 4-vinilfenola

Component	**Mass/volume per 1000 mL of medium**
YNB w/o amino acids and ammonium sulfate (Sigma-Aldrich)	4.0 g
Ammonium sulfate (Sigma-Aldrich)	5.0 g
Yeast Synthetic Drop-Out Medium Supplements without uracil (Sigma)	3.0 g
Galactose (Sigma-Aldrich)	20 g
Glucose (Sigma-Aldrich)	20 g
10 % (w/v) 4-vinylphenol (Sigma-Aldrich)	100 µL
dH_2O	Up to final volume of 1000 mL

All of the powdery constituents were weighed into 1000 mL volumetric flask and 900 mL of dH_2O was added. Flask was shaken until of the constituents completely dissolved. 10 % solution of 4-vinylphenol was added and filled with dH_2O up to 1000 mL mark. Medium was then filter sterilized and stored at 4°C until needed.

3.1.2.6 Storage media

Composition of liquid media for storage of wild-type yeast cultures was based on liquid YPD medium (Raspor and Smole Možina, 1993) and supplemented with 20 % (w/v) glycerol. Composition of medium is listed in Table 15.

Table 15: Liquid storage media for wild-type yeast cultures
Preglednica 15: Tekoče gojišče za shranjevanje ne-transformiranih kultur kvasovk

Component	**Mass per 250 mL of medium**
Glucose (Sigma-Aldrich)	5 g
Peptone (Biolife)	5 g
Yeast extract (Biolife)	2.5 g
Glycerol (Kemika)	50 g
dH_2O	Up to final volume of 250 mL

All of the reagents were mixed in 500 mL laboratory flask and shaken till all of the constituents completely dissolved. Solution was sterilized in autoclave at 121°C, 1.1 bar for 20 minutes. After cooling down, the medium was stored at 4°C until needed.

Medium for storage of transformed *E. coli* cells was based on liquid LB medium, as recommended by Invitrogen and supplemented with 20 % (w/v) of glycerol. Composition of medium is listed in Table 16.

Table 16: Composition of *E. coli* storage medium
Preglednica 16: Sestava tekočega gojišča za shranjevanje bakterij vrste *E. coli*

Component	**Mass per 250 mL of medium**
Tryptone (Sigma-Aldrich)	2.5 g
NaCl (Sigma-Aldrich)	2.5 g
Yeast Extract (Biolife)	1.25 g
Glycerol (Kemika)	50 g
dH_2O	Up to final volume of 250 mL

All of the reagents were mixed in 500 mL laboratory flask and shaken till all of the constituents completely dissolved. Solution was sterilized in autoclave at 121°C, 1.1 bar for 20 minutes. After cooling down, the medium was stored at 4°C until needed.

Medium for storage of transformed *S. cerevisiae* cells was based on medium for cultivation of *S. cerevisiae* transformants (SC-URA) as recommended by Invitrogen and supplemented with 20 % (w/v) glycerol. Composition of medium is listed in Table 17.

Table 17: Composition of liquid SC-URA medium for storage of yeast transformants
Preglednica 17: Sestava tekočega gojišča SC-URA za shranjevanje transformiranih kvasovk

Component	**Mass per 250 mL of medium**
YNB w/o amino acids and ammonium sulfate (Sigma-Aldrich)	1.0 g
Ammonium sulfate (Sigma-Aldrich)	1.25 g
Yeast Synthetic Drop-Out Medium Supplements without uracil (Sigma)	0.7 g
Glucose (Sigma-Aldrich)	5 g
Glycerol (Kemika)	50 g
dH_2O	Up to final volume of 250 mL

All of the reagents, except for glycerol, were mixed in 500 mL laboratory flask and shaken till all of the constituents completely dissolved. Glycerol was sterilized in autoclave at 121°C, 1.1 bar for 20 minutes and added to other reagents. Medium was stored at 4°C until needed.

3.1.3 PCR, RFLP and molecular cloning reagents

This chapter lists reagents and materials that were used for PCR, RFLP and molecular cloning experiments.

3.1.3.1 PCR reagents

Table 18: Polymerases used in PCR amplifications
Preglednica 18: Seznam polimeraz, uporabljenih pri PCR reakcijah

Purpose	**Polymerase**	**Manufacturer**
General PCR reactions	GoTaq polymerase	Promega, USA
pYES2.1 molecular cloning	*Pfu* polymerase	Fermentas
pRS426 molecular cloning	Phusion High-Fidelity DNA polymerase	Thermo Scientific, USA
PCR amplification for sequencing	Phusion High-Fidelity DNA polymerase	Thermo Scientific, USA

Table 19: 50x Tris Acetic acid EDTA buffer (TAE)
Preglednica 19: 50x založna raztopina tris ocetna kislina EDTA pufra

Component	**Mass/volume per 100 mL of solution**
Tris base (Merck)	1 g
Acetic acid (Sigma-Aldrich)	5 tablets
PBS buffer	Up to final volume of 100 mL

TAE buffer was stored at 4°C until needed.

Table 20: Agarose gel preparation
Preglednica 20: Priprava agaroznega gela

Component	**Mass/volume per 100 mL agarose gel**			
	1 % agarose	**1.5 % agarose**	**2 % agarose**	**2.5 % agarose**
Agarose (Sigma-Aldrich)	1 g	1.5 g	2 g	2.5 g
1x TAE buffer	100 mL	100 mL	100 mL	100 mL

Agarose gel of desired density was prepared with boiling in microwave oven with 1x TAE buffer until agarose completely dissolved. Solution was cooled to 60°C and mixed with 5 µL of SYBR Safe® DNA gel stain (Invitrogen, USA) – the only exception was RFLP analysis, where gel was stained with ethidium bromide (Sigma-Aldrich, Germany) instead. Gel was cast into mini-cell and left at room temperature until it polymerized.

Other reagents and materials:
1. 6x DNA loading dye (Thermo Scientific, USA)
2. SYBR Safe® DNA gel stain (Invitrogen, USA)
3. GeneRuler Ladder Mix; 100 – 10000 bp (Thermo Scientific, USA)
4. High Pure PCR Product Purification Kit (Roche, Switzerland)

3.1.3.2 Reagents for RFLP analysis

RFLP (Restriction Fragment Length Polymorphism) analysis was performed using following restriction enzymes enzymes as recomeded by Guillamón et al. (1998):

- *Cfo*I (Fermetas)
- *Hae*III (Fermetas)
- *Hin*fI (Fermentas)

3.1.3.3 Plasmids used for heterologous expression

Two different plasmids were used for heterologous expression in *S. cerevisiae*. Plasmid, used in most of the experiments was pYES2.1 TOPO which was obtained in pYES2.1 TOPO® TA Expression Kit (Invitrogen, USA) in linearized form.

The second plasmid that was used in our heterologous expression studies – pRS426 – was donated by dr. Jean-Marc Daran from Industrial Microbiology group at TU Delft (The Netherlands).

3.1.3.4 Control plasmids

Plasmid that was used for positive control of heterologous expression was donated by dr. Krešimir Gjuračić from Acies Bio d.o.o. (Slovenia). Plamid is based on pYES2.1 TOPO® *S. cerevisiae* expression plasmid (Invitrogen, USA) with ORF of *S. cerevisiae RAD59* gene (SGD ID: S000002217) as an expressed insert. Plasmid map is enclosed in Annex K.

3.1.3.5 Plasmid isolation

Isolation of plasmid DNA from transformed *E. coli* cells was performed using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Germany) according to manufacturer's specifications. Plasmids were eluted with 50 µL of elution buffer.

3.1.3.6 Reagents for ligation of pRS426-based plasmids

pRS426 based expression plasmids were constructed using Gibsons Assembly® Master Mix (NEB, USA) according to manufacturers specifications.

3.1.3.7 Chemical transformation reagents

This chapter lists materials and reagents used for chemical transformation of *S. cerevisiae* as recommended by Gietz and Woods (2002) as follows.

Carrier DNA for transformation of *S. cerevisiae* was prepared by mixing of 20 mg of deoxyribonucleic acid from salmon testes (Sigma-Aldrich, Germany) with 10 mL of ddH_2O and heated until fully dissolved to obtain final concentration of 2 mg/mL. Solution was then sterilized in autoclave at 121°C, 1.1 bar for 20 minutes, dispensed into 1 mL aliquots and stored at -20°C until needed.

50 % (w/v) polyethylene glycol (PEG) 3350 was prepared by dissolving of 50 g of PEG 3350 (Sigma-Aldrich, Germany) in 100 mL of ddH_2O. Solution was then sterilized in autoclave at 121°C, 1.1 bar for 20 minutes and stored at 4°C until needed.

Solution of 1M lithium acetate (LiAc) was prepared by dissolving 6.59 g of LiAc (Sigma-Aldrich, Germany) in 100 mL of ddH_2O. Solution was mixed until powder fully dissolved. 50 mL of 1M LiAc solution was then mixed with 450 mL ddH_2O to obtain 0.1 M LiAc solution. Both solutions were sterilized in autoclave at 121°C, 1.1 bar for 20 minutes and stored at 4°C until needed.

3.1.4 Oligonucleotide primers

This chapter lists all of the oligonucleotide primers that were used in our experiments.

Table 21: Oligonucleotide primers for amplification of *B. bruxellensis* vinylphenol reductase gene as proposed by Tchobanov et al. (2008)
Preglednica 21: Oligonukleotidni začetniki za pomnoževanje gena za vinilfenol reduktazo kvasove *B. bruxellensis* osnovanih na sekvenci, ki jo je predlagal Tchobanov in sod. (2008)

Reference	Gene	Name	Tm (°C)	Nucleotide sequence (5'-3')
Tchobanov et al. (2008)	VPR	VPR_F	65.9	ATGCCTCTCATGACAATTTCAGACT
		VPR_R	67.1	CTGCTGATCATTCTGAGCGTAGAAG
		VPR_ORF2	64.3	ATGACAATTTCAGACTCTGTCAAGG
		VPR_TAG	65.1	CTACTGCTGATCATTCTGAGCGTAG
This study	VPR	Carb_Y_F	63.7	ATGTCTTTTATAGTAGCTAGCTAGCAG GAGTATACG
		Carb_Y_R	65.6	CTGCTGATCATTCTGAGCGTAGA

Table 22: Oligonucleotide primers for amplification of internal transcribed spacer sequence of fungal rDNA (Guillamón et al., 1998)
Preglednica 22: Oligonukleotidni začetniki za pomnoževanje ITS regije rDNA gliv (Guillamón in sod., 1998)

Reference	Gene	Name	Tm (°C)	Nucleotide sequence (5'-3')
Guillamón et al. (1998)	ITS sequence of fungal rDNA	ITS1	68.5	TCCGTAGGTGAACCTGCGG
		ITS4	61.5	TCCTCCGCTTATTGATATGC

Table 23: Oligonucleotide primers supplied by Invitrogen for sequencing of pYES2.1 insert
Preglednica 23: Oligonukleptidni začetniki za sekveniranje inserta v pYES2.1 plazmidih (Invitrogen)

Reference	Gene	Name	Tm (°C)	Nucleotide sequence (5'-3')
pYES2.1 TOPO® TA Expression Kit (Invitrogen)	-	GAL1	51.5	AATATACCTCTATACTTTAACGTC
		V5	66.0	ACCGAGGAGAGGGTTAGGGAT

Table 24: Oligonucleotide primers for amplification of alternative vinylphenol reductase candidate genes
PReglednica 24: Oligonukleotidni začetniki za pomnoževanje alternativnih kandidatnih genov za encim vinilfenol reduktaza

Reference	**Gene**	**Name**	**Tm (°C)**	**Nucleotide sequence (5'-3')**
Granato et al. (2014)	SOD	SOD_F	61.1	ATGGTTTTTATTCAAAGTTATAAGTATTCAA
		SOD_R	63.0	GGCAAAGATTTTCGAAGCAT
		SOD_TAG	62.1	TTAGGCAAAGATTTTCGAAGC
Tchobanov et al. (2008); Granato et al. 2014)	TPI	TPI_F	66.8	ATGGCTAGAACATTTTTCGTCGG
		TPI_R	65.7	CTGTCTTGACTTAATGATGTCAGCG
This study	Putative cytochrome p450 monooxygenase	PCp450M_F	65.1	ATGTTAGAAGAATTACTGCAAAAAGAATCAG
		PCp450M_R	65.7	CTCTTTAGATTCGATTGGGTCCAG
This study	p450	p450_F	64.8	ATGGAAAGGCCCACACG
		p450_R	64.4	ATCAAGGCGCGATGGAT

Table 25: Oligonucleotide primers for pRS426 based expression plasmid construction
Preglednica 25: Oligonukleotidni začetniki za konstrukcijo ekspresijskega plazmida, osnovanega na plazmidu pRS426

Reference	**Gene**	**Name**	**Tm (°C)**	**Nucleotide sequence (5'-3')***
Addgene pRS416, 2014	pRS426 plasmid backbone	pRS426_fwd	61.4	agattactctaacgcctcagccatcatcggtaatagctcgaattgctgagaacccgtgacAGCTGTTTCCTGTGTGAAATTG
		pRS426_rev	61.3	gttgaacattcttaggctggtcgaatcatttagacacgggcatcgtcctctcgaaaggtgTAGGGTGATGGTTCACGTAG
Pavlović and Hörz (1988)	*S. cerevisiae GPD* promoter sequence	60bp_B+pGPD_fwd	55.4	ctacgtgaaccatcaccctacacctttcgagaggacgatgcccgtgtctaaatgattcgaccagcctaagaatgttcaacAGTTTATCATTATCAATACTCGC
		60bp_B+pGPD_rev	56.6	tgagaggcatATCCGTCGAAACTAAGTTC
Tchobanov et al. (2008)	VPR	VPR_fwd	57.7	ttcgacggatATGCCTCTCATGACAATTTC
		VPR_rev	57.3	aattacatgaCTACTGCTGATCATTCTGAG
Russo and Sherman (1989)	*S. cerevisiae CYC1* terminator sequence	tCYC1+60bp_H_fwd	58.0	tcagcagtagTCATGTAATTAGTTATGTCACGC
		tCYC1+60bp_H_rev	62.9	atttcacacaggaaacagctgtcacgggttctcagcaattcgagctattaccgatgatggctgaggcgttagagtaatctGGCCGCAAATTAAAGCCTTC

*Uppercase letters indicate primer sequence, while lowercase letters indicate homologous region used in Gibsons Assembly reactions or synthetic spacer sequence

Table 26: Oligonucleotide primers for verification of correct assembly of pRS426 based expression plasmid
Preglednica 26: Oligonukleotidni začetniki za potrditev pravilne sestave inserta plazmida, osnovanega na plazmidu pRS426

Reference	Gene	Name	Tm (°C)	Nucleotide sequence (5'-3')
This study	-	GPD_seq_F	66.5	CTATCAGGGCGATGGCCCACTAC
		VPR_seq_R	66.3	AGTGAAGTCAATCCTAGGCAAGTTCTTGG
This study	-	VPR_seq_F	51.8	CGGTAGGTATTGATTGTAATTC
		CYC_seq_R	52.9	CAGTATAATGTTACATGCGTACAC

All oligonucleotide primers, listed in this chapter were obtained from Sigma-Aldrich (Germany) and diluted to 100 nM as specified by manufacturer. Primers were further diluted to 10 nM working stock concentration and stored at -20°C.

3.1.5 HPLC reagents

This chapter lists reagents used for preparation of HPLC mobile phases as well as standard solution used for preparation of standard curves.

3.1.5.1 Volatile phenols mobile phase

The concentration of volatile phenols was measured with Waters column (130 Å; 3.5 µm; 4.6 mm x 150 mm; 1/pkg) with 27 % acetonitrile used as mobile phase. For preparation of mobile phase, 270 mL of acetonitrile (Sigma) was poured into 1000 mL volumetric flask and filed the flask up to the 1000 mL mark with ddH_2O. Mobile phase was degassed for 20 minutes before usage.

3.1.5.2 Standard solutions for preparation of calibration curves

The exact concentrations of 4-vinylphenol and 4-ethylphenol were calculated with use of their respective measurement curves.

3.1.5.2.1 4-vinylphenol standard curve

4-vinylphenol was supplied by as 10 % solution (w/v) in propylene glycol by manufacturer (Sigma-Aldrich, Germany). 100 DNA from candidate strains was isolated with "MasterPure™ Yeast DNA Purification Kit" (Epicentre, Madison, WI, USA) according to manufacturer's specifications of solution was dissolved into 9.9 mL of ddH_2O to obtain concentration of 1 g/L. Solution was diluted 100x (in two 10x dilution steps) to obtain final concentration of 10 mg/L. This concentration was further diluted 4 times in 1:1 ratio (500 µL of solution was mixed with 500 µL of ddH_2O), to obtain following concentrations for standard curve preparation: 10 mg/L, 5 mg/L, 2.5 mg/L, 1.25 mg/L and 0.625 mg/L. Standard curve is presented in the Annex A1.

3.1.5.2.2 4-ethylphenol standard curve

100 mg of 4-ethylphenol (Sigma-Aldrich, Germany) was added to 10 mL volumetric flask and dissolved in 1 mL of 100 % ethanol (Sigma-Aldrich). Flask was then filled to 10 mL mark with ddH_2O to obtain concentration of 10 g/L. Solution was diluted 1000x (in three 10x dilution steps) to obtain final concentration of 10 mg/L. This concentration was further diluted four times in 1:1 ratio (500 µL of solution was mixed with 500 µL of ddH_2O), to obtain following concentrations for standard curve preparation: 10 mg/L, 5 mg/L, 2.5 mg/L, 1.25 mg/L and 0.625 mg/L. Standard curve is presented in the A2.

3.1.6 Western Blot reagents

This chapter lists reagents and buffers used for Western blot analysis, as well as buffers used for protein isolation and SDS-PAGE.

3.1.6.1 Protein isolation reagents

Reagents for protein isolation were prepared as described by Méchin et al. (2007) and modified as follows.

Table 27: Composition of 0.25 M NaOH solution with 1 % 2-mercaptoethanol
Preglednica 27: Sestava 0.25 M raztopine NaOH z dodatkom 1 % 2-mercaptoetanola

Component	**Mass/volume per 100 mL of solution**
NaOH (Sigma-Aldrich)	1 g
2-mercaptoethanol (Sigma-Aldrich)	1 mL
dH_2O	Up to final volume of 100 mL

0.25 M NaOH solution with 1 % 2-mercaptoethanol was dissolved in 100 mL of dH_2O just before start of protein isolation.

Table 28: Composition of protein resuspension buffer
Preglednica 28: Sestava pufra za resuspendiranje proteinov

Component	**Mass/volume per 100 mL of solution**
SDS (Sigma-Aldrich)	1 g
Complete Mini Protease Inhibitor cocktail (Roche)	5 tablets
PBS buffer	Up to final volume of 100 mL

Protein suspension buffer was prepared just before the start of protein isolation and stored at 4°C.

50 % (w/v) trichloroacetic acid solution was prepared by dissolving 5 g of trichloroacetic acid (Merck, Germany) in 10 mL of ddH_2O. Solution was prepared just before the start of protein isolation and stored at 4°C.

Other reagents for protein isolation:

- Acetone (Sigma-Aldrich, Germany)

3.1.6.2 SDS-PAGE and protein transfer buffers

Table 29: Composition of 10x SDS-PAGE buffer (CSH Protocols, 2006)
Preglednica 29: Sestava 10x založne raztopine pufra za SDS-PAGE (CSH Protocols, 2006)

Component	**Mass per 1000 mL of solution**
Tris base (Merck)	30.3 g
Glycine (Sigma-Aldrich)	144 g
Sodium dodecyl sulfate (Sigma-Aldrich)	10 g
dH_2O	Up to final volume of 1000 mL

Stored at room temperature.

Table 30: Composition of 10x protein transfer buffer stock
Preglednica 30: Sestava 10x založne raztopine pufra za prenos proteinov

Component	**Mass per 1000 mL of solution**
Tris base (Merck)	30.3 g
Glycine (Sigma-Aldrich)	144.1 g
dH_2O	Up to final volume of 1000 mL

Solution was sterilized in autoclave at 121°C, 1.1 bar for 20 minutes and stored at 4°C until needed.

Table 31: Composition of 10x Tris base saline (TBS) buffer
Preglednica 31: Sestava 10x založne raztopine za TBS pufer

Component	**Mass per 1000 mL of solution**
Tris base (Merck)	12.1 g
NaCl (Sigma-Aldrich)	87.8 g
dH_2O	Up to final volume of 1000 mL

Solution was sterilized in autoclave at 121°C, 1.1 bar for 20 minutes and stored at 4°C until needed.

Table 32: Composition of protein transfer buffer
Preglednica 32: Sestava pufra za prenos proteinov

Component	**Volume per 1000 mL of solution**
10x Protein transfer buffer stock	100 g
Methanol (Sigma-Aldrich)	200 g
dH_2O	Up to final volume of 1000 mL

Stored at 4°C.

Table 33: Composition of Ponceau S solution for detection of membrane bound proteins
Preglednica 33: Sestava raztopine Ponceau S za detekcijo proteinov, vezanih na membrano

Component	**Volume per 30 mL of solution**
1 % acetic acid	30 mL
Ponceau S (Sigma-Aldrich)	0.03 g

Stored at room temperature.

Table 34: Composition of 1x TBS buffer supplemented with 3 % powdered milk (TBSM)
Preglednica 34: Sestava 1x raztopine TBS pufra z dodatkom 3 % mleka v prahu

Component	**Volume per 200 mL of solution**
10x TBS buffer	20 mL
Instant powdered milk (Pomurske Mlekarne)	6 g
dH_2O	Up to final volume of 200 mL

Solution was prepared just before blocking of nitrocellulose membrane.

Table 35: 6x SDS sample buffer
Preglednica 35: Sestava 6x založne raztopine SDS pufra

Component	**Volume/mass per 10 mL of solution**
1M Tris-HCl pH 6.8	3.75 mL
Sodium dodecyl sulfate (Sigma-Aldrich)	1.2 g
DTT (Sigma-Aldrich)	0.93 g
Glycerol (Kemika)	6 mL
Bromophenol Blue (Sigma-Aldrich)	0.002 g
dH_2O	Up to final volume of 10 mL

First, 100 mL of 1M Tris (Merck) solution was prepared and pH adjusted to 6.8. Tris-HCl solution was then sterilized in autoclave at 121°C, 1.1 bar for 20 minutes. 6x SDS sample buffer was prepared with mixing all of the components and storing them as 1 mL aliquots at -20°C until needed.

Other materials and reagents used:

- Protran 0.45 µm nitrocellulose membrane (Whatman, USA)
- Anti-V5-HRP conjugated antibody (Invitrogen, USA)
- Filter papers (Bio-Rad, USA)
- Pierce™ DAB Substrate Kit (Thermo Scientific, USA)
- Unstained protein molecular weight marker (Fermentas)

3.1.7 Other reagents

Phosphate-buffered saline (PBS) solution was prepared with dissolving 1 phosphate-buffered saline (Dulbecco A) tablet (Oxoid, UK) per 100 mL of dH_2O and sterilizing it in in autoclave at 121°C, 1.1 bar for 20 minutes. PBS solution was stored at 4°C until needed.

3.1.8 Laboratory materials and equipment

Frequently used laboratory equipment and laboratory materials used in our experimental work are listed in the following tables.

Table 36: Table of frequently used materials
Preglednica 36: Preglednica pogosto uporabljenega potrošnega materiala

Item	**Manufacturer**
Microcentrifuges (1.5 and 2.0 mL)	Eppendorf, Germany
50 mL microcentrifuges	TPP, Switzerland
15 mL microcentrifuges	TPP, Switzerland
Magnets	Brand, Germany
Automatic pipette dispensers	Gilson, France
96 Micro-well microtiter plates	Nunc, ThermoFischer Scientific, USA
Disposable syringes	BD, USA
HPLC vials with screw-caps	Supelco, Sigma-Adrich, Germany
Erlenmeyer flasks (100, 150, 200 and 250 mL)	Schott Duran, Germany
Laboratory flasks (250, 500 and 1000 mL)	IsoLab, Germany
Phenex 0.2 μm RC filters	Phenomoenex, Italy
96-well PCR plates with cover strips	Nippon Genetics, Japan
Cryo.s 2 mL cryo vials	Greiner Bio-One, Germany
8-well PCR strips with covers	Brand, Germany
Burker-Türk hemocytometer	Brand, Germany
Nalgene® bottle-top sterile filter units	Nalgene, Sigma-Aldrich, Germany
Sterile plastic petri dishes	Labortehnika Golias, Slovenija
Sterile plastic inoculation loops	Labortehnika Golias, Slovenija

Table 37: Table of equipment used for experimental work
Preglednica 37: Preglednica opreme, ki smo jo uporabljalo tekom eksperimentalnega dela

Equipment	**Name**	**Manufacturer**
Laminar flow cabinet	PIO LFVP 12	Iskra, Slovenija
Set of automatic pipettes		Gilson, France
PCR apparatus	GeneAmp DNA thermal cycler	Perkin Elmer, USA
Centrifuge – large	3K30	Sigma, USA
Centrifuge – small	MiniSpin	Eppendorf, Germany
Autoclave		Sutjeska, Yugoslavija
Incubator – cooling/heating	BTES_frigomat	Termo Iskra, Slovenija
Incubator		Kambič, Slovenija
Scale	Sartorius excellence	Sartorius, Switzerland
Precise scale	ME204	Mettler Toledo, USA
Shaker	Multitron HT	Inforst HT, Switzerland
Dust chamber		Iskra, Slovenija
Refrigerator		Gorenje, Slovenija
Freezer (-80°C)	HERAfreeze HFU T Series	Thermo Scientific, USA
Heater and magnetic stirrer	RCT basic	IKA, Germany
Vortex	Lab Dancer	IKA, Germany
Water bath		Kambič, Slovenija
HPLC software	Eurochrom 2000	Knauer, Germany
Microscope	DM4000 B	Leica, Germany
Microscope camera	Leica DFC290	Leica, Germany
Picture analysis software	ImageJ	Schneider et al., 2012
Software for plasmid construction	Unipro UGENE	Okonechnikov et al., 2012
Software for plasmid construction	NEBuilder® Assembly Tool	NEB, USA
HPLC apparatus		Knauer, Germany
Microwave oven	Sanyo	Sanyo, USA
Drying cabinet	SO-250	Elektromedicina Slovenija
pH meter	Seven Multi	Mettler Toledo, USA
Spectrophotometer	Lambda Bio Plus	Perkin-Elmer, USA
HPLC auto-sampler	Midas	Spark, The Netherlands
HPLC capillary column	ZB-FFAP	Phenomenex, Italy
Spectrofluorometric microtiter plate reader	Safine 2	Tecan, Switzerland
HPLC fluorescence detector	RF-551	Shimidzu, Japan
HPLC column for volatile phenol separation	XBridge Phenyl column	Waters, USA
Gel imaging system	Gel Doc	Bio-Rad, USA
Agarose gel electrophoresis system	Wide Mini-Sub Cell GT	Bio-Rad, USA
Protein electrotransfer apparatus	Mini Trans-Blot Cell	Bio-Rad, USA
Vacuum pump	Air KKDet	Cole Parmer, USA

3.2 METHODS

All of the methods that were used in our experimental work are listed in this chapter. These methods include isolation and characterization of novel *B. bruxellensis* strain, fermentation experiments for determination of specific conversion rate of *B. bruxellensis* strains, *in-silico* vector design and subsequent construction. Methods for heterologous expression of vinylphenol reductase, confirmation of expression and activity of heterologous enzyme are also included in this chapter. The flowchart representing the course of the experiments is presented in Figure 2.

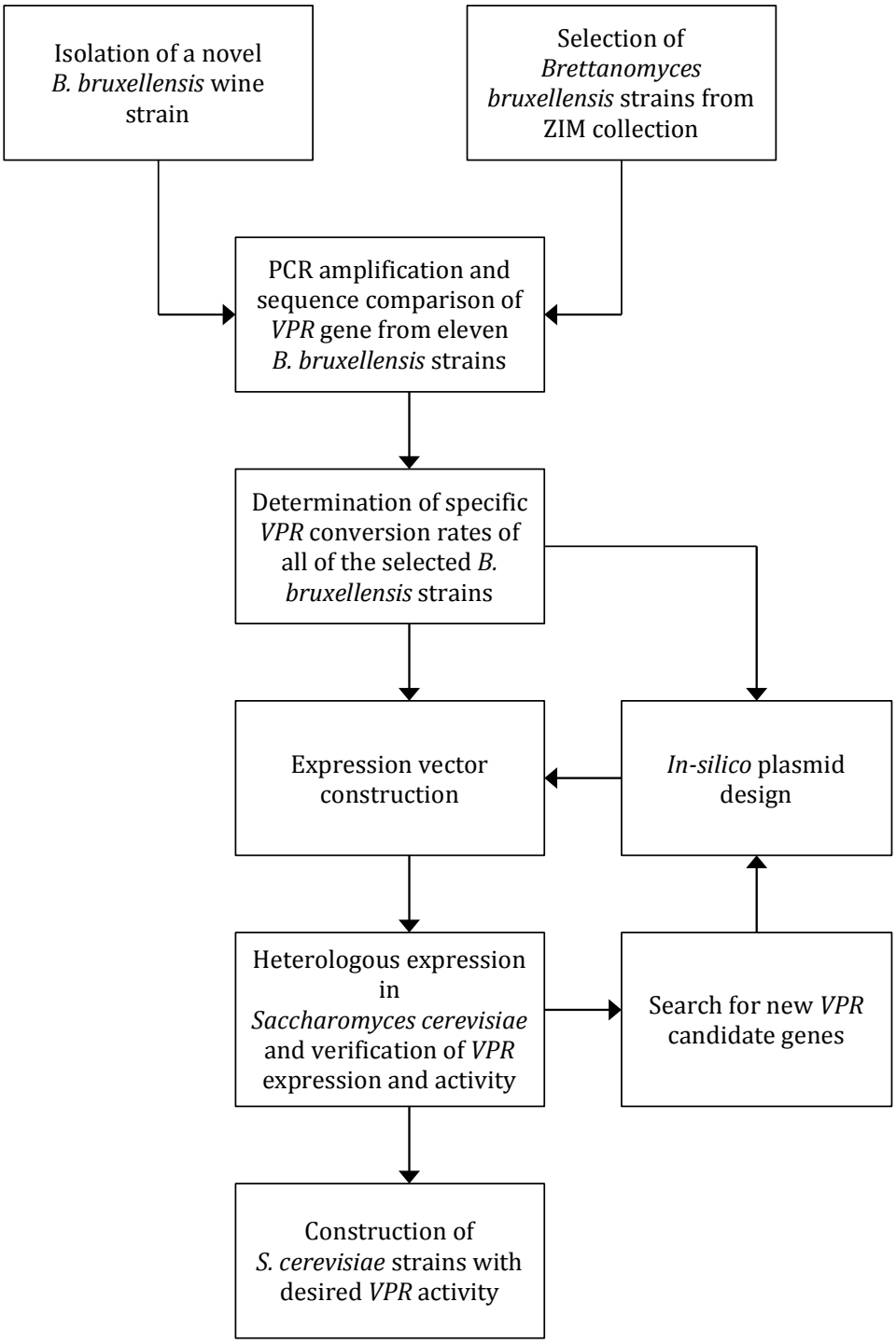

Figure 2: Flow-chart representing course of the experiments (*VPR* – vinylphenol reductase)
Slika 2: Blokovna shema poteka eksperimentalnega dela (*VPR* – vinilfenol reduktaza)

3.2.1 Isolation and identification of novel *B. bruxellensis* strain

This chapter lists all the methods, used for isolation and identification of novel *B. bruxellensis* strain.

3.2.1.1 Isolation of novel *B. bruxellensis* strain

A novel *B. bruxellensis* strain was isolated from wine must imported from Montenegro according to a method developed by Rodrigues et al. (2001). 6 mL of sterile DBDM (*Dekkera Brettanomyces* Differential Medium) was transferred to sterile glass tubes and inoculated with 1 mL of vortexed wine must sample. Experiment was performed in two replicates. Tubes were incubated for 2 weeks at 28°C. Test tubes that changed colour from blue to yellow (indicating the drop of pH) were used for further analysis. Tubes were vortexed and 100 µL of the suspension was transferred and spread onto OGYE agar plates. Plates were then incubated for 5 days at 28°C. After incubation, the colonies that exhibited colony and cell morphology, characteristic for *B. bruxellensis* were used for further analysis.

3.2.1.2 Isolation of genomic DNA from *B. bruxellensis*

DNA from candidate *B. bruxellensis* strains was isolated with "MasterPure™ Yeast DNA Purification Kit" (Epicentre, USA) according to manufacturer's specifications. In final step, DNA was dissolved in 35 µL of molecular biology grade H_2O. Concentration of DNA was measured with Lambda Bio Plus (Perkin Elmer, USA) spectrophotometer with built-in settings. Calibration for background correction was done with 1.5 µL of molecular biology grade water. Samples were stored at -20°C until needed.

3.2.1.3 Identification of *B. bruxellensis* with RFLP method

Identification of novel *B. bruxellensis* was performed with RFLP (Restriction Fragment Length Polymorphisem) analysis of two ribosomal internal transcribed spacers ITS1 and ITS2 as described by Guillamón et al. (1998) and modified as follows. 20 µL PCR reaction mixture was prepared according to polymerase manufacturer's specification (GoTaq polymerase; Promega, USA) with 100 ng of genomic DNA used as a template and primer concentration of 0.5 µM (each). Primers used in this experiment are listed in Table 22. PCR reaction was performed in thermocycler as follows: 5 min of initial denaturation at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55.5°C for 30 s, extension at 72°C for 60 s and 1 cycle of final extension at 72°C for 7 min.

After PCR reaction, enzymatic restriction of PCR amplicons was performed with enzymes *Cfo*I, *Hae*III and *Hin*fI (all Fermentas) according to manufacturer's specification and modified as follows. Three 10 µL reaction mixtures were prepared (one for each restriction enzyme) for each sample as follows:

1. 1 µL of enzyme buffer (Buffer R for *Hinf*I and *Hae*III; Buffer Tango for *Cfo*I)
2. 0.2 µL of *Cfo*I, *Hae*III or *Hin*fI enzyme (1 U)
3. 3.8 µL of molecular biology reagent grade water
4. 5 µL of PCR product

The digestion reaction mixtures were incubated for three hours at 37°C in thermocycler. After the completion of restriction reaction, 10 µL of each restriction reaction was stained with 2 µL of 6x loading dye solution and loaded onto 2.5 % agarose gel. Electrophoresis was performed at 120 V (constant) and 400 mA for 40 min in 1x TAE buffer. After electrophoresis, restriction fragments were visualized under UV light after ethidium bromide staining (15 min of ethidium bromide staining and 10 min of equilibration in distilled H_2O). Fragment sizes were estimated by comparison using DNA size marker (Thermo Scientific, USA). Colonies that tested positive were deposited in ZIM culture collection (Biotechnical faculty, Slovenija).

3.2.2 Determination of specific conversion rate of 4-vinylphenol to 4-ethylphenol of *B. bruxellensis* strains

This chapter lists methods and protocol that were used to obtain specific conversion rates of different *B. bruxellensis* strains.

3.2.2.1 Inoculum preparation

Inoculum experiment was designed specifically for our experiments as follows. Eleven *B. bruxellensis* strains were used in this experiment: ZIM 700, 701, 702, 703, 704, 705, 1762, 1764, 2306, 2512 and CBS 2499 with *S. cerevisiae* ZIM 1927 as a negative control. Starter cultures of single strains were prepared in 100 mL of sterile YPD broth in 150 mL Erlenmeyer flasks at 28°C with shaking at 160 rpm for 48 hours for *B. bruxellensis* strains and 24 hours for *S. cerevisiae* control. Cells were collected in sterile 50 mL microcentrifuge tubes and centrifuged for 3 minutes at 2000 g. Supernatant was discarded and the cells were washed three times with 20 mL of sterile PBS buffer. Finally, cells were resuspended in 5 mL of YNB medium (supplemented with 20 g/L glucose and 6 µg/L 4-vinylphenol). After determination of metabolically active cell concentration in 1 mL of suspension with hemocytometer (as described in Chapter 1.2.2.3), we inoculated YNB medium (supplemented with 20 g/L glucose and 6 µg/L 4-vinylphenol) to a final concentration of 1×10^6 metabolically active cells/mL.

3.2.2.2 Fermentations in defined synthetic medium

Fermentation experiments were performed as described by Hixson et al. (2012) and modified as follows. Fermentations were performed in 250 mL Erlenmeyer flasks were filled with sterile YNB medium (supplemented with 20 g/L glucose and 6 µg/L 4-vinylphenol) to final volume of 200 mL, closed with sterile rubber stoppers with CO_2 outlet. Fermentation vessels were inoculated with 1×10^6 metabolically active cells/mL. Fermentations were performed for 6 days at 28°C, with sampling times at day 0, 1, 2, 4 and 6. All fermentations got performed in triplicates. Before sampling, flasks were shaken, until sediment was fully suspended. 2 mL of sample was retrieved into sterile 2 mL microcentrifuge tubes and frozen at -80°C for later analysis with HPLC. Additional 100 µL of suspension was withdrawn for determination of concentration of metabolically active cells (as described in Chapter 1.2.2.3).

3.2.2.3 Determination of metabolically active cells with microscope

Concentration of metabolically active cells was determined as described by Zupan et al. (2013). Each Erlenmeyer flask was shaken, until the sediment got fully suspended. 100 µL was withdrawn from each flask and diluted in 1:1 (v/v) ratio with methylene blue solution (Painting and Kirsop, 1990). Suspension was vortexed and 20 µL was transferred into a 100 µm deep Burker-Türk hemocytometer (Brand, Germany). Cells were observed with a microscope with attached camera (Leica DFC290) under bright-field setting, maximized gamma correction and maximally closed aperture, which resulted in light spots. That represented viable cells on a dark background. 10x magnification objective was used to cover as much area as possible. Images were documented using Leica application Suite version 3.7.0 (Switzerland).

A total number of metabolically active cells was determined with automated image analysis with ImageJ software (Schneider et al., 2012), also described by Zupan et al. (2013). Image analysis proceeded in exact sequence:

- A set of images was downloaded into program with threshold adjusted for metabolically active cells.
- Images were then processed by the *Fill holes* and *Watershed* tool in order to correct for weakly labeled cells and to separate buds respectively.
- *Analyze particles* tool was used to selectively count cells, based on their size (set at 8 - ∞) and circularity (set at 0.3 -1.0), which enabled the program to discard any artifacts present in the sample.

Concentration of yeast cells was calculated, considering the volume, according to Bürker-Türk chamber depth (100 µm) and the surface covered by the microscope image (the volume in our case was 3.24×10^{-5} mL). Sample image is presented in Annex B.

3.2.2.4 Determination of 4-vinylphenol and 4-ethylphenol concentration with HPLC

For determination of 4-vinylphenol and 4-ethylphenol concentrations, 2 mL microcentrifuges, containing samples were defrosted at room temperature and centrifuged at 4000 g for 5 minutes. The HPLC analysis was performed as described by Kosel et al. (2014). Supernatant was filtered through 0.22 µm filters (Phenomenex, Italy). Filtrates were placed in HPLC vials and analyzed with High-Performance Liquid Chromatography (HPLC). The apparatus (Knauer, Berlin, Germany) comprised of Midas autosampler (Spark, Emmen, The Netherlands), two Knauer K1001 WellChrom Pumps with 10 mL pump heads, Knauer dynamic mixing chamber and a Shimadzu RF-551 fluorescence detector (Schimadzu, Kyoto, Japan). Separation was carried out with an XBridge Phenyl column (130 Å, 3.5 µm, 4.6 mm × 150 mm, 1/pkg; Waters, Milford, USA) using a 27 % acetonitrile as the mobile phase at flow rate of 1 mL/min. External standards of 4-vinylphenol and 4-ethylphenol, were used for determination of standard curves and subsequent quantification of sample concentrations (preparation of external standards is described in Chapter 3.1.5). The detector was set to high sensitivity at an excitation wavelength of 280 nm and emission wavelength of 333 nm. Retention time of 4-vinylphenol was 12 minutes and 14 minutes for 4-ethylphenol.

3.2.2.5 Calculation of specific conversion rates

The data from the measurement of viable cells and metabolite concentrations was used to calculate the substrate (4-vinylphenol) consumption rates and product (4-ethylphenol) as described by Zupan et al. (2013) and modified for the purpose of our experiments.

The consumption/production rates of volatile phenols (VP; 4-vinylphenol/4-ethylphenol) between two time points were calculated as:

$$R_{VP} = \frac{(c_{VP(t_2)} - c_{VP(t_1)})}{\left(\frac{c_{t_1} + c_{t_2}}{2}\right) \times (t_2 - t_1)} \times 10^9 \text{ [pg/cells/day] (Zupan et al. 2013)} \quad \ldots (1)$$

Legend:

R_{VP} – Specific consumption/production rate of 4-vinvlphenol/4-ethylphenol at specified time point
$c_{VP(t_1)}$ – Concentration µg/L) of volatile phenol at time point 1
$c_{VP(t_2)}$ – Concentration (µg/L) of volatile phenol at time point 2
c_{t_1} – Concentration of metabolically active cells in 1 mL at time point 1
c_{t_2} – Concentration of metabolically active cells in 1 mL at time point 2
t_1 – Time point 1 (day)
t_2 – Time point 2 (day)

It has to be pointed out, that there were five time points (0, 1, 2, 4 and 6 days) and R_{VP} was calculated for each day and for each volatile phenol (4-vinylphenol and 4-ethylphenol) separately. When all of the R_{VP} were calculated, the results were used for construction of graph of relative consumption rate of 4-vinylphenol and graph of relative formation rate of 4-ethylphenol. Differences between the curve profiles among different strains of *B. bruxellensis* indicated the efficiency of conversion of the tested strain.

3.2.3 Search for additional candidates for vinylphenol reductase genes

Methods for discovery of new candidates for vinylphenol reductase gene of *B. bruxellensis* are listed in this chapter as well as the methods used for the preparation of PCR amplicons for sequencing and their comparison.

3.2.3.1 Search for additional candidate genes

Due to discrepancy between publications describing identification of vinylphenol reducatse gene of *B. bruxellensis* (Godoy et al., 2008; Tchobanov et al., 2008; Granato et al., 2014), additional literature check was performed to find vinylphenol reducate activity in other organisms. Sequences for reported candidate genes were retrieved from EMBL European Bioinformatics Institute web page (McWilliam et al., 2013). Retrieved sequences were used to run nucleotide BLAST (Altschul et al., 1990). All of the parameters were left at default settings, except for organism, which was set to *B. bruxellensis* (taxid: 5007) and search algorithm, which was set to be optimized for somewhat similar sequences (blastn).

When the results of the BLAST search were obtained, selected sequences with highest similarity and E-value ≤ $1E^{-4}$ were retrieved and used for further experiments.

3.2.3.2 Design of oligonucleotide primers used for sequencing

Oligonucleotide primers that were used to obtain PCR amplicons of vinylphenol reductase candidate for sequencing reaction were designed manually with Unipro UGENE software (Okonechnikov et al., 2012) because of the low quality of the deposited sequences. For primer design, nucleotide sequences for whole genes as well as their 1000 bp upstream and 1000 bp downstream regions were imported into program. All of the primers were designed to capture the longest open reading frame (ORF) of selected genes, with forward primer targeted at upstream region of ORF and reverse primer targeted at downstream region of ORF. All of the designed primers are listed in Chapter 3.1.4.

3.2.3.3 Amplification of selected candidate genes

In order to obtain PCR amplicons of candidate genes, genes were amplified with PCR reaction. 50 µL PCR reaction mixture was prepared according to polymerase manufacturer's specifications (Phusion High Fidelity DNA polymerase, ThermoFischer Scientific, USA) with 100 ng of genomic DNA used as a template and primer concentration of 0.5 µM (each). Primers used are listed in Chapter 3.1.4. PCR reaction was performed in thermocycler as follows: 5 min of initial denaturation at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 75 s and 1 cycle of final extension for 7 min. To test, if all of the PCR reactions were completed successfully, 5 µL of reaction mixtures were stained with 1 µL of 6x loading dye solution (Thermo Scientific, USA) and loaded onto 1 % agarose gel, that was pre-stained with SYBR® Safe DNA gel stain (Invitrogen, USA) according to manufacturer's specifications. Electrophoresis conditions were 100 V (constant) and 400 mA for 30 min. Gels were visualized under UV light and size of the fragments was estimated using DNA size marker (Thermo Scientific, USA).

After the presence and size of PCR amplicons were confirmed, they were purified with High Pure PCR Product Purification Kit (Roche). Purified amplicons were eluted with 50 µL of elution buffer. After purification, samples were loaded onto 96-well PCR plates, sealed and stored at -20°C.

3.2.3.4 Sequencing of vinylphenol reductase candidate genes

To determine the differences between nucleotide sequences of different candidate genes, purified PCR amplicons of these genes were sent for sequencing to Macrogen Inc. (Seoul, Republic of South Korea). Samples were sequenced using standard sequencing service with sequencing algorithms optimized for samples, longer then 500 bp with oligonucleotide primers, designed in Chapter 3.1.4. All of the samples were sequenced in both directions.

3.2.3.5 Comparison of vinylphenol reductase candidate genes

Nucleotide sequences of vinylphenol reductase gene of selected *B. bruxellensis* candidates were compared using EMBL-EBI ClustalW2 online sequence comparison software (McWilliam et al., 2013). Longest ORF sequences were further imported into Unipro UGENE program (Okonechnikov et al., 2012) and aligned with ClustalW algorithm. After the alignment of sequences, sequence identity percentages (compared to reference sequence) were retrieved. The alignment was then used further to construct dendrogram with built-in software. For dendrogram construction PHYLIP Neighbor Joining method was selected with F84 distance matrix.

After nucleotide sequence comparison, nucleotide sequences were translated into protein sequences with SIB ExPASy online software (Gasteiger et al., 2003) and compared again with EMBL-EBI MUSCLE online software (McWilliam et al., 2013) to check, if the differences in nucleotide sequences resulted in change of amino acid composition.

3.2.4 *In-silico* vector construction

In-silico plasmid design for all of the plasmids was performed with Unipro UGENE software (Okonechnikov et al., 2012).

3.2.5 Plasmid construction and transformation of *E. coli* and *S. cerevisiae*

This chapter lists all the methods used for vector and vector insert construction as well as techniques used for *E. coli* and *S. cerevisiae* transformation.

3.2.6 Isolation of genomic DNA from *B. bruxellensis*

DNA from candidate strains was isolated with "MasterPure™ Yeast DNA Purification Kit" (Epicentre, USA) according to manufacturer's specifications (as already described in Chapter 3.2.1.2). In final step, DNA was dissolved in 35 µL of dH_2O and kept at -20°C.

3.2.6.1 Oligonucleotide primer design for the PCR amplification of candidate genes for plasmid construction

Oligonucleotide primers for cloning of candidate vinylphenol reductase gene of *B. bruxellensis* were based on nucleotide sequences, obtained in Chapter 3.2.3. To obtain the whole ORF sequence, primers for pYES2.1-based plasmid were designed manually with the help Unipro UGENE software (Okonechnikov et al., 2012) to check for suitable T_m, possible dimers and secondary structures. Stop codon was omitted from all of the reverse primers for pYES2.1 cloning reactions, except for vinylphenol reductase genes proposed by Tchobanov et al. (2008) and Granato et al. (2014) of which two reverse oligonucleotide primers were created – one with without stop codon and one with stop codon. All of the created primers are listed in Chapter 3.1.4.

Oligonucleotide primers for construction pRS426-based plasmid were created using NEBuilder® Assembly Tool (NEB, USA) and its respective plasmid map recreated with Unipro UGENE software (Okonechnikov et al., 2012).

3.2.6.2 PCR amplification of plasmid inserts for pYES2.1-based plasmids

For cloning of selected ORF's into vector of choice, these sequences were amplified using PCR reaction. Each reaction was set up as 50 µL reaction mixture (*Pfu* polymerase, Fermentas) with 100 ng of genomic DNA used as a template and primer concentration of 0.5 µM (each). Primers used are listed in Chapter 3.1.4. PCR reaction was performed in thermocycler as follows: 5 min of initial denaturation at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 75 s and 1 cycle of final extension for 7 min. Because *Pfu* polymerase (Fermentas) leaves blunt ends, addition of 0.5 U of *Taq* polymerase (GoTaq polymerase, Promega) was needed with 15 min incubation at 72°C to get the 3' adenine overhangs on PCR amplicons as suggested by pYES2.1 TOPO® TA Expression Kit Instruction Manual (Invitrogen, USA).

To test, if all of the PCR reactions were completed successfully, 5 µL of reaction mixtures were stained with 1 µL of 6x loading dye solution (Thermo Scientific, USA) loaded onto 1 % agarose gel, that was pre-stained with SYBR® Safe DNA gel stain (Invitrogen, USA) according to manufacturer's specifications. Electrophoresis conditions were 100 V (constant) and 400 mA for 30 min. Gels were visualized under UV light and size of the fragments was estimated using DNA size marker (Thermo Scientific, USA). Sample images of the PCR amplicons are presented in Annex D.

After the presence and size of PCR amplicons was confirmed, they were purified with High Pure PCR Product Purification Kit (Roche). Purified PCR amplicons were eluted with 50 µL of elution buffer.

3.2.6.3 Cloning reaction of pYES2.1-based plasmids and transformation into *E. coli*

The cloning and *E. coli* transformation reaction was performed according to manufacturer's instructions (pYES2.1 TOPO® TA Expression Kit; Invitrogen, USA) and modified as follows. Reaction mixture comprised of 2 µL of purified PCR amplicon (see Chapter 3.2.5.3), 1 µL salt solution, 1 µL pYES2.1 TOPO vector solution (Invitrogen, USA) and 1 µL of PCR grade water. Reaction mix was gently mixed and incubated for 5 minutes at room temperature and after incubation placed on ice. 2 µL of reaction mixture was then removed and added to 1 vial (per PCR amplicon) of 50 µL chemically competent TOP10 One Shot® *E. coli* cells (supplied by the manufacturer; Invitrogen), gently mixed and incubated on ice for additional 15 min. After incubation on ice, *E. coli* suspension was heat-shocked in water bath for 30 s at 42°C and immediately placed on ice for 2 min. After 2 min, 250 µL of room temperature SOC medium was added to each vial and gently mixed. Vials were transferred to incubator, where they were shaken in horizontal position at 37°C for 60 min at 220 rpm. After the incubation, 20 µL of each transformation reaction was plated on pre-warmed LB agar plates containing 50 µg/mL ampicillin and incubated overnight at 37°C.

3.2.6.4 Cloning reaction of pRS426-based plasmids and transformation into *E. coli*

In order to construct plasmid pRS426 plasmid backbone, *S. cerevisiae GPD* promoter and *CYC1* terminator sequences as well as the *B. bruxellensis VPR* sequence had to be amplified. DNA from candidate *B. bruxellensis* strains as well as *S. cerevisiae* ZIM 1927

was isolated with "MasterPure™ Yeast DNA Purification Kit" (Epicentre, USA) according to manufacturer's specifications and eluted with 35 µL of molecular biology grade H_2O. For PCR amplification 50 µL PCR reaction mixtures were prepared according to polymerase manufacturer's specifications (Phusion High Fidelity DNA polymerase, ThermoFischer Scientific, USA) with 100 ng of genomic DNA used as a template and primer concentration of 0.5 µM (each). Primers used in this experiment are listed in Chapter 3.1.4. PCR reaction was performed in thermocycler as follows: 5 min of initial denaturation at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 75 s and 1 cycle of final extension for 7 min.

To test, if all of the PCR reactions were completed successfully, 5 µL of reaction mixtures were stained with 1 µL of 6x loading dye solution (Thermo Scientific, USA) loaded onto 1 % agarose gel, that was pre-stained with SYBR® Safe DNA gel stain (Invitrogen, USA) according to manufacturer's specifications. Electrophoresis conditions were 100 V (constant) and 400 mA for 30 min. Gels were visualized under UV light and size of the fragments was estimated using DNA size marker (Thermo Scientific, USA).

After the presence and size of PCR amplicons was confirmed, they were purified with High Pure PCR Product Purification Kit (Roche). Purified PCR amplicons were eluted with 50 µL of elution buffer. Concentration of DNA was measured with Lambda Bio Plus (Perkin Elmer, USA) spectrophotometer with built-in settings. Calibration for background correction was done with 1.5 µL of molecular biology grade water.

Ligation of pRS426 backbone, *GPD* promoter, *CYC1* terminator and *VPR* gene was achieved in one step with Gibsons Assembly® Master Mix (NEB, USA) according to manufacturers specifications. Transformation of *E. coli* proceeded as described in previous chapter.

To verify the correct orientation of the assembled expression cassette, 20 µL PCR reaction was set up using GoTaq polymerase (Promega, USA) according to the manufacturers specifications as listed below. Primers used in this PCR reaction are listed in Table 26 and were designed to cover short region between end of the promoter sequence and the beginning of *VPR* gene as well as the region between end of the *VPR* sequence and beginning of the terminator sequence. PCR reaction was performed in thermocycler as follows: 5 min of initial denaturation at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 45°C for 30 s, extension at 72°C for 30 s with another round of 95°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 30 s and 1 cycle of final extension at 72°C for 7 min.

To test, if all of the PCR reactions were completed successfully, 5 µL of reaction mixtures were stained with 1 µL of 6x loading dye solution (Thermo Scientific, USA) loaded onto 1 % agarose gel, that was pre-stained with SYBR® Safe DNA gel stain (Invitrogen, USA) according to manufacturer's specifications. Electrophoresis conditions were 100 V (constant) and 400 mA for 30 min. Gels were visualized under UV light and size of the fragments was estimated using DNA size marker (Thermo Scientific, USA). Sample images of the plasmid construction are presented in Annex E.

3.2.6.5 Amplification, isolation and verification of pYES2.1 based plasmid DNA

After incubation, 5-10 colonies were picked from each transformation reaction to confirm integration of PCR amplicon into plasmid (pYES2.1 TOPO; Invitrogen, USA) vector as recommended by manufacturer (Invitrogen, USA). Each of 5-10 colonies was transferred from LB agar plates containing 50 µg/mL ampicillin to 20 µL PCR reaction mixture using pipette tip; the same pipette tip was used to streak the transformed *E. coli* colony onto fresh LB agar plate containing 50 µg/mL ampicillin.

Verification with PCR was performed according to polymerase manufacturer's (GoTaq, Promega) recommendations in 20 µL reaction mixture with transformed *E. coli* used as a DNA template and addition of 0.5 µM of forward primer that was used for the amplification of the cloned gene and 0.5 µM of V5 reverse primer, which in case of the successful plasmid integration amplified the open reading frame as well as the part of the terminator region. PCR reaction was performed in thermocycler as follows: 5 min of initial denaturation at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 75 s and 1 cycle of final extension for 7 min.

To test, if the PCR amplicons were integrated, 5 µL of reaction mixtures were stained with 1 µL of 6x loading dye solution (Thermo Scientific, USA) and loaded onto 1 % agarose gel, that was pre-stained with SYBR® Safe DNA gel stain according to manufacturer's specifications. Electrophoresis conditions were 100 V (constant) and 400 mA for 30 min. Gels were visualized under UV light and size of the fragments was estimated using DNA size marker (Thermo Scientific, USA).

E. coli transformants, that yielded positive results (appropriately sized PCR amplicons) were used to inoculate 7 mL of LB medium, containing 50 µg/mL ampicillin in sterile 15 mL microcentrifuge tube. Microcentrifuge tubes were then transferred to incubator for overnight incubation at 37°C with light shaking (50 rpm). After incubation, microcentrifuges were vortexed and 1.8 mL of cell suspension was transferred into sterile 2 mL microcentrifuge tubes and centrifuged for 1 min at 12000 rpm. Supernatant was discarded. From that point on, all of the steps for isolation of plasmid DNA were performed with GenElute™ Plasmid Miniprep Kit according to manufacturer's (Sigma-Aldrich) specifications. In final step, the plasmid DNA was eluted with 50 µL of elution solution. Isolated plasmid DNA was stored at -20°C till needed.

Furthermore, 850 µL of *E. coli* transformant suspension was transferred to 2 mL cryovials and 150 µL of sterile glycerol was added. Cryovials were then closed, appropriately labelled, vortexed until glycerol dissolved and stored at -80°C.

Of those plasmids, that already had the size and the integration of PCR amplicons confirmed, plasmids selected for transformation into *S. cerevisiae* were sent for sequencing to confirm the correct orientation of PCR amplicon.

Samples were sent for sequencing to Macrogen Inc. (Seoul, Republic of South Korea). Standard sequencing service was selected with sequencing algorithms optimized for samples, longer than 500 bp. 25 µL of selected plasmid DNA samples was transferred to 96-well plates and when all of the samples were transferred, the plate was sealed.

Primers for sequencing (*GAL1* forward primer and V5 primer) were recommended by pYES2.1 TOPO® TA Expression Kit manufacturer (Invitrogen, USA) and enclosed to sequencing plates. All of the samples were sequenced in both directions. When sequencing results became available, they were imported into Unipro UGENE software (Okonechnikov et al., 2012) and checked for correct orientation.

3.2.6.6 Transformation of *S. cerevisiae*

Transformation of yeast cells was performed as first described by Ito et al. (1983), improved by Gietz and Woods (2002) and modified as follows. One colony of selected *S. cerevisiae* strain (T334 or YPH250) was transferred into 100 mL of sterile YPD medium in 150 mL Erlenmeyer flask and transferred to shaker for overnight incubation at 30°C and 220 rpm. Next day, cells were counted under the microscope using ImageJ software as described previously in Chapter 3.2.2.3 - grey scale, bright-field microscopy with half-closed aperture and increased gamma correction was used to uniformly darken all of the cells to capture the total cell number (Zupan et al., 2013).

When the cells reached $2x10^7$ cells/mL, they were harvested by centrifugation for 5 min at 3000 g; supernatant was discarded. Cells were then washed with 25 mL of sterile water, centrifuged for 5 min at 3000 g; supernatant was discarded. The pellet was resuspended in 1 mL of 100 mM LiAc solution and transferred into a sterile 1.5 mL microcentrifuges. After cells were spun down and supernatant was discarded, the cells were resuspended in 100 mM LiAc solution to final volume of 500 µL. Afterwards, 50 µL aliquots were distributed to fresh sterile 1.5 mL microcentrifuge tubes, cells were spun down and supernatants were discarded. Before the transformation mixture was prepared, carrier DNA was boiled for 5 min and transferred to ice. Transformation reagents were then added in the exact order as follows:

- 240 µL PEG-4000 solution (50 % w/v)
- 36 µL 1M LiAc solution,
- 50 µL carrier DNA solution (2 mg/mL),
- 10 µL plasmid DNA,
- 24 µL sterile dH_2O

Reaction mixture was mixed with gentle aspiration till the pellet was fully suspended. Transformation proceeded with heat shock in water bath at 42°C for 40 minutes. After heat shock, reaction mixtures were centrifuged for 60 s at 6000 rpm and supernatant was discarded. Cells were then resuspended by gentle aspiration in 1 mL of sterile water. 100 µL of suspension was transferred and streaked onto SC-URA plates. Plates were incubated for 4 days at 30°C. Because both of the *S. cerevisiae* strains (T334 and YPH250) used for transformation lacked gene for uracil synthesis, only cells that acquired plasmid carrying complementing *URA* gene, could grow on these plates. After incubation period, 5 colonies from each plate were transferred to fresh SC-URA plates and used for further experiments. Among these five colonies, three colonies were stored in cryovials with SC-URA medium supplemented with 20 % glycerol and stored at -80°C.

3.2.7 Heterologous expression of vinylphenol reductase in *S. cerevisiae* and confirmation of *in-vivo* enzyme activity through fermentation experiments

This chapter lists methods and procedures that were used for heterologous expression and confirmation of *in-vivo* enzyme activity of vinylphenol reductase through fermentation experiments, using *S. cerevisiae* transformants, prepared in Chapter 1.2.5.

3.2.7.1 Induction of heterologous expression

Induction of expression was performed according to manufacturer's instructions (pYES2.1 TOPO® TA Expression Kit; Invitrogen) and modified as follows. One colony of selected transformed *S. cerevisiae* strain was transferred to sterile 150 mL Erlenmeyer flask, containing 100 mL SC-URA medium, closed with sterile foam stopper and aluminium foil and incubated overnight on shaker at 30°C and 220 rpm. After incubation, the cell suspension was transferred into a sterile 50 mL microcentrifuges and centrifuged for 3 minutes at 2000 g. Supernatant was discarded and cells were resuspended in 100 mL of one of the following SC-URA media:

- SC-URA medium supplemented with 2 % glucose, 2 % galactose and 10 mg/L 4-vinylphenol for experiments with *S. cerevisiae* T334 strain
- SC-URA medium supplemented with 2 % galactose and 10 mg/L 4-vinylphenol for experiments with *S. cerevisiae* YPH250 strain

Suspensions were transferred back to their respective Erlenmeyer flasks and incubated on shaker at 28°C and 160 rpm. Two different controls were used for these experiments – flasks inoculated with *S. cerevisiae* (T334 or YPH250) transformed with plasmid without insert served as a negative control – and flasks inoculated with *B. bruxellensis* ZIM 2512, which served as a positive control. After induction, 1.8 mL samples were taken at 0, 3, 6, 24 and 48 h time point from each flask and transferred to sterile 2 mL microcentrifuges. Samples were centrifuged for 5 min at 4000 g. Supernatant was placed into separate sterile 2 mL centrifuge tube and frozen at -20°C till needed for further analysis. Microcentrifuges containing cell pellets were frozen at -80°C till needed for further analysis.

3.2.7.2 Assessment of conversion of 4-vinylphenol to 4-ethylphenol with HPLC analysis

For determination of 4-vinylphenol and 4-ethylphenol concentrations, 2mL microcentrifuges, containing samples were defrosted at room temperature. The HPLC analysis was performed as described by Kosel et al. (2014). Supernatant was filtered through 0.22 μm filters (Phenomenex, Italy). Filtrates were placed in HPLC vials and analysed with High-Performance Liquid Chromatography (HPLC) as described in Chapter 1.2.2.4 with following additions. External standards of 4-vinylphenol and 4-ethylphenol (all Sigma-Aldrich) were used for determination of standard curves and subsequent quantification of sample concentrations (preparation of external standards is described in Chapter 3.1.5). The detector was set to high sensitivity at an excitation wavelength of 280 nm and emission wavelength of 333 nm. Retention time of 4-vinylphenol was 12 minutes and 14 minutes for 4-ethylphenol.

3.2.8 Confirmation of gene expression by Western blot

This chapter lists all the methods that were used for isolation of proteins as well as SDS-PAGE electrophoresis, transfer of proteins and their detection with antibodies on nitrocellulose membrane.

3.2.8.1 Protein isolation

Proteins for confirmation of heterologous expression were isolated from cell pellets, collected during experiments, described in Chapter 1.2.6.2. Proteins were isolated from samples, collected 6 hours after induction and isolated as described by Méchin et al. (2007) and modified as follows. Frozen samples were suspended in 1 mL solution of 0.25 M NaOH/1 % 2-mercaptoethanol and placed on ice for 10 min. After incubation, 160 µL of 50 % (w/v) trichloroacetic acid solution was added and samples were placed on iced for another 10 min. Samples were then resuspended in 1 mL of acetone, which was stored at -20°C followed by centrifugation at maximum speed for 10 min. After centrifugation, supernatant was removed and remaining protein pellets were left on ice to dry in laminar-flow cabinet. Pellets were then resuspended by aspiration in 100 µL of protease inhibitor cocktail solution and stored -80°C till needed for further analysis.

3.2.8.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was performed as first described by Shapiro et al. (1967) and modified as follows. Before SDS-PAGE was performed, samples were defrosted, placed on ice and mixed with 20 µL of 6x SDS sample buffer. Samples were then incubated for 10 min at 95°C, followed by centrifugation at 14000 rpm for 10 min.

To separate proteins according to their size, polyacrylamide gel was prepared. Gel was composed of stacking (12.5 % acrylamide) and separating gel (15 % acrylamide). Composition of gel is described in Table 38. It has to be stressed, that TEMED solution was added to the gel solution directly after casting and that each part of the gel was cast separately. Separating gel has been cast first and after polymerization (which took 20-30 minutes) the stacking gel was cast. After polymerization of stacking gel had been completed, gel was transferred to SDS-PAGE electrophoresis apparatus and 30 µL of each sample was loaded. 7 µL of protein size standard (Fermentas) was loaded onto acrylamide gel so the molecular mass of the protein could be estimated. Electrophoresis was performed in SDS-PAGE running buffer for 45 minutes at 25 mA.

Table 38: Preparation of acrylamide gel for SDS-PAGE
Preglednica 38: Priprava akrilamidnega gela za SDS-PAGE

Reagent	**Separating gel (µL)**	**Stacking gel (µL)**
30 % bis-acrylamide	2820	625
1M Tris pH8.8	1875	/
1M Tris pH 6.8	/	1250
10 % SDS solution	75	50
APS	22.5	15
TEMED	11.25	7.5
dH2O	2135	3075

3.2.8.3 Transfer of protein to nitrocellulose membrane

After the SDS-PAGE run had been completed, gels were taken from their casts and put onto nitrocellulose membrane and compiled for wet transfer as recommended by "Mini Trans-Blot Cell" (Bio-rad) manufacturer's instructions. Transfer was performed at 140-170 mA for 75 min. Sample images are presented in Annex M.

3.2.8.4 Detection of target protein tagged with V5 epitope

Detection of target proteins tagged with V5 proteins was performed as described by Harlow and Lane (1988) and modified as follows. After transfer of proteins to nitrocellulose membrane from acrylamide gel, membranes were incubated in Ponceau S solution for 10 minutes to check if proteins were transferred successfully. When proteins were visualized, size marker bands were marked with a pen and membranes were washed 3 times with light agitation for 10 min with of dH_2O. Membranes were transferred to TBSM blocking buffer and gently agitated for 40 min. Membranes were then transferred to 50 mL centrifuge tubes with 10 mL of TBSM solution mixed with 2 µL of "Anti-V5-HRP Antibody" (Invitrogen, USA). Hybridization occurred during overnight incubation at 4°C. Next day, membranes were washed 3 times for 10 minutes with TBS solution with light agitation. Finally, membranes were transferred to a clean tray with mixture of 2.5 mL of DAB and 22.5 mL of stable peroxide solution as recommended by manufacturer (Thermo Scientific, USA). Tray with reaction solution and membranes was incubated for 10 minutes in dark space till bands developed. Membranes were washed under the water, dried and checked for bands.

4 RESULTS AND DISCUSSION

This section features the results of our experimental work. For the sake of clarity, the discussion of the results is also featured in this chapter.

4.1 ISOLATION OF A NOVEL *Brettanomyces bruxellensis* STRAIN

Plating of grape must imported from Montenegro yielded many yeast colonies, however only those with proper colony morphology were selected and checked under microscope. Colonies with promising cell morphology (Smith, 2011) were plated on fresh plates and checked with RFLP analysis. From all of the tested samples, only one yielded characteristic RFLP profile (Guillamón et al., 1998), which is enclosed in the Annex C. The isolated strain was deposited in ZIM culture collection (Biotechnical faculty, Ljubljana, Slovenia) under designation ZIM 2512 and used for our experimental work.

4.2 DETERMINATION OF VINYLPHENOL REDUCTASE ACTIVITY OF THE SELECTED *B. bruxellensis* STRAINS

To determine the *in-vivo* activity of vinylphenol reductase of different *B. bruxellensis* strains, we decided to measure the *in-vivo* activity through fermentation experiments. This was done as described by (Zupan et al., 2013) – we measured metabolically active cells and the change of concentration 4-vinylphenol and 4-ethylphenol. Results were used for calculation of relative consumption rate of 4-vinylphenol and relative formation rate of 4-ethylphenol (both displayed as pg/cell/day). The growth and consumption/production curves can are shown in Figures 3 and 4. Relative consumption and production rates are depicted in Figure 5.

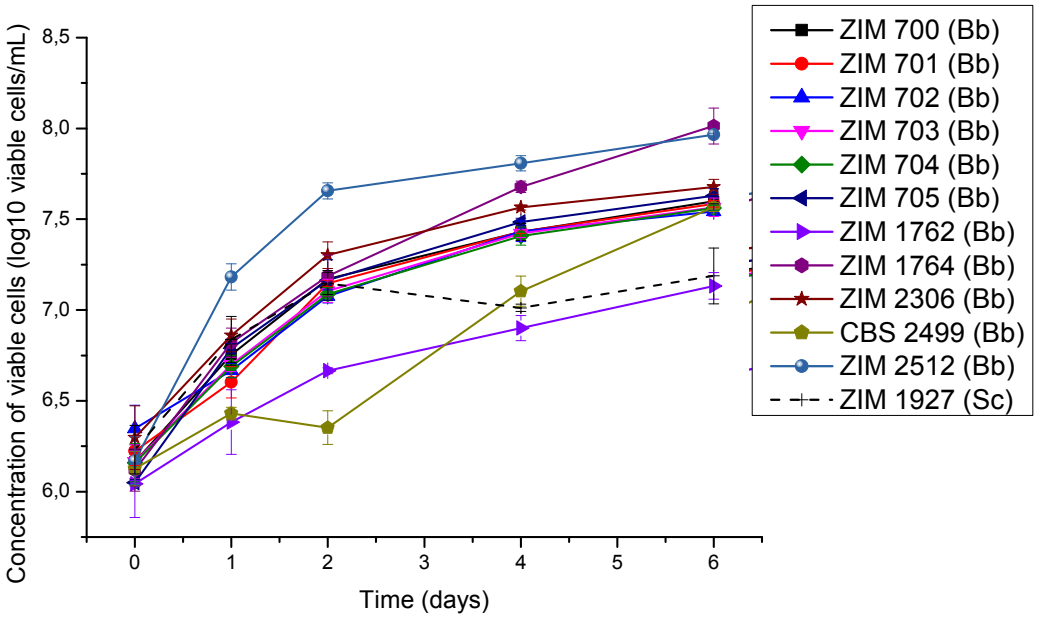

Figure 3: Growth curves during 6 day fermentation in static 250 mL Erlenmeyer flasks in 200 mL of YNB medium supplemented with 20 g/L glucose and 6 mg/L 4-vinylphenol under microaerobic conditions. Each growth curve represents either one of the *B. bruxellensis* strains or *S. cerevisiae* control strain.
Slika 3: Graf rastnih krivulj tekom 6 dnevne fermentacije v statičnih 250 ml Erlenmejericah z 200 ml gojišča YNB z dodatkom 20 g/L glukoze in 6 mg/L 4-vinilfenola v mikroaerobnih pogojih. Vsaka izmed krivulj predstavlja posamezen sev *B. bruxellensis*, oziroma kontrolo inokulirano s *S. cerevisiae*.

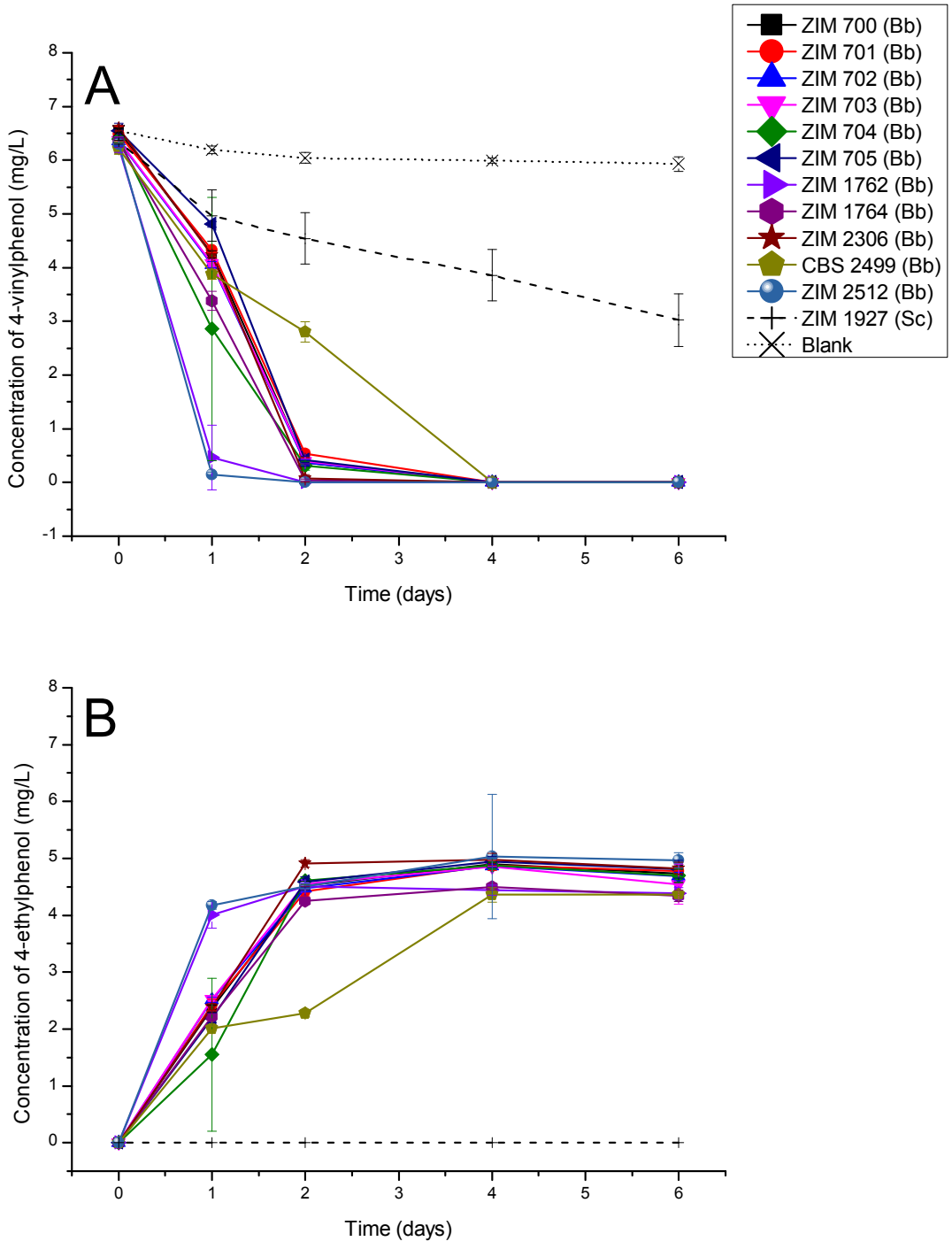

Figure 4: Consumption curves of 4-vinylphenol (A) and production curves of 4-ethylphenol (B) during 6 day fermentation in static 250 mL Erlenmeyer flasks in 200 mL of YNB medium supplemented with 20 g/L glucose and 6 mg/L 4-vinylphenol under microaerobic conditions. Each curve represents either one of the *B. bruxellensis* strains or *S. cerevisiae* control strain or control flask without inoculum.

Slika 4: Graf porabe 4-vinilfenola (A) in nastanka 4-etilfenola (B) tekom 6 dnevne fermentacije v statičnih 250 ml Erlenmejericah z 200 ml gojišča YNB z dodatkom 20 g/L glukoze in 6 mg/L 4-vinilfenola v mikroaerobnih pogojih. Vsaka izmed krivulj predstavlja posamezen sev *B. bruxellensis*, oziroma kontrolo inokulirano s *S. cerevisiae*, oziroma brez inokuluma.

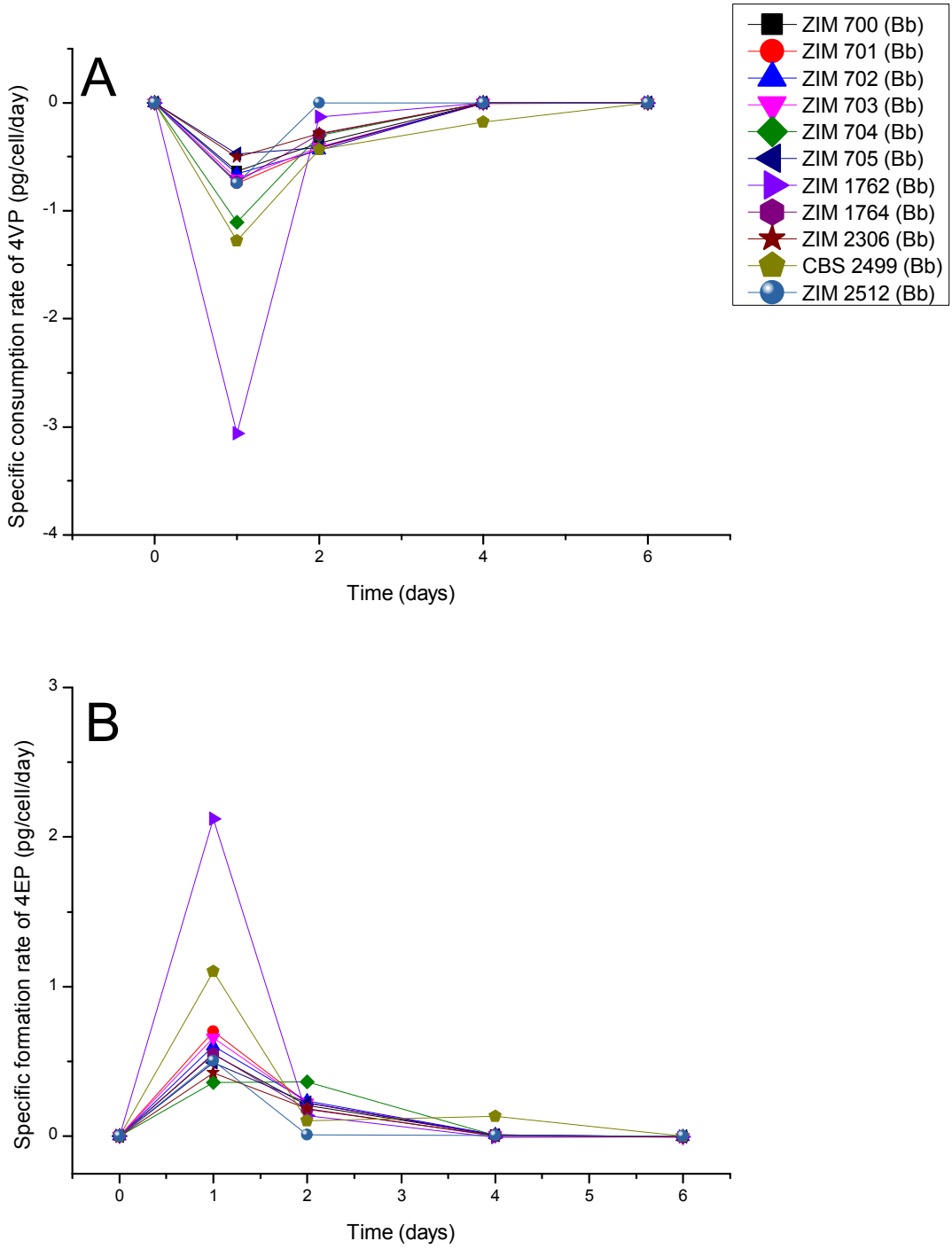

Figure 5: Consumption rates of 4-vinylphenol (A) and specific production rates of 4-ethylphenol (B) during 6 day fermentation in static 250 mL Erlenmeyer flasks in 200 mL of YNB medium supplemented with 20 g/L glucose and 6 mg/L 4-vinylphenol under microaerobic conditions. Each curve represents one of the *B. bruxellensis* strains.

Slika 5: Graf specifične porabe 4-vinilfenola (A), oziroma nastajanja 4-etilfenola (B) tekom 6 dnevne fermentacije v statičnih 250 ml Erlenmejericah z 200 ml gojišča YNB z dodatkom 20 g/L glukoze in 6 mg/L 4-vinilfenola v mikroaerobnih pogojih. Vsaka izmed krivulj predstavlja posamezen sev *B. bruxellensis*.

Although *S. cerevisiae* control (ZIM 1927) was first to reach the lag phase, as it can be seen on Figure 3, all of the *B. bruxellensis* strains managed to produce higher concentration of metabolically active cells till day 6 (with the exception of ZIM 1762) then the *S. cerevisiae* control. Among tested *B. bruxellensis* strains, five distinct growth curve profiles can be seen – ZIM 2512 exhibited the fastest growth in the first two days but. ZIM 1764 followed and on the final day even surpassed ZIM 2512 in terms of final concentration of metabolically active cells. All of the Slovenian isolates followed (ZIM 700, 701, 702, 703, 704, 705 and ZIM 2306) and exhibited very similar growth curve profiles. The strains that exhibited slowest formation of metabolically active cells were CBS 2499 and ZIM 1762. Although these results cannot be directly compared with other authors' experiments, due to the difference in experimental conditions, many experiments lead to similar conclusions that *B. bruxellensis* can reach higher cell concentrations compared to *S. cerevisiae* (Blomqvist et al., 2010). We decided to perform fermentations with low concentrations of 4-vinylphenol because of the toxicity of the substrate, as suggested by Stead (1995) and Kosel et al. (2014). Furthermore, microaerobic conditions for these fermentation experiments were chosen because of the Custers effect (Scheffers, 1961) which was first observed and described precisely in *B. bruxellensis*.

Consumption curves of 4-vinylphenol on Figure 4A revealed three distinct profiles of 4-vinylphenol. The fastest strain to consume all of the available 4-ethylphenol were ZIM 2512 and ZIM 1762, which managed to consume all of the available 4-vinylphenol till day two. They were followed by strains ZIM 700, 701, 702, 703, 704, 705, 1764 and ZIM 2306, which exhibited very similar consumption profiles and consumed all of the available substrate till day four. The strain that exhibited the slowest consumption of 4-vinylphenol was CBS 2499. 4-vinylphenol production curves show inverted but similar trends as 4-ethylphenol production curves. Fermentations with *S. cerevisiae* control strain (ZIM 1927) also exhibited depletion of 4-vinylphenol although no 4-ethylphenol was detected. We noticed that the conversion of 4-vinylphenol to 4-ethylphenol did not occur in 1:1 ratio, as described by Edlin et al. (1995) which was noticed also by Harris et al. (2008) - they attributed the loss of 4-ethylphenol to alternative conversion pathways. We noticed that there was 26 to 31 % less 4-ethylphenol produced then 4-vinylphenol consumed. When we compared absolute consumption/production curves with *S. cerevisiae* control, 52 % reduction of 4-vinylphenol was observed with no 4-ethylphenol formed – this phenomenon was described as adsorption to the yeast cell wall of *S. cerevisiae* by Pradelles et al., (2008), Salameh et al. (2008) and Morata et al. (2013). Fermentations without inoculum showed 9 % depletion of 4-vinylphenol, which we attributed to volatility of the precursor.

Figure 5 depicts relative consumption rates of 4-vinylphenol (A) and relative consumption rates of 4-ethylphenol (B). Both graphs show similar although inverted trends. Three major consumption/production profiles can be seen from the graphs – ZIM 1762 exhibited more then 2-fold faster consumption/conversion rates then strain CBS 2499 which had the second highest consumption/conversion rates. All of the other strains (ZIM 700, 701, 702, 703, 704, 705, 1764 and ZIM 2306) comprised the group with low conversion/consumption rates.

Difference in the activity of vinylphenol reductase among different *B. bruxellensis* isolates was observed and confirmed by Godoy et al. (2009) and Granato et al. (2014). This has

been contributed mainly to the large genetic diversity of *B. bruxellensis* (Curtin et al., 2007; Harris et al., 2008; Agnolucci et al., 2009; Borneman et al., 2014; Crauwels et al., 2014). We decided to assess the activity of *B. bruxellensis* through the calculation of metabolically active cells (Zupan et al., 2013) as only metabolically active cells retain vinylphenol reductase ability as demonstrated by Zuehlke and Edwards (2013). These curves helped us to discern the differences among tested *B. bruxellensis* strains in their ability to convert 4-vinylphenol to 4-ethylphenol, which could be misleading if only absolute production/consumption curves would be taken into account. For example – strain ZIM 2512 was among the first strains to convert all of the available 4-vinylphenol into 4-ethylphenol but when the concentration of metabolically active cells were taken into consideration (ZIM 2512 was one of the fastest growing strains), ZIM 2512 actually fell into the group with lowest 4-ethylphenol conversion rates. With this finding we demonstrated, that there are differences in the activity of vinylphenol reductase activity among different *B. bruxellensis* strains. As these strains fell into three categories in terms of 4-vinylphenol to 4-ethylphenol conversion speed, we decided to choose only one strain from each of these categories for further experiments. ZIM 1762 was used as a representative for fast converting strains, CBS 2499 as a representative for medium fast converting strains and ZIM 701 as a representative for slow converting strains.

With these experiments, we answered on one of the hypotheses – we proved that there are indeed differences in the activity of the vinylphenol reductase among different *B. bruxellensis* strains.

4.3 NUCLEOTIDE SEQUENCE COMPARISON OF VINYLPHENOL REDUCTASE OF THE SELECTED *B. bruxellensis* STRAINS

Longest ORF sequences of vinylphenol reductase gene as proposed by Tchobanov et al. (2008) of selected *B. bruxellensis* candidates were compared using EMBL-EBI ClustalW2 online sequence comparison software (McWilliam et al., 2013). Sample alignment region, aligned in Unipro UGENE (Okonechnikov et al., 2012) is presented in the Annex F.

Comparison of the longest open reading frames of vinylphenol reductase gene showed, that there indeed are differences in vinylphenol reductase sequences among different strains. Comparison of obtained longest ORF sequences to the reference ORF sequence from *B. bruxellensis* AWRI 1499 (GenBank ID: EIF45903.1) revealed that there is 99 % sequence identity with strains CBS 2499, ZIM 701, 704, 703 and ZIM 2306; 98 % sequence identity with strains ZIM 702, 700 and ZIM 1764. Strains ZIM 1762, 2512 and ZIM 705 had 89 %, 79 % and 76 % sequence identity with AWRI 1499 reference strain due to early stop codon (ZIM 1762 and ZIM 2512) or shorter ORF (ZIM 705). Since all of the strains performed the conversion of 4-vinylphenol to 4-ethylphenol we can conclude that the active part of the enzyme was not affected by shorter open reading frame or early stop codons. We could also conclude that gene variant of ZIM 1762 might be the most catalytically effective since ZIM 1762 had the highest relative conversion rate. This confirms the hypothesis that there are differences in the nucleotide sequences of vinylphenol reductase gene among different *B. bruxellensis* strains that might affect the activity of the vinylphenol reductase. Dendrogram representing distances among vinylphenol reductase sequences of different *B. bruxellensis* strains is presented in Figure 6.

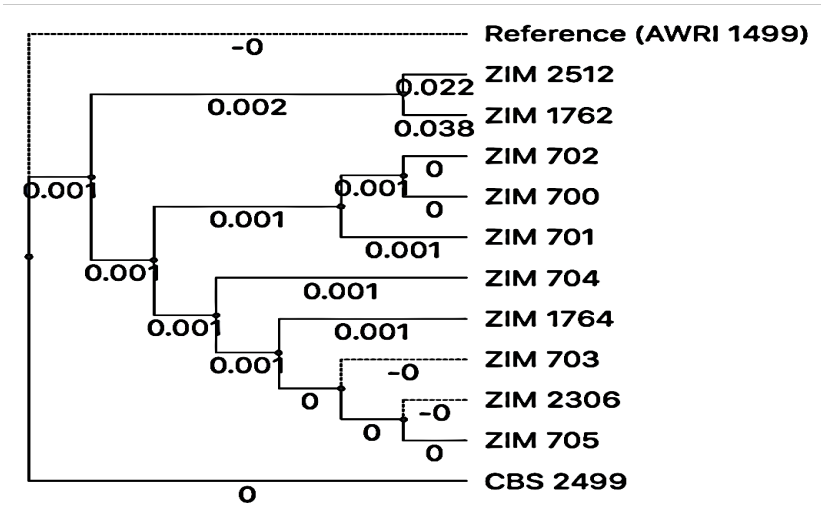

Figure 6: Dendrogram representing distances among vinylphenol reductase longest ORF sequences of the selected *B. bruxellensis* strains performed in Unipro UGENE software with PHYLIP Neighbour Joining method.
Slika 6: Dendrogram razdalj med nukleotidnimi sekvencami različnih sevov *B. bruxellensis* najdaljših odprtih bralnih okvirjev encima vinilfenol reduktaze. Dendrogram je bil izrisan z uporabo metode PHYLIP v programu Unipro UGENE.

After nucleotide sequence comparison, nucleotide sequences were translated into protein sequences with SIB ExPASy online software (Gasteiger et al., 2003) and compared again with EMBL-EBI MUSCLE online software (McWilliam et al., 2013) to check, if the differences in nucleotide sequences resulted in change of amino acid composition. Amino acid sequence alignment is enclosed in the Annex G.

Amino acid sequence comparison from different *B. bruxellensis* strains showed, that the difference in the nucleic acid sequence resulted not only in the length of the final protein, but in amino acid changes as well.

4.4 *IN-SILICO* PLASMID DESIGN

Plasmids for expression of vinylphenol reductase of *B. bruxellensis* were designed in Unipro UGENE software (Okonechnikov et al., 2012). List of the designed plasmids is listed in Table 39.

Table 39: Table of pYES2.1 and pRS426-based plasmids created in this study, listing their respective plasmid backbones and gene insert sources
Preglednica 39: Preglednica plazmidov, osnovanih na plazmidih pYES2.1 in pRS426, ki so bili sestavljeni tekom eksperimentalnega dela

No.	Plasmid name	Backbone	Genotype	Gene sources
1.	pYES2.1_VPR_ORF1_V5	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*VPR*-T(*CYC1*)-V5	*VPR* - *B. bruxellensis*
2.	pYES2.1_VPR_ORF1	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*VPR*-T(*CYC1*)	*VPR* - *B. bruxellensis*
3.	pYES2.1_VPR_ORF2_V5	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*VPR*-T(*CYC1*)-V5	*VPR* - *B. bruxellensis*
4.	pYES2.1_VPR_ORF2	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*VPR*-T(*CYC1*)	*VPR* - *B. bruxellensis*
5.	pYES2.1_VPR_ORF3_V5	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*VPR*-T(*CYC1*)-V5	*VPR* - *B. bruxellensis*
6.	pYES2.1_VPR_ORF3	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*VPR*-T(*CYC1*)	*VPR* - *B. bruxellensis*
7.	pRS426_VPR	pRS426	2 µm ori, *URA3*, B-P(GPD)-VPR(B.b.)-T(CYC1)-H	Promoter and terminator - *S. cerevisiae* EC1118; *VPR* - *B. bruxellensis*

As it can be seen from the table, most of the plasmids were based on the pYES2.1 TOPO® (Invitrogen) plasmid, sequence of which was obtained from manufacturer's website (Invitrogen, 2015). We decided to conduct most of our experiments with pYES2.1 based plasmids, because they offer simple and reliable insertion of desired gene as well as control of expression with integrated *GAL1* promoter of *S. cerevisiae* which allows induction of the expression in presence of galactose and absence of glucose (Flick and Johnston, 1990). Furthermore, pYES2.1 plasmid has integrated V5 epitope sequence, which enables the expression of the protein of choice fused with V5 epitope in the absence of stop codon of the core protein sequence. This allows the detection of target protein with specific antibodies. Nucleotide sequence for gene of vinylphenol reductase of *B. bruxellensis*, as proposed by Tchobanov et al. (2008), was taken as the core insert. pYES2.1 based plasmids were designed in six different versions – three plasmids (pYES2.1_VPR_ORF1_V5, pYES2.1_VPR_ORF2_V5 and pYES2.1_VPR_ORF3_V5) containing three longest reading frames without stop codon and similar three plasmids (pYES2.1_VPR_ORF1, pYES2.1_VPR_ORF2 and pYES2.1_VPR_ORF3) containing stop codon. Plasmid maps of pYES2.1-based plasmids are presented in Figures 7, 8 and 9.

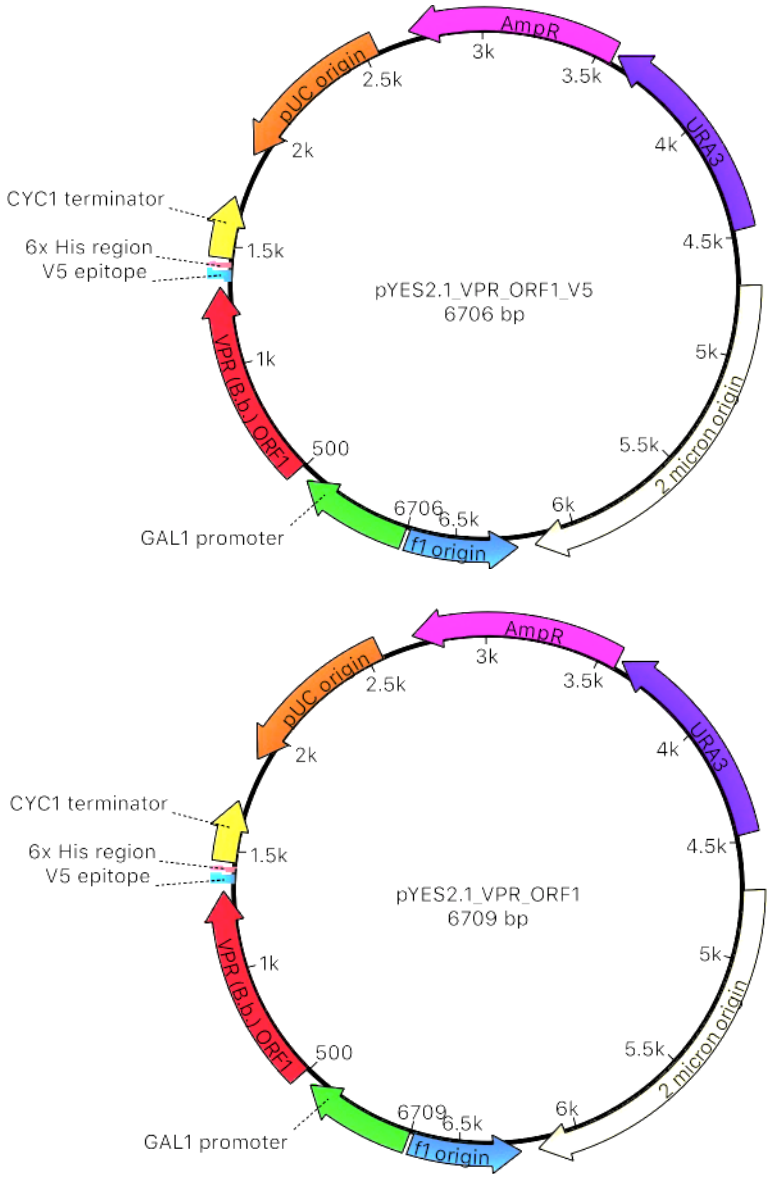

Figure 7: Plasmid maps, constructed with Unipro UGENE software, representing plasmids pYES2.1_VPR_ORF1_V5 and pYES2.1_VPR_ORF1 for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae*.
Slika 7: Shemi plazmidov pYES2.1_VPR_ORF1_V5 in pYES2.1_VPR_ORF1 za visoko inducibilno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae*, izrisani s programom Unipro UGENE.

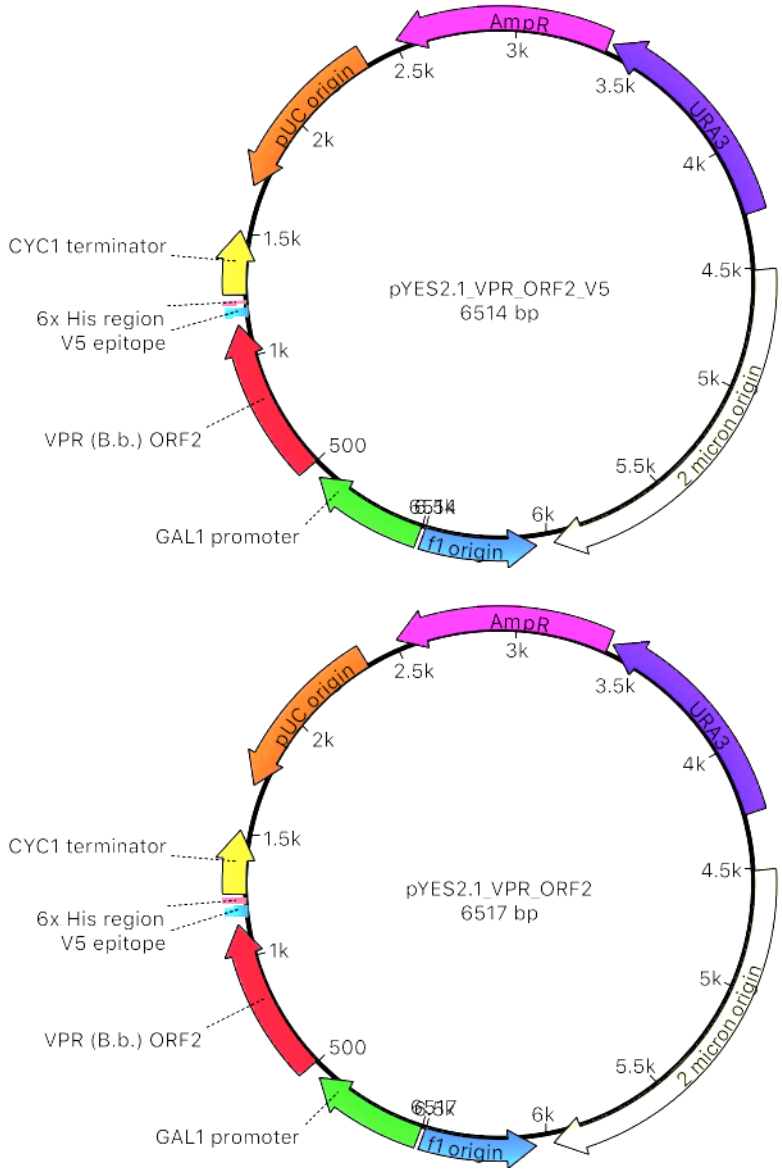

Figure 8: Plasmid maps, constructed with Unipro UGENE software, representing plasmids pYES2.1_VPR_ORF2_V5 and pYES2.1_VPR_ORF2 for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae*.
Slika 8: Shemi plazmidov pYES2.1_VPR_ORF2_V5 in pYES2.1_VPR_ORF2 za visoko inducibilno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae*, izrisani s programom Unipro UGENE.

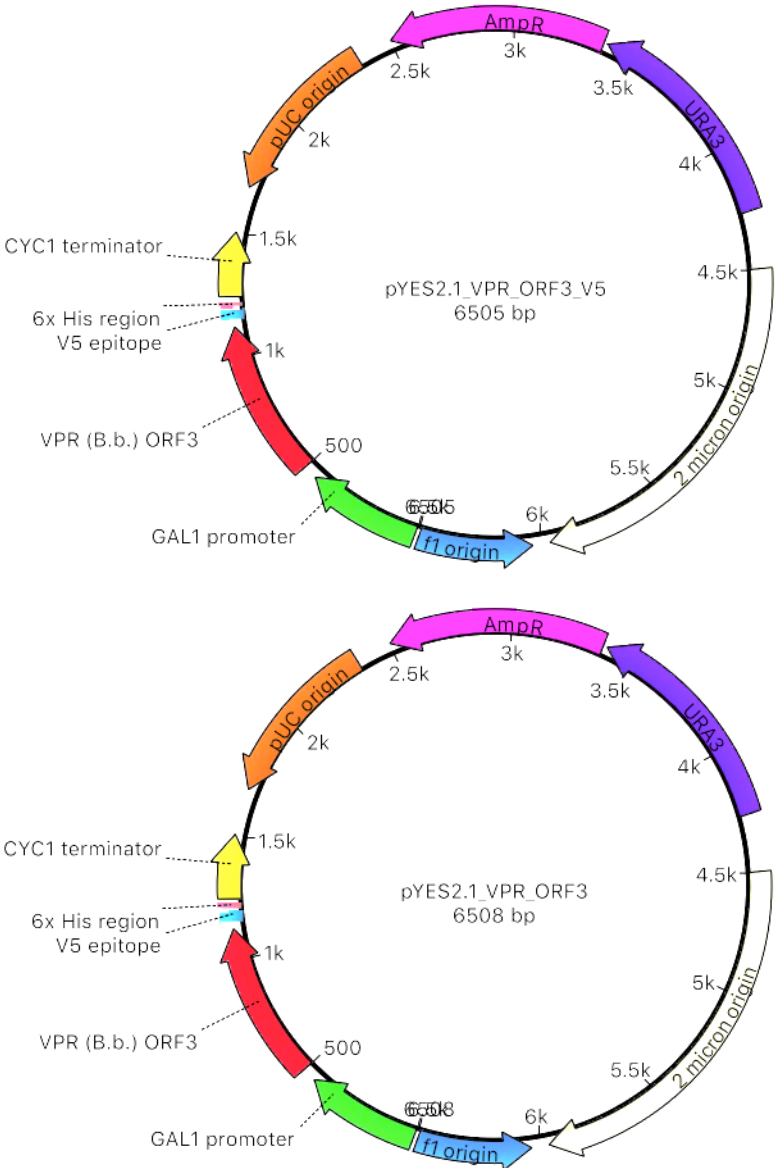

Figure 9: Plasmid maps, constructed with Unipro UGENE software, representing plasmids pYES2.1_VPR_ORF3_V5 and pYES2.1_VPR_ORF3 for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae*.
Slika 9: Shemi plazmidov pYES2.1_VPR_ORF3_V5 in pYES2.1_VPR_ORF3 za visoko inducibilno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae*, izrisani s programom Unipro UGENE.

Plasmids for positive and negative control of expression were also based on the pYES2.1 plasmid. Plasmid for positive control had *S. cerevisiae RAD59* (SGD ID: S000002217) gene integrated into pYES2.1 backbone. For negative control of gene expression, pYES2.1 plasmids that had circularized without the insertion of desired gene were used. Their respective maps are included in Annex K.

Core nucleotide sequence for construction of pRS426-based plasmid was obtained from Addgene vector database (Addgene pRS426, 2014). *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (*GPD*; SGD ID: S000003424) promoter and *S. cerevisiae*

cytochrome c (*CYC1*; SGD ID: S000003809) terminator sequences were obtained from *Saccharomyces* genome database website (Cherry et al., 2012).
To prove the principle, pRS426 plasmid was chosen for the construction of a plasmid with high expression because it is one of the best characterized and most commonly used plasmid vectors for heterologous overexpression in *S. cerevisiae* due to 2 micron origin, which allows the cell to maintain the plasmid in high copy numbers (Sikorski and Hieter, 1989; Christianson et al., 1992; Mumberg et al., 1995). *S. cerevisiae GPD* promoter (Musti et al., 1983) was chosen for the expression of *VPR* of *B. bruxellensis* because it is one of the best characterized and studied promoters for high constitutive expression (Mumberg et al., 1995; Teste et al., 2009; Partow et al., 2010; Blazeck et al., 2012; Sun et al., 2012; Weinhandl et al., 2014). The same reasoning was applied when we chose *CYC1* terminator (Zaret and Sherman, 1982), which is a terminator of choice, when expressing single genes in *S. cerevisiae* (Mumberg et al., 1995; Partow et al., 2010). The plasmid map of pRS426_VPR is presented in Figure 10.

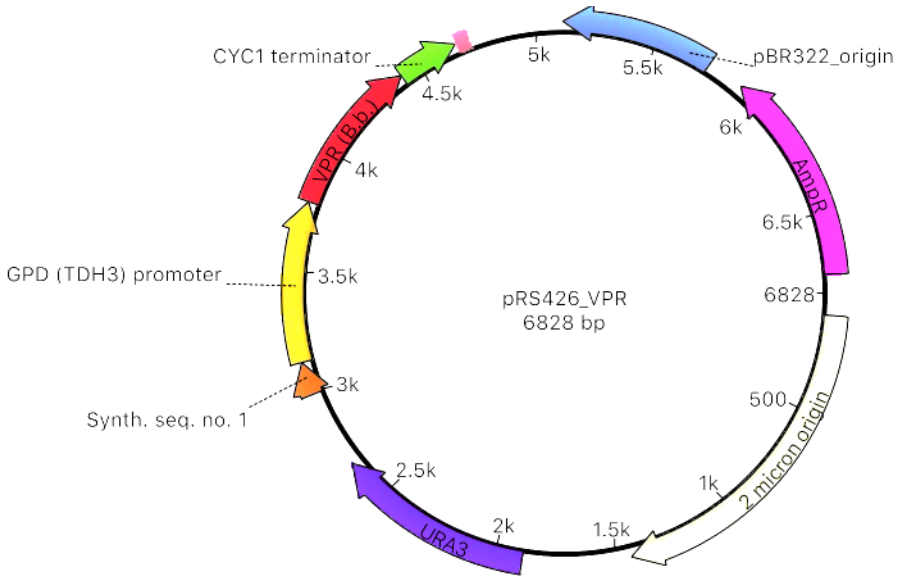

Figure 10: Plasmid map, constructed with Unipro UGENE software, representing pRS426_VPR plasmid for high constitutive expression of vinylphenol reductase gene of *B. bruxellensis* in *S. cerevisiae.*
Slika 10: Shema plazmida pRS426_VPR za visoko konstitutivno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae* izrisan s pomočjo programa Unipro UGENE.

Furthermore, we designed additional plasmids to achieve our goal – low constitutive expression of vinylphenol reductase of *B. bruxellensis* in *S. cerevisiae*. We decided to design our expression plasmids on pRS416 – core nucleotide sequence was obtained from addgene vector database (Addgene pRS 416, 2014). pRS416 is one of the most commonly used plasmids, that is maintained in cell in low copy number and is a plasmid of choice, when low expression of the desired product is needed (Sikorski and Hieter, 1989; Mumberg et al., 1995) due to *CEN6*/*ARS4* sequence, which enable plasmid to behave like yeast chromosome (Newlon, 1988). Features of the constructed plasmids are presented in Table 40.

Table 40: Table of pRS416-based plasmids designed in this study
Preglednica 40: Preglednica plazmidov, dizajniranih na osnovi plazmida pRS416

No.	Plasmid name	Backbone	Genotype
1.	pRS416_P(GPD)_VPR	pRS416	CEN6/ARS4, *URA3*, P(GPD)-*VPR*-T(CYC1)
2.	pRS416_P(CYC1)_VPR	pRS416	CEN6/ARS4, *URA3*, P(CYC1)-*VPR*-T(CYC1)
3.	pRS416_P(CIT2)VPR	pRS416	CEN6/ARS4, *URA3*, P(CIT2)-*VPR*-T(CYC1)
4.	pRS416_P(ADH1)_VPR	pRS416	CEN6/ARS4, *URA3*, P(ADH1)-*VPR*-T(CYC1)

We decided to design four different pRS416-based plasmids. All of the designed plasmids contained *B. bruxellensis VPR* gene and *S. cerevisiae CYC1* terminator, for the reasons mentioned in the previous paragraphs. The designed plasmids differed among themselves in choice of *S. cerevisiae* promoters with constitutive expression. Although there have been many efforts to characterize (Mumberg et al., 1995; Teste et al., 2009; Partow et al., 2010; Weinhandl et al., 2014) and even to design synthetic promoters (Stagoj et al., 2006; Nevoigt et al., 2007; Blazeck et al., 2012; Blount et al., 2012; Shen et al., 2012; Montiel et al., 2015) for strong constitutive expression, the promoters for low constitutive expression have been mainly used as references. Nevertheless, many of them have been characterized and we decided to design four pRS416-based plasmids with four different promoters. First plasmid contained *S. cerevisiae GPD* promoter for the reasons listed in the paragraph above to obtain single-to-low copy control plasmid with high constitutive expression. Based on the literature, we also decided to design pRS416-based plasmid containing *S. cerevisiae CYC1* promoter (pRS416_P(CYC1)_VPR) for low constitutive expression (SGD ID: S000003809) characterized by Guarente et al. (1984) and tested by Mumberg et al. (1995) and Blount et al. (2012). Next pRS416-based plasmid (pRS416_P(CIT2)_VPR) was designed with *S. cerevisiae* citrate synthase (*CIT2*; SGD ID: S000000598) promoter, which is also a promoter with low constitutive expression, characterized by Chen and Lopes (2010) and Blount et al. (2012). The final pRS416-based plasmid contained *S. cerevisiae* alcohol dehydrogenase (*ADH1*; SGD ID: S000005446) promoter, a promoter with low constitutive expression, which was first characterized by Denis et al. (1983) and tested by Mumberg et al. (1995), Partow et al. (2010), Blount et al. (2012), Sun et al. (2012) and Weinhandl et al. (2014).

It has to be stressed, that all of pRS416-based plasmids contained unique restriction sites (*Nae*I) upstream and downstream (*Eco*RI, *Xho*I and *Bam*HI) of the constructed expression cassette for the excision and construction of the integrative expression cassettes. These plasmids were constructed only *in-silico* due to the course and results of the experimental work. Their respective plasmid maps are presented in Figures 11 and 12.

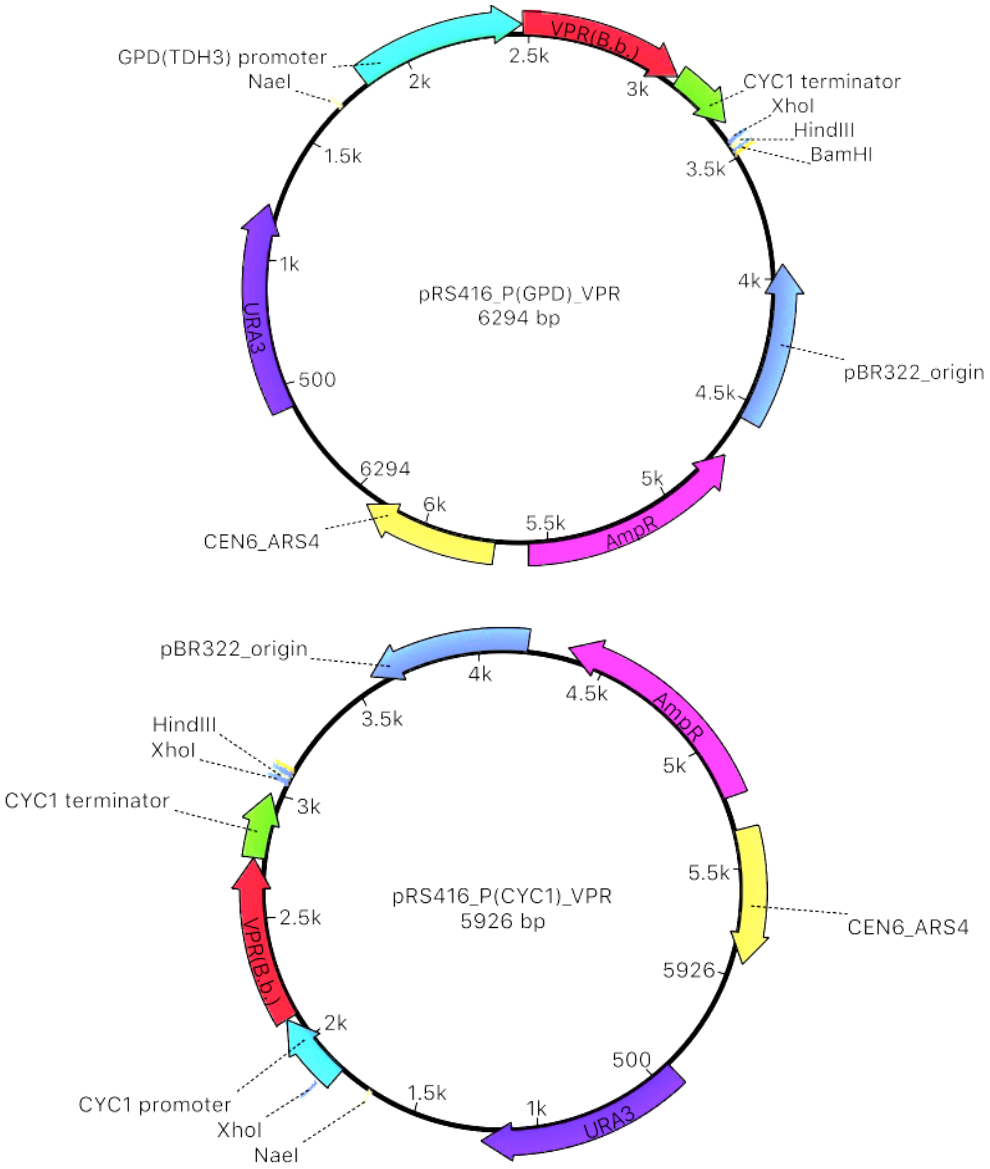

Figure 11: Plasmid maps, constructed with Unipro UGENE software, representing pRS416_P(GPD)_VPR and pRS416_P(CYC1)_VPR plasmids for high (GPD) and low (CYC1) constitutive expression of vinylphenol reductase gene of *B. bruxellensis* in *S. cerevisiae.*
Slika 11: Shema plazmidov pRS416_P(GPD)_VPR in pRS416_P(CYC1)_VPR za visoko (GPD) in nizko (CYC1) konstitutivno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae.*

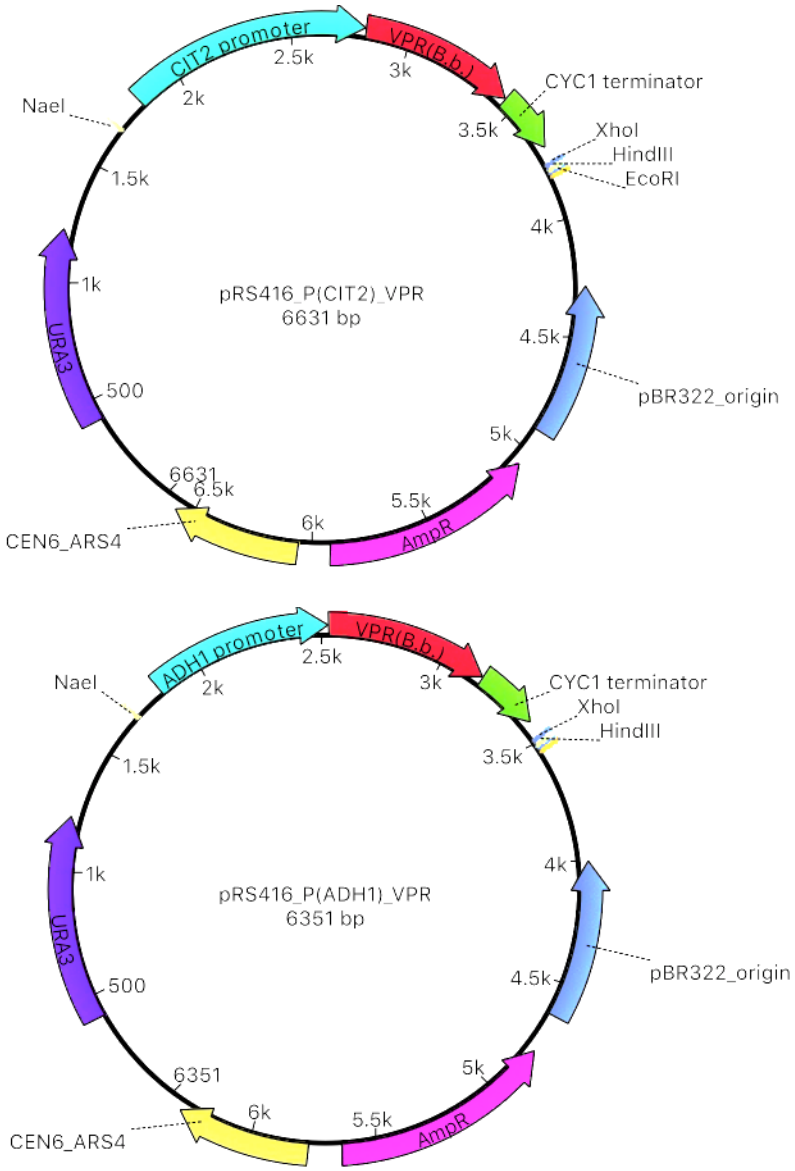

Figure 12: Plasmid maps, constructed with Unipro UGENE software, representing pRS416_P(CIT2)_VPR and pRS416_P(ADH1)_VPR plasmids for low constitutive expression of vinylphenol reductase gene of *B. bruxellensis* in *S. cerevisiae*.
Slika 12: Shema plazmidov pRS416_P(CIT2)_VPR in pRS416_P(ADH1)_VPR za nizko konstitutivno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae*.

The final plasmid was designed for the expression vinylphenol reductase from *B. bruxellensis* in industrial (wine) strain of *S. cerevisiae*. Although even centromere-based yeast plasmids are lost through generations of *S. cerevisiae* division (Amin and Pearlman, 1987), we decided to design *in-silico* pRS416-based plasmid to test the expression of vinylphenol reductase in wine strain of *S. cerevisiae*. The plasmid was based on pRS416_P(CIT2)_VPR plasmid, design of which is described in previous paragraphs. Since industrial strains are usually prototrophic (Mülleder et al., 2012) and for industrial application, such as wine fermentation, require that these strains to stay prototrophic to perform the desired function. We decided to replace the *URA3* auxotrophic marker with *Kan*MX4 dominant marker, conferring resistance to geneticin (Wach et al., 1994) which

would enable the maintenance of plasmids through generations. However, resistance markers are not allowed to be present in the organisms for such applications (Cebollero et al., 2006), so we decided to include the flippase recognition target (FRT) sequence upstream of *Kan*MX cassette as well. FRT sequence is a part of FLP-FRT site directed recombination, which involves the recombination of sequences between short FRT sites by the flippase (FLP). The recombination is only possible between two identical FRT sequences (Schlake and Bode, 1994). Since only 45 bp homology on both sides of the PCR cassette is needed for efficient targeted integration in *S. cerevisiae* (Manivasakam et al., 1995) and the modern technology allows us to synthetize primers up to 120 bp long, we could design upstream primer with the second identical FRT sequence, which would result in the PCR based integrative expression cassette with two FRT sequences allowing the self-excision of the dominant marker. This way, the construct would enable us to perform the *in-vitro* testing of the *S. cerevisiae* transformed with plasmid as well as the construction of the integrative cassette, which would be able excise the *Kan*MX marker upon the integration into genome. Plasmid map of the constructed plasmid designated as pRS416_ura3Δ::KanMX4 is presented in Figure 13.

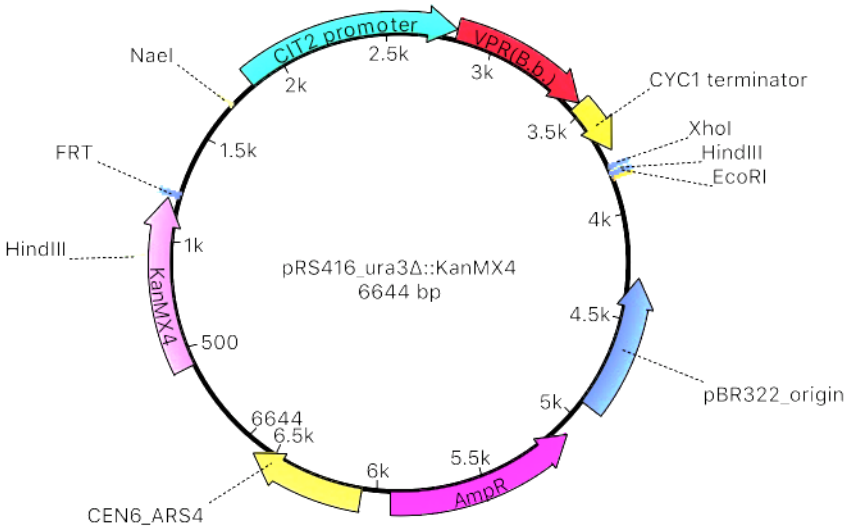

Figure 13: Plasmid maps, constructed with Unipro UGENE software, representing pRS416_ura3Δ::KanMX4 plasmid for low constitutive expression of vinylphenol reductase from *B. bruxellensis* in *S. cerevisiae.*
Slika 13: Shema plazmida pRS416_ura3Δ::KanMX4 za nizko konstitutivno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae.*

The fact that the most of the wine strains of *S. cerevisiae* are mostly diploid or aneuploid, and occasionally polyploid (Snow, 1983) would allow us to integrate our PCR-based expression cassette into *URA3* locus – integration into the same locus was used by Husnik et al. (2006) to engineer *S. cerevisae* with the ability to perform malolactic fermentation which was later approved by U.S. Food and Drug Administration (FDA) to be used in wine industry in U.S.A., Canada and Moldova (Chambers and Pretorius, 2010).

4.5 FERMENTATION EXPERIMENTS

To test the *in-vivo* activity of heterologously expressed vinylphenol reductase of *B. bruxellensis* described by Tchobanov et al. (2008), we performed the fermentation experiments as described in Chapter 3.2.6. We decided to first perform trials with plasmids containing longest open reading frame of vinylphenol reductase gene transformed into *S. cerevisiae* T334 strain, which has the mutation in *GAL1* repressor that enables the induction of expression of protein of choice in presence of glucose as soon as the galactose is added to medium although it cannot utilize galactose as a carbon source due to mutation in galactokinase (*GAL1*) enzyme (Lewis et al., 1999). We also decided to perform the fermentations with three different plasmid constructs (designated as pYES2.1_VPR_ORF1), each containing *VPR* gene of a different *B. bruxellensis* strain. We used plasmid constructs that carried *VPR* genes from strains with different specific conversion rates as determined in Chapter 4.2 – ZIM 701 (low specific 4-vinylphenol conversion rate; plasmid designated as pYES_VPR_ORF1_701), CBS 2499 (medium fast specific 4-vinylphenol conversion rate; pYES_VPR_ORF1_2499) and ZIM 1762 (high specific conversion rate; pYES_VPR_ORF1_1762). Graphs representing the consumption of 4-vinylphenol are presented in Figure 14.

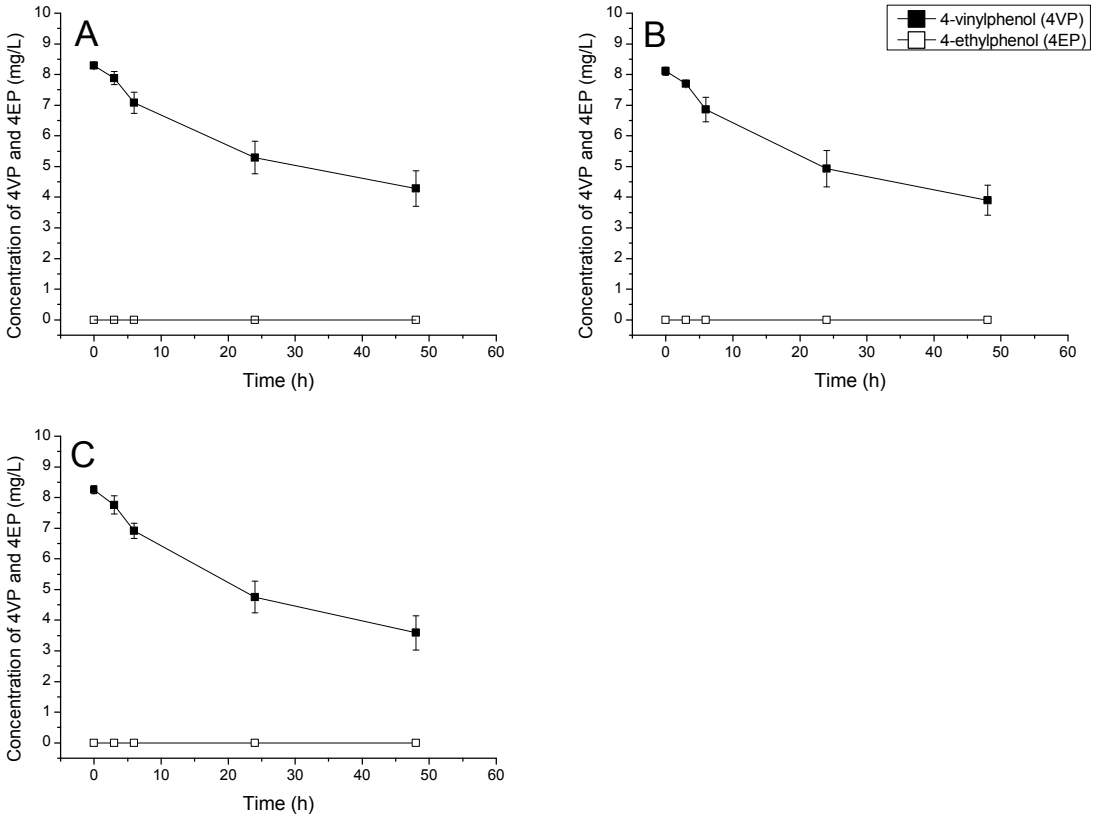

Figure 14: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES_VPR_ORF1_V5_701 (A), pYES_VPR_ORF1_V5_2499 (B) and pYES_VPR_ORF1_V5_1762 (C).
Slika 14: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L glukoze in galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* T334, ki so vsebovale plazmide pYES_VPR_ORF1_V5_701 (A), pYES_VPR_ORF1_V5_2499 (B) in pYES_VPR_ORF1_V5_1762 (C).

As can be seen on Figure 14, no formation of 4-ethylphenol has been detected to indicate the activity of the vinylphenol reductase enzyme, although Western blot analysis confirmed the expression of the heterologously expressed product. Graphs showed the depletion of 4-vinylphenol without the formation of 4-ethylphenol – similar observations were made with the control fermentations with *S. cerevisiae* transformed with plasmids that have circularized without the insertion of target. Up to 57 % loss of 4-vinylphenol was observed due to the volatility of the substrate and cell adsorption, as observed in the fermentations with transformants containing plasmids without the *VPR* gene (see Annex L). Fermentations with *B. bruxellensis* ZIM 2512, which served as a positive control showed, that up to 61 % of 4-vinylphenol was converted into 4-ethylphenol in the first 24 hours (see Annex L).

After the longest open reading frame of the *VPR* enzyme failed to produce the desired results, we considered trying the fermentations with the second and third longest open reading frames of the *VPR* enzyme. We constructed three plasmids containing second

(designated as pYES2.1_VPR_ORF2) and third longest (designated as pYES2.1_VPR_ORF3) open reading frames, containing *VPR* genes from strains with different specific conversion rates – ZIM 701 (pYES2.1_VPR_ORF2_701; pYES2.1_VPR_ORF3_701), CBS 2499 (pYES2.1_VPR_ORF2_2499; pYES2.1_VPR_ORF3_2499), and ZIM 1762 (pYES2.1_VPR_ORF2_1762; pYES2.1_VPR_ORF3_1762). Fermentations were performed with *S. cerevisiae* T334 strain transformants. The results of the fermentation experiments are depicted in Figures 15 and 16.

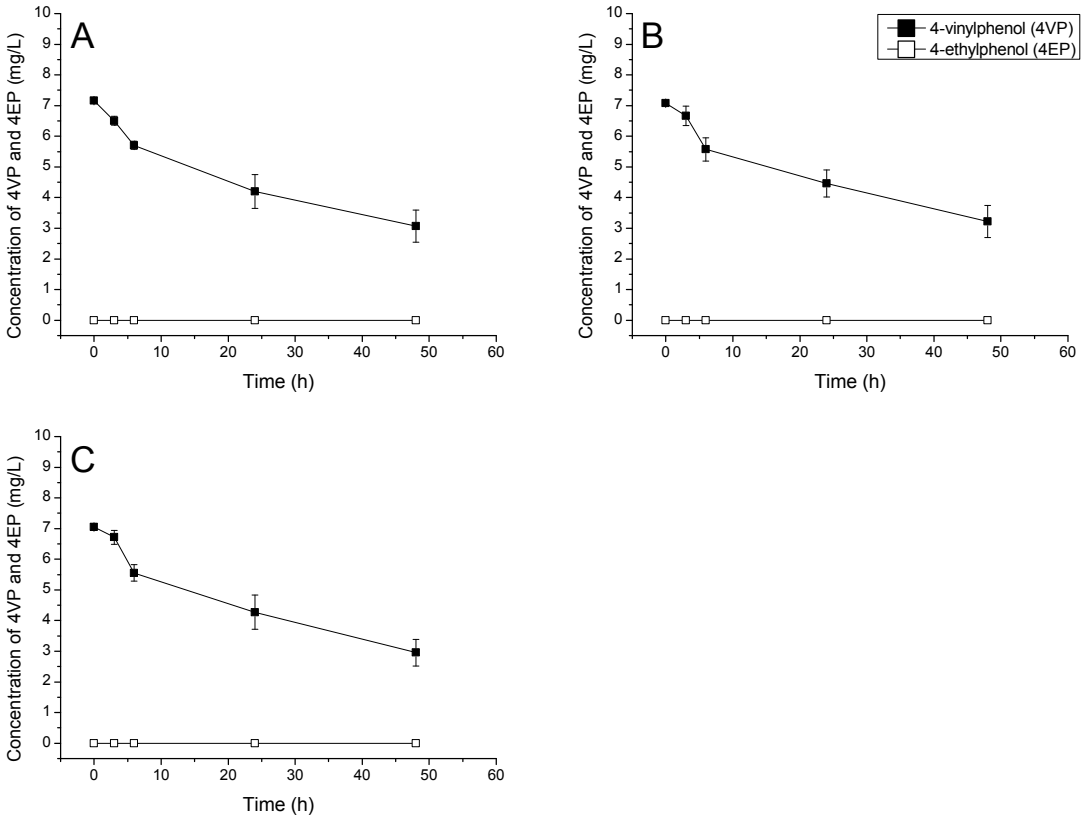

Figure 15: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_VPR_ORF2_V5_701 (A), pYES2.1_VPR_ORF2_V5_2499 (B) and pYES2.1_VPR_ORF2_V5_1762 (C).
Slika 15: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L glukoze in galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* T334, ki so vsebovale plazmide pYES2.1_VPR_ORF2_V5_701 (A), pYES2.1_VPR_ORF2_V5_2499 (B) in pYES2.1_VPR_ORF2_V5_1762 (C).

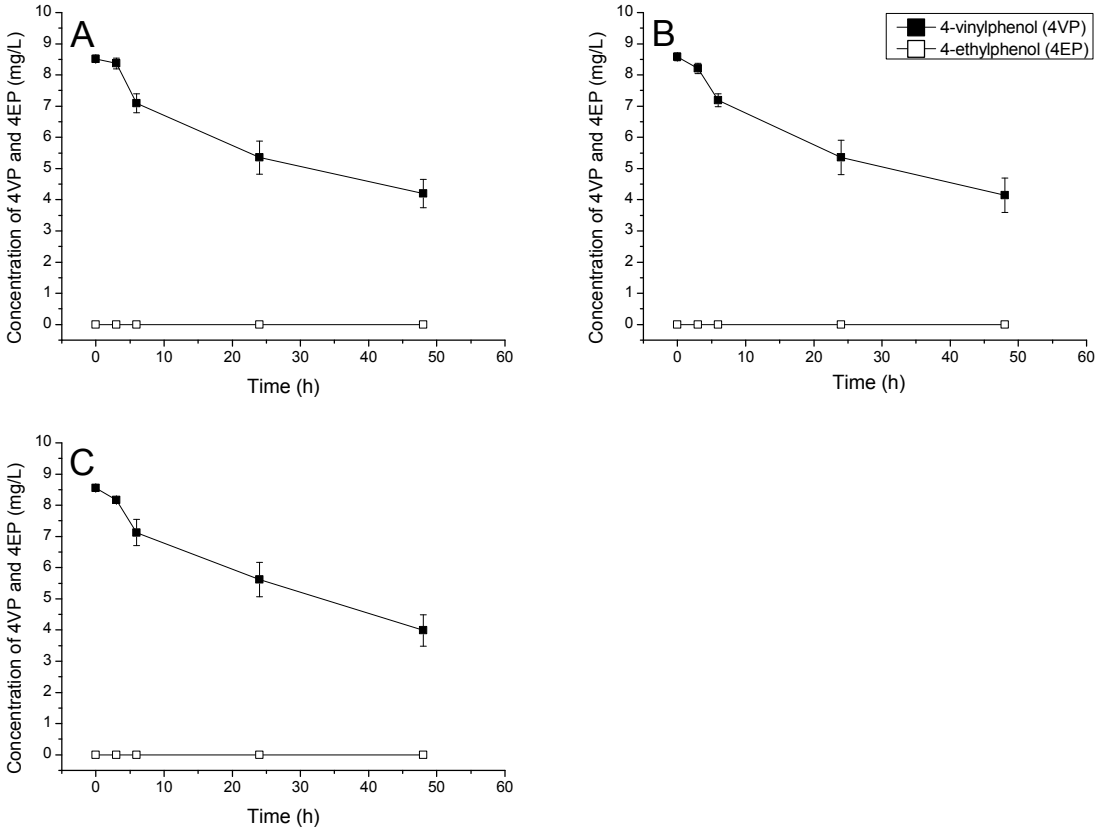

Figure 16: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_VPR_ORF3_V5_701 (A), pYES2.1_VPR_ORF3_V5_2499 (B) and pYES2.1_VPR_ORF3_V5_1762 (C).
Slika 16: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L glukoze in galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* T334, ki so vsebovale plazmide pYES2.1_VPR_ORF3_V5_701 (A), pYES2.1_VPR_ORF3_V5_2499 (B) in pYES2.1_VPR_ORF3_V5_1762 (C).

As can be seen from Figures 15 and 16, no formation of 4-ethylphenol has been detected to indicate the activity of the vinylphenol reductase enzyme, although Western blot analysis confirmed the expression of the heterologously expressed product. Although depletion of 4-vinylphenol has been observed in the control fermentation experiments – we attributed it to the volatility of 4-vinylphenol and to the adsorption to *S. cerevisiae* cell walls, as discussed in previous paragraphs.

After these experiments, we considered that the V5 epitope for antibody detection and 6x histidine tail that are fused to the expressed enzyme might be blocking its active site, so we designed plasmids each of the previously expressed open reading frames of vinylphenol reductase of *B. bruxellensis*. We used only reading frames of the CBS 2499, which had the second highest specific 4-ethylphenol production rate, this time containing stop codon, which prevented the expression of V5 epitope and 6x histidine tail. Plasmids were designated as pYES2.1_VPR_ORF1_2499, pYES2.1_VPR_ORF2_2499 and

pYES2.1_VPR_ORF3_2499 and were transformed in *S. cerevisiae* T334 strain. Results of the fermentation experiments can be seen in Figure 17.

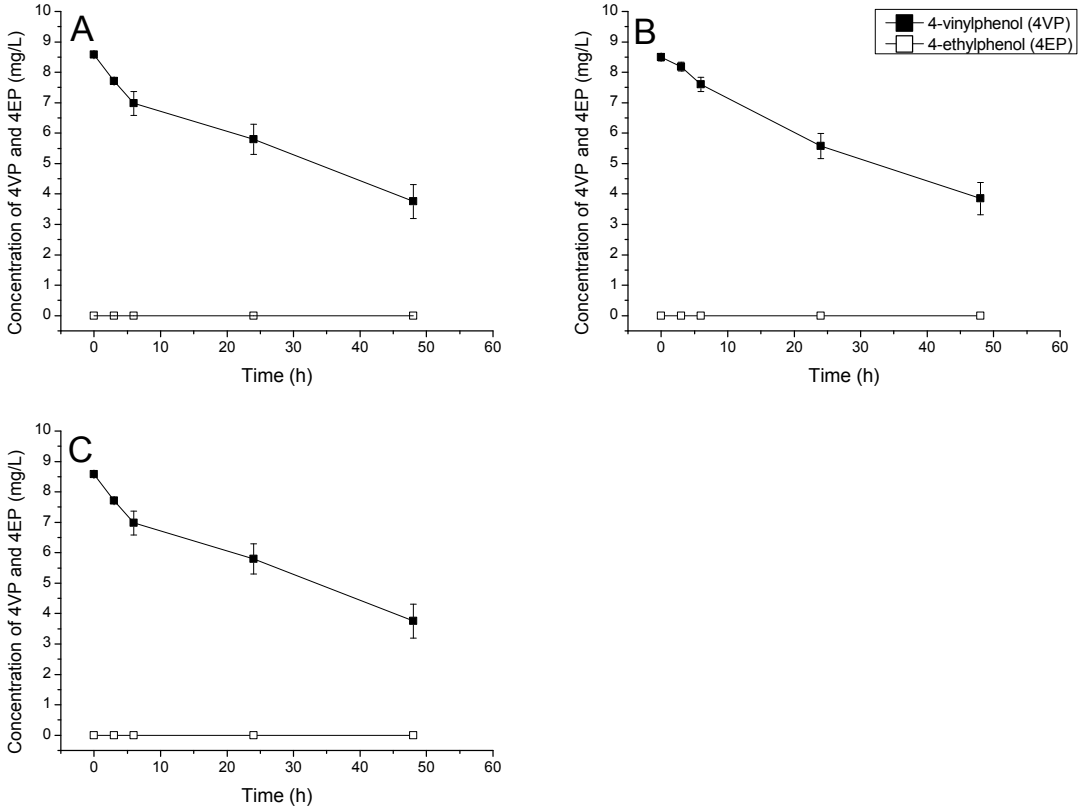

Figure 17: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for monitoring activity of heterologously expressed *B. bruxellensis* vinylphenol reductase gene in *S. cerevisiae*, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_VPR_ORF1_2499 (A), pYES2.1_VPR_ORF2_2499 (B) and pYES2.1_VPR_ORF3_2499 (C).
Slika 17: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L glukoze in galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* T334, ki so vsebovale plazmide pYES2.1_VPR_ORF1_2499 (A), pYES2.1_VPR_ORF2_2499 (B) in pYES2.1_VPR_ORF3_2499 (C).

The graphs in Figure 17 show no formation of 4-ethylphenol that would indicate the activity of the vinylphenol reductase enzyme. Due to inclusion of stop codon into open reading frames of the proposed vinylphenol reductase enzyme, we could not confirm the expression of vinylphenol reductase enzyme – nevertheless, these constructs were based on plasmids, which expression was already confirmed. Similar levels of depletion of 4-vinylphenol has been observed in the control fermentation experiments – we attributed it to the volatility of 4-vinylphenol and to the adsorption to *S. cerevisiae* cell walls, as discussed in previous paragraphs.

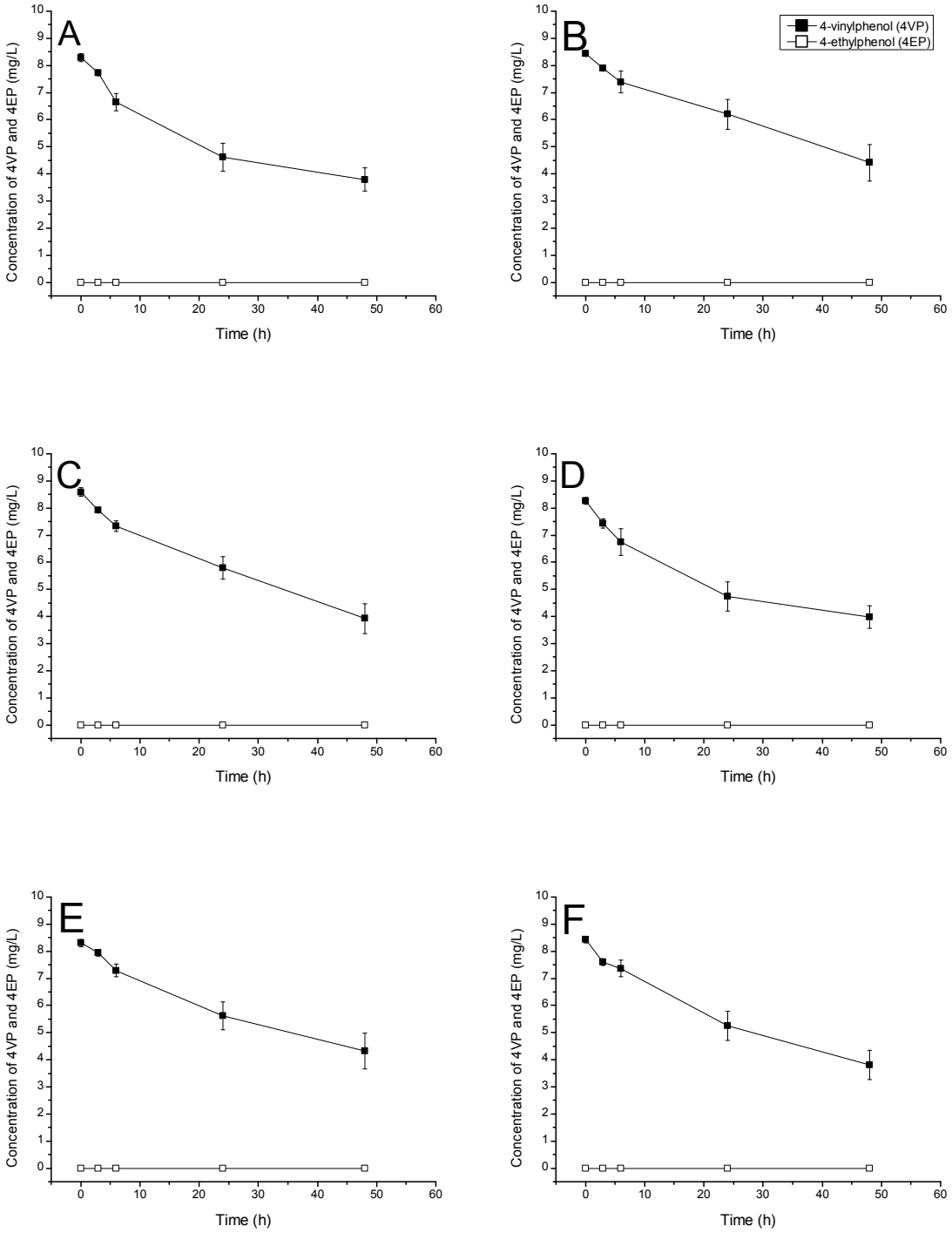

Figure 18: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* YPH250 transformants, containing plasmids pYES2.1_VPR_ORF1_V5_2499 (A), pYES2.1_VPR_ORF2_V5_2499 (B), pYES2.1_VPR_ORF3_V5_2499 (C), pYES2.1_VPR_ORF1_2499 (D), pYES2.1_VPR_ORF2_2499 (E) and pYES2.1_VPR_ORF3_2499 (F).

Slika 18: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* YPH250, ki so vsebovale plazmide pYES2.1_VPR_ORF1_V5_2499 (A), pYES2.1_VPR_ORF2_V5_2499 (B), pYES2.1_VPR_ORF3_V5_2499 (C), pYES2.1_VPR_ORF1_2499 (D), pYES2.1_VPR_ORF2_2499 (E) in pYES2.1_VPR_ORF3_2499 (F).

Finally, we considered that the strain for heterologous expression might cause improper processing of the heterologously expressed vinylphenol reductase enzyme, so we decided to try to perform the experiments with already constructed plasmids and to transform them into different *S. cerevisiae* strains. We used pYES_VPR_ORF1_V5_2499, pYES_VPR_ORF2_V5_2499, pYES_VPR_ORF3_V5_2499, pYES_VPR_ORF1_2499 (A), pYES_VPR_ORF2_2499 (B) and pYES_VPR_ORF3_2499 (C) and transformed them into *S. cerevisiae* YPH250 strain. Results of the fermentation experiments can be seen in Figure 18.

As can be seen from Figure 18, no formation of the 4-ethylphenol which would indicate the *in-vivo* activity of heterologously expressed vinylphenol reductase enzyme was observed. Similar levels of depletion of 4-vinylphenol were also observed in the control fermentation and we attributed it to the volatility of 4-vinylphenol and to the adsorption to *S. cerevisiae* cell walls as discussed in the previous paragraphs.

After our failure to demonstrate the *in-vivo* activity of the enzyme, we first considered that there might be a problem with the expression due to different codon usage. However, when the codon usage of *S. cerevisiae* was compared with the codon usage of *B. bruxellensis* using the Graphical Codon Usage Analyzer (GCUA) online software (GCUA, 2006), we found only minor differences between the codons used between both organisms (see Annex H). Furthermore, Western blot analysis confirmed that the product was expressed so we decided not to use the synthetic gene with optimized codons for expression in *S. cerevisiae*. At that point we decided to review the literature again to check if we missed any piece of information that might indicate what could have gone wrong.

The vinylphenol reductase enzyme described by Tchobanov et al. (2008) was isolated and characterised by capturing the fractions of *B. bruxellensis* cell lysate, which exhibited the vinylphenol reductase activity, and further purified with acrylamide gel electrophoresis. Amino acid sequence of the peptide fragments of vinylphenol reductase was determined by mass-spectrometry and compared with NCBI BLAST programs (blastp and tblastn algorithms) against the genome sequence of *B. bruxellensis* CBS 2499, which had 40 % coverage (Woolfit et al., 2007) at the time of the publication of the article. In the same year, Godoy et al. (2008) also published the characterisation of vinylphenol reductase of *B. bruxellensis*, which exhibited different kinetic properties when compared to the enzyme characterised by Tchobanov et al. (2008). Unfortunately, Godoy et al. (2008) did not provide any additional information which would help us identify their vinylphenol reductase candidate. These two publications showed indications that *B. bruxellensis* might posses an alternative pathway for 4-vinylphenol conversion.

When the first whole genome sequence of *B. bruxellensis* AWRI 1499 was published by Curtin et al. (2012), their article briefly mentioned that the vinylphenol reductase described by Tchobanov et al. (2008) lacked NAD(H) or NADP(H) binding sites, which were regarded by Godoy et al. (2008) as crucial for efficient substrate conversion. Quite a few years passed until further evidence concerning the nature of this enzyme was provided. Granato et al. (2014) published an article characterising a novel candidate for *B. bruxellensis* vinylphenol reductase enzyme. The main function of this newly described vinylphenol reductase enzyme is to act as Cu/Zn superoxide dismutase (*SOD*), which authors determined by sequence comparison with available genome sequences of

B. bruxellensis. According to Granato et al. (2014), the ability to convert vinylphenols to ethylphenols is the moonlighting property of this enzyme.

Because of this new information, we decided to design new plasmids containing new vinylphenol reductase candidate as proposed by Granato et al. (2014). Due to the discrepancy in the published literature we also decided to try to find other candidates for this elusive enzyme.

4.6 SEARCH FOR ADDITIONAL VINYLPHENOL REDUCTASE CANDIDATES

Due to our failure to demonstrate the *in-vivo* activity of the vinylphenol reductase of *B. bruxellensis* described by Tchobanov et al. (2008) and with the new evidence, provided by Granato et al. (2014), indicating that Cu/Zn superoxide dismutase (*SOD*) of *B. bruxellensis* is the enzyme that catalyzes reduction of 4-vinylphenol to 4-ethylphenol, we decided to compare *SOD* sequences of the *B. bruxellensis* strains. In addition, we decided to try to find additional nucleotide sequences coding for enzymes that might possess the ability to reduce 4-vinylphenol to 4-ethylphenol.

4.6.1 Comparison of *SOD* sequences

Nucleotide sequences of Cu/Zn superoxide dismutase gene with vinylphenol reductase activity as proposed by Granato et al. (2014) of selected *B. bruxellensis* candidates were compared using EMBL-EBI ClustalW2 online sequence comparison software (McWilliam et al., 2013). Sample alignment region, aligned in Unipro UGENE (Okonechnikov et al., 2012) is presented in Annex I.

Comparison of the longest open reading frames of Cu/Zn superoxide dismutase gene showed, that there indeed are differences in Cu/Zn superoxide dismutase sequences among different strains. Comparison of obtained sequences to the reference ORF sequence from *B. bruxellensis* AWRI 1499 (GenBank ID: EIF47273.1) revealed that there is 96 % sequence identity with strains ZIM 700, 701, 702, 703, 704, 1762, 1764, 2306 and CBS 2499; 95 % sequence identity with strain ZIM 705 and 87 % sequence identity with strain ZIM 2512. Although there were differences in the nucleotide sequences, they did not result in the length of the longest open reading frame but might nevertheless result in the change of amino acid composition and the activity of the enzyme. Dendrogram representing distances among vinylphenol reductase sequences of different *B. bruxellensis* strains is presented in Figure 19.

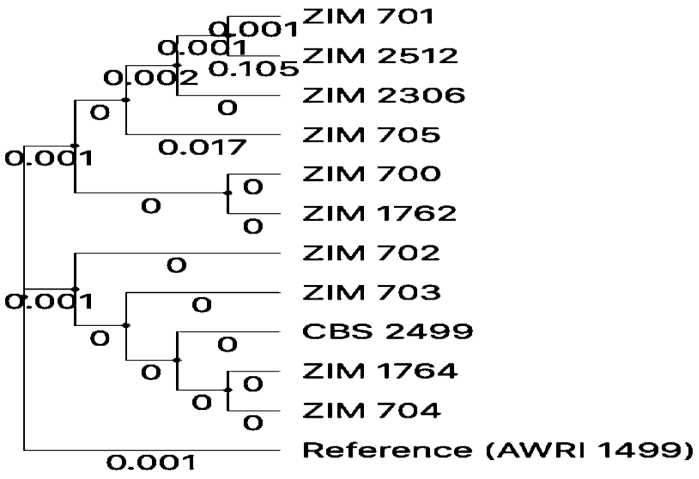

Figure 19: Dendrogram presenting distances among Cu/Zn superoxide dismutase longest ORF sequences of different *B. bruxellensis* strains performed in Unipro UGENE software with PHYLIP Neighbour Joining method.
Slika 19: Dendrogram razdalj med nukleotidnimi sekvencami različnih sevov *B. bruxellensis* najdaljših odprtih bralnih okvirjev encima Cu/Zn superoksid dismutaze. Dendrogram je bil izrisan z uporabo metode PHYLIP v programu Unipro UGENE.

After ORF sequence comparison, nucleotide sequences were translated into protein sequences with SIB ExPASy online software (Gasteiger et al., 2003) and compared again with EMBL-EBI MUSCLE online software (McWilliam et al., 2013) to check, if the differences in nucleotide sequences resulted in change of amino acid composition. Amino acid sequence alignment is enclosed in Annex J.

Amino acid sequence comparison from different *B. bruxellensis* strains showed, that the difference in the nucleic acid sequence resulted in the amino acid changes, which might affect the catalytic activity of the enzyme.

4.6.2 Search for other candidates with possible vinylphenol reductase activity

The first additional candidate that we considered as possible enzyme with vinylphenol reductase function was triosephosphate isomerase (*TPI*) of *B. bruxellensis*. We decided to assess this enzyme based on reports by both Tchobanov et al. (2008) and Granato et al. (2014). In both publications, mass spectra of the purified HPLC fractions that exhibited the vinylphenol reductase activity, included peptide fragments of the *TPI* enzyme in addition to the characterized enzyme. Based on these research publications, we decided to clone, express and assess the activity of the *TPI* of *B. bruxellensis*.

We further decided to search the scientific literature for other candidates for vinylphenol reductase. There are only few microbial species reported that can perform the conversion of 4-vinylphenol to 4-ethylphenol, most notably bacteria of *Lactobacillus* sp. (Barthelmebs et al., 2000; Couto et al., 2006; Kridelbaugh et al., 2010; Buron et al., 2011; Buron et al., 2012; Fras et al., 2013; de Castro et al., 2015). Although there are several whole genome sequences of the bacteria from *Lactobacillus* sp. available, enzyme that performs the function of vinylphenol reductase has yet to be identified. The only other microorganism

that has been reported to produce 4-ethylphenol is *Pichia guilliermondii* (Barata et al., 2006; Martorell et al., 2006; Lopes et al., 2009; Lopes et al., 2009; Sáez et al., 2010) but unfortunately, candidates for the vinylphenol reductase have yet to be characterized.

Since we could not obtain any suitable candidates from the microbial world, we considered other organisms. Literature search revealed that mice (*Mus musculus*) can metabolize styrene compounds, such as 4-vinylphenol and that 4-ethylphenol can frequently be found in urine of mice (Cavaggioni et al., 2006, 2008). Interestingly, some off-flavours that *B. bruxellensis* has been known to produce are described as mouse urine or mousiness (Grbin and Henschke, 2000) although these off-flavours are contributed to tetrahidropyridine compounds (Snowdon et al., 2006). Nevertheless, two genes, that mediate the degradation of 4-vinylphenol in mice and rats had been identified by Carlson et al. (2001). Both genes – CYP2E1 and CYP2F2 – belong to the cytochrome p450 family, function as oxidoreductases and have been reported to function only with the addition of NADPH (Carlson et al., 2001). Similar observations were made for all of the proposed vinylphenol reductase candidates by Godoy et al. (2008), Tchobanov et al. (2008) and Granato et al. (2014). When CYP2E1 (UniProtKB: Q05421) and CYP2F2 (UniProtKB: P33267) protein sequences were ran against *B. bruxellensis* protein sequences with NCBI BLAST blastp search, two results with e-score<10^{-4} were retrieved. The first one was putative cytochrome p450 monooxygenase (GenBank: EIF48136.1; PCp450M) and the second one was cytochrome p450 (GenBank: EIF47012.1; Cp450). We considered both of these sequences as possible candidates for vinylphenol reductase genes.

4.7 DESIGN OF ADDITIONAL VECTORS

Plasmids for expression of vinylphenol reductase candidates from *B. bruxellensis* were designed in Unipro UGENE software (Okonechnikov et al., 2012). List of the designed plasmids is listed in Table 41.

Table 41: Table of pYES2.1-based plasmids created in this study, listing their respective plasmid backbones and gene insert sources
Preglednica 41: Preglednica plazmidov, osnovanih na plazmidu pYES2.1, ki so bili sestavljeni tekom eksperimentalnega dela

No.	Plasmid name	Backbone	Genotype	Gene sources
1.	pYES2.1_SOD_V5	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*SOD*(B.b.)-T(*CYC1*)-V5	*SOD* - *B. bruxellensis* ZIM 701, ZIM 1762, CBS 2499
2.	pYES2.1_SOD	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*SOD*(B.b.)-T(*CYC1*)	*SOD* - *B. bruxellensis* ZIM 701, ZIM 1762, CBS 2499
3.	pYES2.1_TPI_V5	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-*TPI*(B.b.)-T(*CYC1*)-V5	*TPI* - *B. bruxellensis* ZIM 701, ZIM 1762, CBS 2499
4.	pYES2.1_Cp450_V5	pYES2.1	2 µm ori, *URA3*, P(*GAL1*)-Cp450(B.b.)-T(*CYC1*)-V5	Cp450 - *B. bruxellensis* ZIM 1762
5.	pYES2.1_PCp450M_V5	pYES2.1	2 µm ori, URA3, P(GAL1)-pCp450M(B.b.)-T(CYC1)-V5	PCp450M - *B. bruxellensis* ZIM 1762

Nucleotide sequence for vinylphenol reductase of *B. bruxellensis*, as described by Granato et al. (2014), was taken as the core insert (marked as *SOD*). pYES2.1_SOD plasmids were designed in two different versions – pYES2.1_SOD_V5 without stop codon that enabled us detection of V5 epitope with specific antibodies and pYES2.1_SOD without V5

epitope. Nucleotide sequence for *TPI* core insert for plasmid designated as pYES2.1_TPI_V5 was obtained from the peptide amino acid sequence as published by Tchobanov et al. (2008) and Granato et al. (2014). Last two nucleotide sequences from *B. bruxellensis* for plasmid core cassettes for putative cytochrome p450 monooxygenase (PCp450M) and cytochrome p450 (Cp450) were obtained through comparison of mouse (*M. musculus*) CYP2E1 and CYP2F2 protein sequences as described in previous chapter. These two sequences were used to construct pYES2.1_PCp450M_V5 and pYES2.1_Cp450_V5 plasmids. Candidate genes were amplified from *B. bruxellensis* CBS 2499, which exhibited the second highest specific 4-ethylphenol production rate. Plasmid maps of the designed and constructed plasmids are presented in Figures 20, 21 and 22.

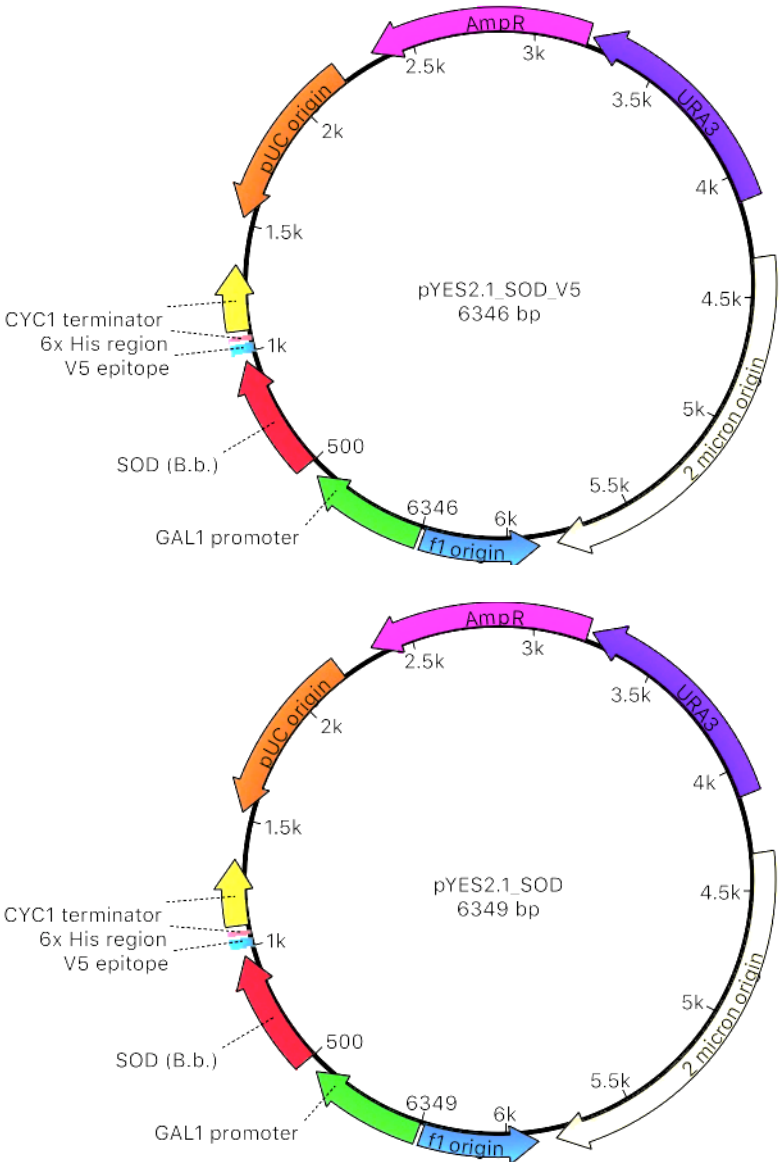

Figure 20: Plasmid maps, constructed with Unipro UGENE software, representing pYES2.1_SOD_V5 and pYES2.1_SOD plasmids for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae*.
Slika 20: Shemi plazmidov pYES2.1_SOD_V5 in pYES2.1_SOD za visoko inducibilno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae*, izrisani s programom Unipro UGENE.

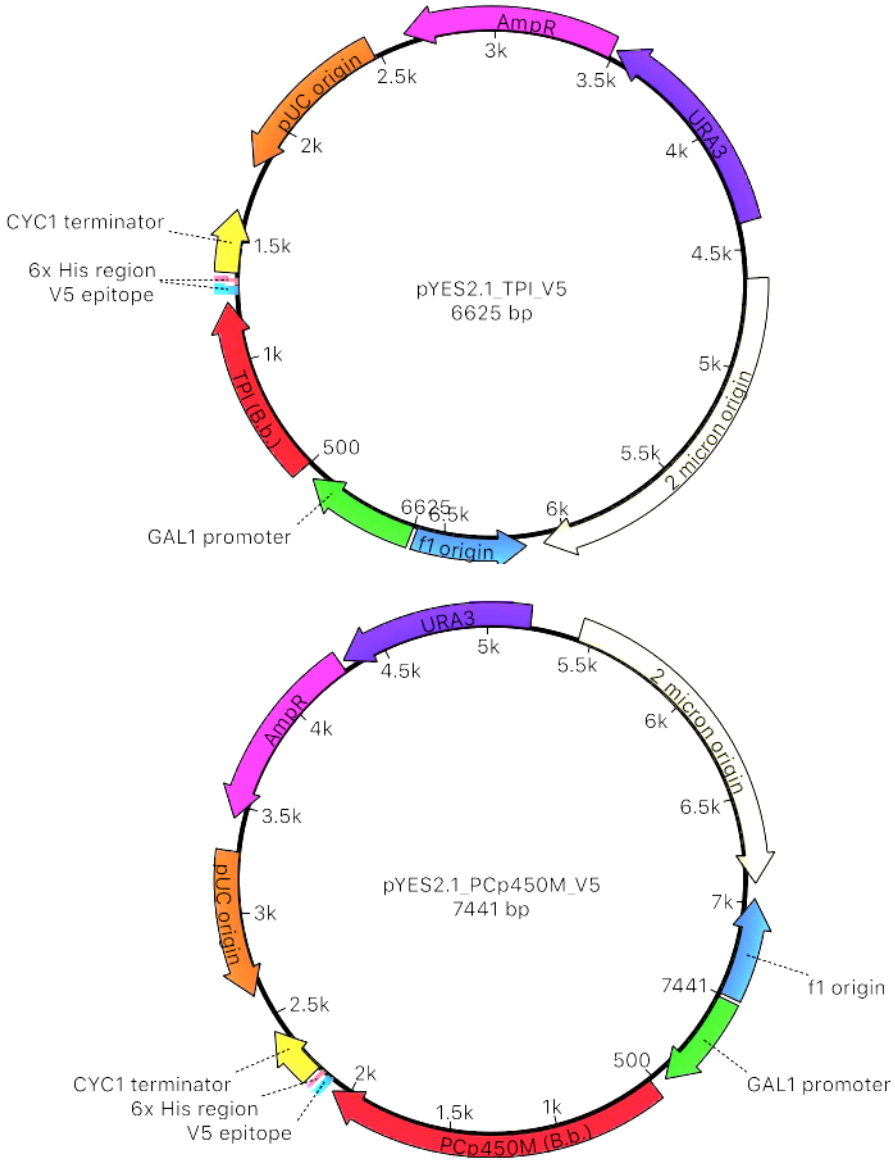

Figure 21: Plasmid maps, constructed with Unipro UGENE software, representing pYES2.1_TPI_V5 and pYES2.1_PCp450M_V5 plasmids for high inducible expression of vinylphenol reductase candidate genes of *B. bruxellensis* in *S. cerevisiae*.
Slika 21: Shemi plazmidov pYES2.1_TPI_V5 in pYES2.1_PCp450M_V5 za visoko inducibilno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae*, izrisani s programom Unipro UGENE.

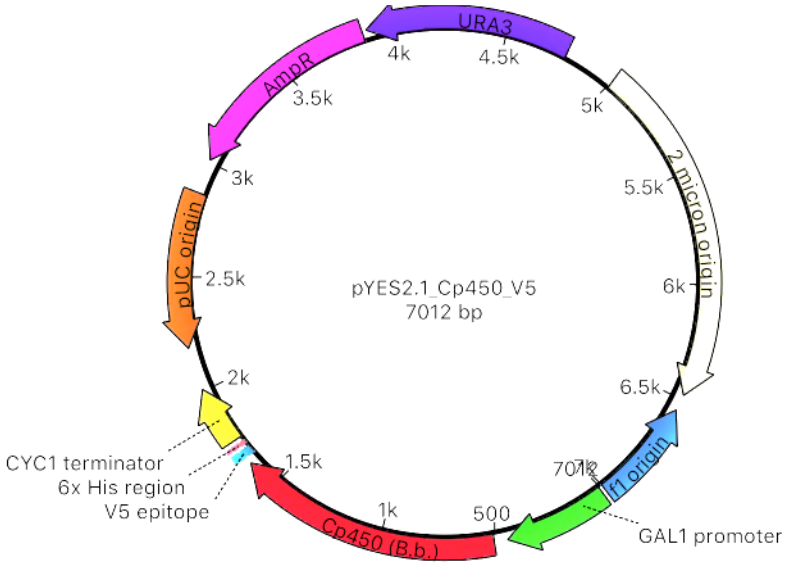

Figure 22: Plasmid map, constructed with Unipro UGENE software, representing pYES2.1_Cp450_V5 plasmid for high inducible expression of vinylphenol reductase candidate gene of *B. bruxellensis* in *S. cerevisiae.*
Slika 22: Shema plazmida pYES2.1_Cp450_V5 za visoko inducibilno izražanje vinilfenol reduktaze kvasovke *B. bruxellensis* v kvasovki *S. cerevisiae*, izrisana s programom Unipro UGENE.

4.8 FERMENTATION EXPERIMENTS WITH NEW VINYLPHENOL REDUCTASE CANDIDATES

To determine, if any of the transformants with candidate vinylphenol reductase exhibit the ability to convert 4-vinylphenol to 4-ethylphenol, we performed the fermentation experiments with *S. cerevisiae* T334 strains, transformed with one of the following plamids, containing the open reading frame of the candidate gene: pYES2.1_SOD_V5, pYES2.1_SOD, pYES2.1_TPI_V5, pYES2.1_PCp450M_V5 and pYES2.1_Cp450_V5. Graphs depicting course of the fermentation experiments are presented in Figures 23 and 24.

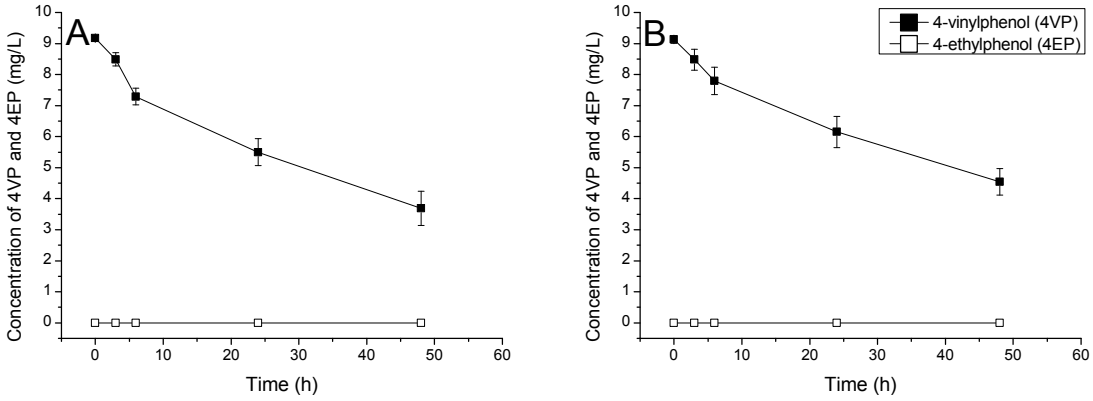

Figure 23: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_SOD_V5 (A) and pYES2.1_SOD (B).
Slika 23: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L glukoze in galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* T334, ki so vsebovale plazmid pYES2.1_SOD_V5 (A) in pYES2.1_SOD (B).

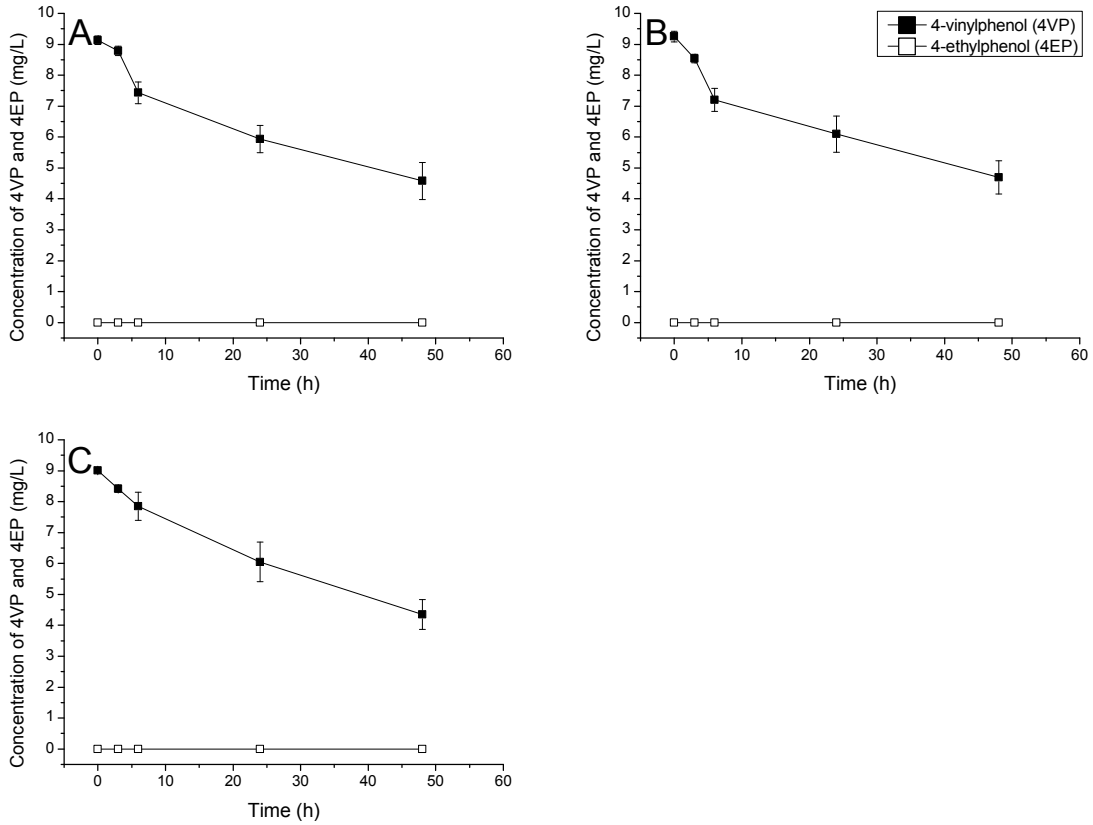

Figure 24: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L glucose and galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* T334 transformants, containing plasmids pYES2.1_TPI_V5 (A), pYES2.1_PCp450M_V5 (B) and pYES2.1_Cp450_V5 (C).
Slika 24: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L glukoze in galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* T334, ki so vsebovale plazmide pYES2.1_TPI_V5 (A), pYES2.1_PCp450M_V5 (B) in pYES2.1_Cp450_V5 (C).

As can be seen on Figures 23 and 24, no formation of 4-ethylphenol has been detected to indicate the activity of the vinylphenol reductase enzyme, although we were able to confirm the expression of the heterologously expressed product. Graphs showed the depletion of of 4-vinylphenol without the formation of 4-ethylphenol – this phenomenon was contributed to the volatility of the product and adsorption to the cell wall of *S. cerevisiae* as already discussed in Chapter 4.5. We decided to perform the same experiments again, this time with plasmids transformed into *S. cerevisiae* YPH250. Course of the fermentation experiments is depicted in Figures 25 and 26.

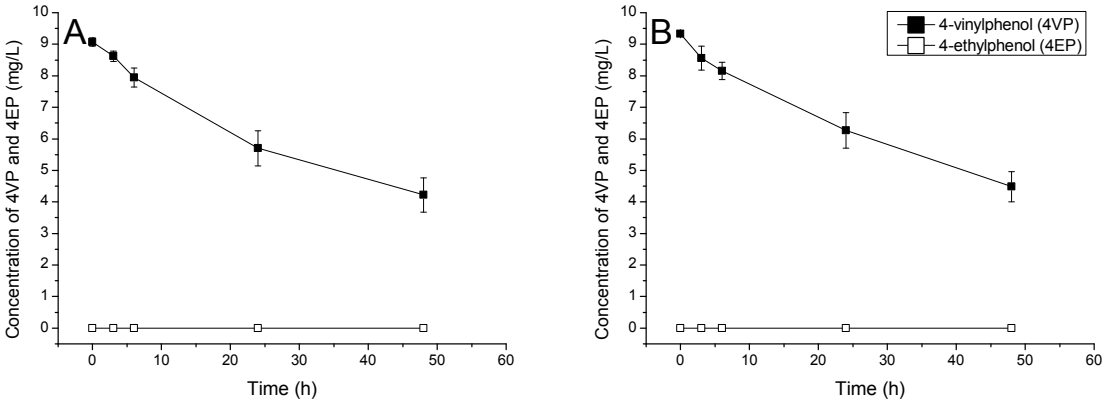

Figure 25: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* YPH250 transformants, containing plasmids pYES2.1_SOD_V5 (A), pYES2.1_SOD (B).

Slika 25: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* YPH250, ki so vsebovale plazmid pYES2.1_SOD_V5 (A) in pYES2.1_SOD (B).

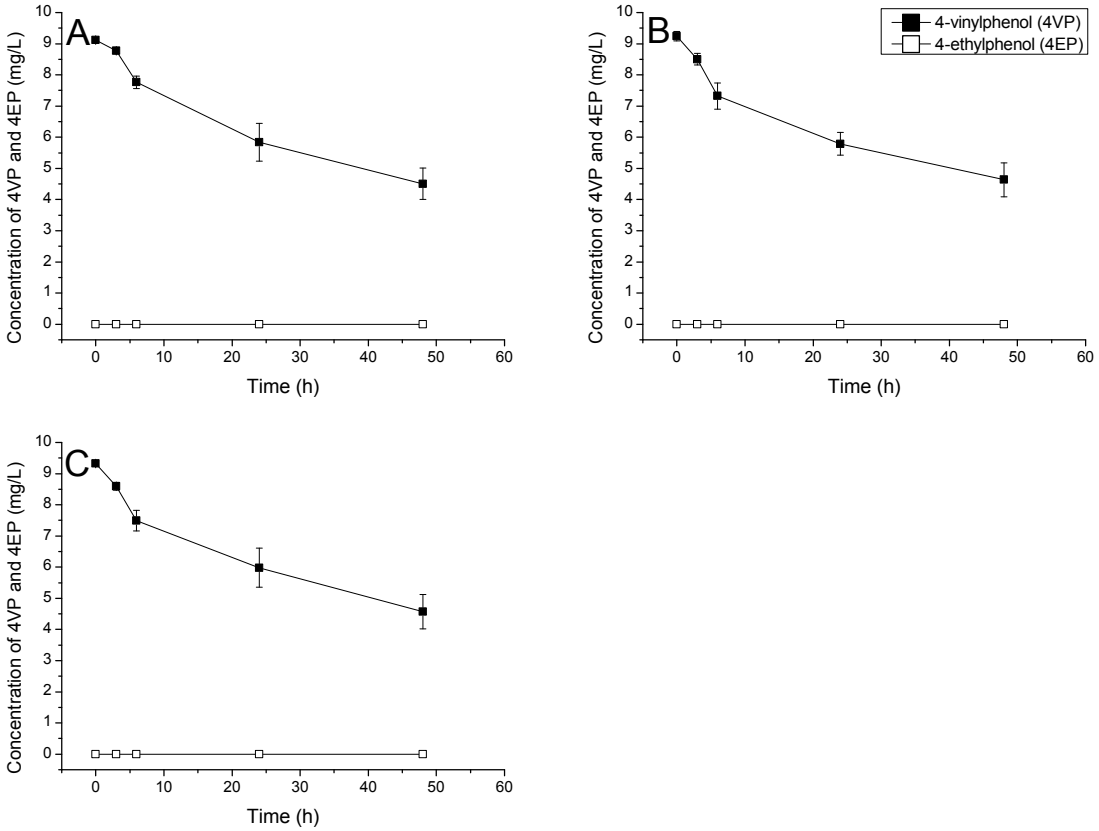

Figure 26: Depletion curves of 4-vinylphenol in fermentation experiments performed in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C on shaker at 160 rpm. Fermentations were performed with *S. cerevisiae* YPH250 transformants, containing plasmids pYES2.1_TPI_V5 (A), pYES2.1_PCp450M_V5 (B) and pYES2.1_Cp450_V5 (C).
Slika 26: Grafi poskusnih fermentacij za spremljanje aktivnosti heterologno izraženega encima vinilfenol reduktaza kavsovke *B. bruxellensis* v kvasovki *S. cerevisiae*. Fermentacije so bile opravljene v 150 ml Erlenmejericah s 100 mL gojišča SC-URA z dodatkom 20 g/L galaktoze ter 10 mg/L 4-vinilfenola na stresalniku pri 28°C in 160 rpm. Fermentacije so bile izvedene s transformantami kvasovke *S. cerevisiae* YPH250, ki so vsebovale plazmide pYES2.1_TPI_V5 (A), pYES2.1_PCp450M_V5 (B) in pYES2.1_Cp450_V5 (C).

As can be seen in Figures 25 and 26, no formation of the 4-ethylphenol, that would indicate the activity of the vinylphenol reductase has been detected. Graphs showed the depletion of 4-vinylphenol without the formation of 4-ethylphenol – this phenomenon was contributed to the volatility of the product and adsorption to the cell wall of *S. cerevisiae* as discussed previously.

4.9 GENERAL DISCUSSION

Since we failed to demonstrate and regulate both the *in-vivo* activity of vinylphenol reductase of *B. bruxellensis* proposed in the literature (Tchobanov et al., 2008; Granato et al., 2014) and the *in-vivo* activity of our own candidates, further experiments would be needed to find out what might have affected the activity of the candidate enzymes.

One of the first steps that should be taken is to perform the *in-vitro* experiments with the candidate enzymes – heterologously express, purify and test the candidate enzymes for their primary activities, such as inhibition of carboxypeptidase Y and dismutation of superoxide radicals, as well as test them for their ability to convert 4-vinylphenol into 4-ethylphenol and to use NAD(P)H as cofactor. A positive result would indicate that folding and possible modifications occurred properly.

There is also a chance of improper folding when *B. bruxellensis* enzymes are expressed in *S. cerevisiae.* However, this possibility does not seem likely since Godoy et al. (2014) reported cloning of functional phenolic acid decarboxylase (*PAD*) gene of *B. bruxellensis* into *S. cerevisiae* without prior codon optimisation. Their study demonstrated that the differences in the codons used should not impede the expression of non-optimized *B. bruxellensis* genes in *S. cerevisiae*. However, there are some factors that might have obstructed the ability of the vinylphenol reductase candidates to function properly. One such factor could be the improper localisation of heterologously expressed enzyme – vinylphenol reductase might originally be located in one of the cellular organelles which can have very different conditions (such as redox potential, pH, cofactor availability to name just a few) compared to its new environment. This possibility might be supported by Harris et al. (2009) who reported that vinylphenol reductase is very unstable once the cell extracts are prepared. The importance of isolation method and the possibility that heterologously expressed vinylphenol reductase might not be as stable and/or function properly without sufficient availability of Cu/Zn at the time of enzyme synthesis was also stressed by Granato et al. (2014). Another factor to consider is possible post-translational processing of the heterologously expressed enzyme, which might occur differently in the host cell and choice of *S. cereveisae* host strain may influence the expression of heterologous enzyme as well. Finally, the last factor that we considered when this work was already in its final stages was the availability of the substrate, in our case 4-vinylphenol, to the candidate enzymes. There is a possibility that 4-vinylphenol might not as readily pass the membrane as its precursor *p*-coumaric acid. This fact should definitely be considered in any further attempts in testing the *in-vivo* activity of vinylphenol reductase.

Another method to demonstrate the activity of the vinylphenol reductase candidates would be to construct *B. bruxellensis* mutants with knock-outs of the proposed genes. Although the transformation and integration of the dominant marker into *B. bruxellensis* had been described by Miklenić et al. (2013), this was done with non-homologous DNA and with random integration into genome. This gave us the possibility to construct the knock-out mutants, but this method is too unspecific and time consuming to be used in such a short time frame with the available resources. However, methods for transformation have recently been improved (Miklenić et al., 2015) as well as tools for the construction of knock-out mutants, namely the CRISPR/Cas9 system (Jinek et al., 2012) which has in

recent years proved to be a very reliable tool for genome editing. With the discovery of autonomous replicating sequences of *B. bruxellensis* (Schifferdecker et al., 2014), the CRISPR/Cas9 system would indeed be a proper tool to prove the proposed functions of these enzymes.

However, owing to the elusive nature of vinylphenol reductase, there is likelihood that the complete enzyme has not been identified yet. In this case, we would first need to consider the function of vinylphenol reductase. It was believed that the main function of this enzyme is to transform volatile phenols into less toxic derivates (Chatonnet et al., 1992). This might even not be true as Harris et al. (2008) demonstrated that 4-ethylphenol might be even more toxic than 4-vinylphenol; furthermore, if that was the case, one would expect to find this enzymatic activity in many more microbial species. A more recent study by Joseph et al. (2013) suggested that vinylphenol reductase might aid in recycling of the oxidized cofactor NAD^+. A more efficient generation of NADH by *B. bruxellensis,* compared to *S. cerevisiae,* has been observed by Rozpędowska et al. (2011) and Tiukova et al. (2013). It has also been reported that *B. bruxellensis* carries additional NADH dehydrogenase complex subunits that are absent in other yeasts (Procházka et al., 2010). Since Godoy et al. (2008), Tchobanov et al. (2008) and Granato et al. (2014) observed higher activity of the vinylphenol reductase activity in the presence of NADH, we could speculate that the enzyme possessing vinylphenol reductase activity could actually be an enzyme composed of multiple subunits, which would explain why it was so difficult to demonstrate the activity of the single isolated enzyme. A research in mice also indicted that even if the subunits identified as the key components in the conversion of the 4-vinylphenol were knocked-out, conversion still proceeded although at a slower pace (Vogie et al., 2004). Finally, another thing to consider is to look for genes that might have been integrated into genome through horizontal gene transfer. One such example is the phenolic acid decarboxylase (*PAD*) gene of *B. bruxellensis*, which converts *p*-coumaric acid into 4-vinylphenol, which is the precursor for 4-ethylphenol. It was Curtin et al. (2012) who already assumed that this gene might contain parts of bacterial origin since no sequence homology with *S. cerevisiae PAD1* gene was found. Godoy et al. (2014) later proved that *PAD* gene of *B. bruxellensis* indeed contains 34 % sequence identity with bacterial homologs. Borneman et al. (2014) performed a survey of four genomes from different *B. bruxellensis* strains and confirmed the presence of sequences that originated in bacteria. Since the ability to form 4-ethylphenol has been confirmed in *Lactobacillus* sp., one of the possible approaches to identify vinylphenol reductase in *B. bruxellensis* is to survey genomic sequences of the available *Lactobacillus* species.

One of the challenges that we encountered during our work was the rapid development of genomic and bioinformatics tools, which sometimes led to inconsistencies and changes of the existing data. We can demonstrate this based on the example of vinylphenol reductase gene of *B. bruxellensis*. The enzyme was first characterised by Tchobanov et al. (2008) through comparison of amino acid sequences with the NCBI BLASTp algorithm which found one match in the genomic sequence of *B. bruxellensis* CBS 2499 (Woolfit et al., 2007) which had at that time 40 % of its genome sequenced and had no previous annotations. When Curtin et al. (2012) published the whole genome sequence of *B. bruxellensis* AWRI 1499, the same nucleotide sequence was automatically annotated as carboxypeptidase Y inhibitor based on sequence similarity with *Pichia angusta*. When the whole genome sequence became accessible, we found out that the sequence published by

Tchobanov et al. (2008) was in fact the second longest ORF, which we took into account in our experiments. If BLAST search would be performed with these sequences today, the results would show that this gene is annotated as carboxypeptidase Y and no longer as carboxypeptidase Y inhibitor, which clearly demonstrates the pace with which this field is evolving. Up to this date, five different *B. bruxellensis* genomes have been sequenced by Curtin et al. (2012), Piškur et al. (2012), Borneman et al. (2014), Crauwels et al. (2014) and Valdes et al. (2014), though only three are currently accessible in the NCBI database, with assembly levels at contig stage (AWRI 1499 and LAMAP2480) or scaffold stage (CBS 2499).

Although none of our attempts managed to demonstrate the *in-vivo* activity of vinylphenol reductase of *B. bruxellensis*, our results contributed a few new pieces of information about physiology and genetics of this interesting organism, which has become important not only in terms of the control of wine quality but also as an industrial organism. This can be seen from the number of the review articles published in the past few years (Curtin and Pretorius, 2014; Schifferdecker et al., 2014; Steensels et al., 2015).

5 CONCLUSIONS

With our experimental work we answered the following hypotheses:

- Various strains of *B. bruxellensis* differ both in the activity of the enzyme as well as nucleotide sequence for vinylphenol reductase enzyme

By the fermentation experiments with available *B. bruxellensis* strains we managed to confirm that there are differences in the vinylphenol reductase activity. Through the calculation of relative consumption rates of 4-vinylphenol and relative production rates of 4-ethylphenol we managed to demonstrate that the ZIM 1762 strain was the most effective strain in terms of formation of 4-ethylphenol. ZIM 1762 was followed by CBS 2499 as the second most effective strain whereas all other strains (ZIM 700, 701, 702, 703, 704, 705, 1764, 2306 and ZIM 2512) exhibited low specific formation rate of 4-ethylphenol. By this experiment we demonstrated the differences in vinylphenol reductase activity among different *B. bruxellensis* strains.

The sequencing results of two published vinylphenol reductase genes showed the differences in the nucleotide sequences among different *B. bruxellensis* strains. Since some of these polymorphisms resulted in the change in amino acid composition or even the length of the enzyme, we can assume that these changes can affect the catalytic activity of the enzyme.

- Active vinylphenol reductase enzyme from *B. bruxellensis* can be expressed in the laboratory strain of *S. cerevisiae*

We managed to successfully express both of the proposed vinylphenol reductase enzymes in the laboratory strain of *S. cerevisiae*. When we performed the fermentation experiments to test the *in-vivo* activity of both candidates, we were not able to confirm the conversion of 4-vinylphenol to 4-ethylphenol. We also tried to find additional gene candidates for vinylphenol reductase in *B. bruxellensis* and through literature search and sequence comparison decided to try to express and assess possible vinylphenol reductase activity of triosephosphate isomerase, putative cytochrome p450 monooxigenase and cytochrome p450 from *B. bruxellensis*. Although the expression of these genes was successful, the *in-vivo* vinylphenol reductase activity of the new candidate genes could not be confirmed.

- Vinylphenol reductase enzyme can also be expressed in the wine strain of *S. cerevisiae* and expression of vinylphenol reductase enzymes can be regulated to such an extent that the concentration of 4-ethylphenol is within the limits of acceptability for wine

Since none of the proposed enzymes exhibited vinylphenol reductase activity under controlled and predictable environment of the laboratory *S. cerevisiae* strains in previous experiments, we concluded after careful consideration that the probability to get a functional industrial strain with desired properties was unrealistic due to the higher genetic complexity of industrial strains. Considering this fact and also the fact that the construction of industrial strain would be too costly and time consuming, we decided not to continue with the development of such a strain based either on vinylphenol reductase sequences published in the literature or candidate sequences obtained in this study.

6 SUMMARY (POVZETEK)

6.1 SUMMARY

Recent studies have shown that alcoholic beverages, such as wine, benefit from the fermentation with multiple different microorganisms in terms of the flavour composition. Concurrently, the popularity of biodynamic and spontaneously fermented wines has stimulated winemakers to produce such wines for the market. However, spontaneous fermentation cannot be managed as well as the inoculated ones and presents a risky operation for the winemaker. Since each vintage is different, use of spontaneous fermentation is no guarantee for success. One of the dangers of spontaneous fermentation is the contamination with *Brettanomyces bruxellensis*, a yeast that is one of the most common causes of wine spoilage due to the production of volatile phenols, acetic acid and biogenic amines. Small concentrations of volatile phenols, e.g. 4-ethylphenol, are even desired in certain types of red wines since they can impart a more complex aromatic profile with smoky and leathery notes. On the contrary, high concentrations impart aromas reminiscent of horse sweat, barnyard and medicinal chemicals. The fact that *B. bruxellensis* is not a traditionally domesticated yeast like *Saccharomyces cerevisiae* makes it inappropriate for use in wine fermentations as a direct starter culture.

In this work, we tried to construct a genetically engineered wine strain of *S. cerevisiae* that would be able to express vinylphenol reductase from *B. bruxellensis*, an enzyme that is responsible for the formation of the 4-ethylphenol through reduction of 4-vinylphenol. *S. cerevisiae* would be engineered in such a way so that the vinylphenol reductase would be expressed at low constitutive levels, which would convert 4-vinylphenol to 4-ethylphenol at acceptable levels for red wine.

To reach our goal, the relative conversion rates of 4-vinylphenol to 4-ethylphenol of different *B. bruxellensis* strains were determined first. The calculation of relative conversion rates showed differences between tested strains, indicating a difference in the activity of vinylphenol reductase. Strain ZIM 1762 proved to be the most efficient converter; it was followed by strain CBS 2499 whereas all other strains tested exhibited low conversion rates. The sequencing of the candidate vinylphenol reductase gene described by Tchobanov et al. (2008) revealed differences in nucleotide sequences. Some of them resulted in the change of the amino acid composition and, in some cases, in the length of the enzyme. The sequences obtained were used to construct plasmids for high inducible expression in *S. cerevisiae*. A fermentation experiment was performed in a synthetic defined medium, supplemented with 4-vinylphenol and galactose as an expression to establish the expression of vinylphenol reductase as well as its *in-vivo* activity. Although multiple open reading frames were tested and the expression of the proposed vinylphenol reductase during fermentation experiments was confirmed, the fermentation experiments did not confirm the *in-vivo* activity of the enzyme.

We decided to repeat the experiment with the Zn/Cu superoxide dismutase gene of *B. bruxellensis*, which was also reported to possess the vinylphenol reductase activity (Granato et al., 2014). Sequencing of Zn/Cu superoxide dismutase gene also revealed differences in the nucleotide sequences among different *B. bruxellensis* strains, which in some cases resulted in the change of amino acid composition but not in the length of the

enzyme. Although the expression of the enzyme during fermentation experiments was confirmed, no vinylphenol reductase activity was detected.

Finally, based on the reviewed literature, we decided to test the triosephosphate isomerase, putative cytochrome p450 monooxigenase and cytochrome p450 of *B. bruxellensis* to find out if any of these enzymes possesses the vinylphenol reductase activity. Fermentation experiments were performed as described before. We confirmed the expression of enzymes during fermentation experiments but, again, no vinylphenol reductase activity was detected in the fermentation broth.

One of the challenges that we faced during our experimental work was the rapid development of genomic and bioinformatics tools. These sometimes led to inconsistencies and changes of the existing data, which was evident in the case of the vinylphenol reductase of *B. bruxellensis*.

The final goal of our work – to construct genetically modified wine strain of *S. cerevisiae* with low constitutive expression of vinylphenol reductase from *B. bruxellensis* – was based on the prediction that we will be able to express and confirm the *in-vivo* activity of vinylphenol reductase enzyme in the laboratory strain of *S. cerevisiae* and then tune its expression in an industrial strain. Since we were not able to confirm the *in-vivo* activity of the enzyme in the specified time frame and with available resources, the construction and evaluation of the new strain was not realised. However, our experiments indicated that further investigations into the nature of this unusual enzyme would be needed to understand and discover its full potential.

6.2 POVZETEK

Aktivnost raznolike združbe mikroorganizmov v fermentaciji alkoholnih pijač, kot je na primer vino, lahko pozitivno vpliva na aromatski profil končnega izdelka (Dashko in sod., 2015). Ravno slednje je spodbudilo porast zanimanja potrošnikov za biodinamična vina in vina, ki so bila proizvedena s spontano fermentacijo, kar je tudi proizvajalce spodbudilo k sami proizvodnji tovrstnih vin. Gre za precej tvegan proces, saj ni vsak letnik primeren za tak tip fermentacije. Eno izmed večjih tveganj spontane fermentacije vina predstavlja kontaminacija s kvasovko *Brettanomyces bruxellensis*, ki zaradi produkcije hlapnih fenolov, ocetne kisline in biogenih aminov spada med najpogostejše povzročitelje kvara vina. Čeprav so majhne koncentracije hlapnih fenolov, med katere spada tudi 4-etilfenol, zaželene v določenih vrstah rdečih vin, je visoka koncentracija 4-etilfenola v vinu nezaželena. Kljub morebitnem pozitivnem doprinosu k aromi pa je uporaba kvasovke *B. bruxellensis* v vinarstvu zaradi zahtevnega nadzora še vedno tvegana in neželena (Oelofse in Pretorius, 2008). Do sedaj je bila sposobnost tvorbe vinilfenolov opisana pri več vrstah mikroorganizmov, med drugim tudi pri kvasovki *Saccharomyces cerevisiae*. Po drugi strani pa je bila sposobnost tvorbe etilfenolov opisana le pri določenih vrstah mikroorganizmov, med katerimi je *B. bruxellensis* edina, ki je sposobna tvorbe etilfenolov v pogojih fermentacije vina. Sposobnost tvorbe etilfenolov kvasovke *B. bruxellensis* je bila pripisana prisotnosti encima vinilfenol reduktaza (Godoy in sod., 2008; Tchobanov in sod., 2008; Granato in sod., 2014).

V zadnjih nekaj letih se je glavni fokus raziskav kvasovke *B. bruxellensis* preselil iz njene sposobnosti kvara vina na njen biotehnološki potencial, saj so raziskave pokazale, da imajo nekateri sevi kvasovk *B. bruxellensis* boljše izkoristke v fermentacijah za pridobivanje bioetanola. Ti izsledki so botrovali k bolj intenzivni usmeritvi raziskav v genetiko in fiziologijo kvasovk *B. bruxellensis*, kar je vodilo do objav prvih popolnih genomskih sekvenc in kmalu za tem tudi prvih transkriptomskih študij (Curtin in sod., 2012; Tiukova in sod., 2013; Steensels in sod., 2015). Kljub hitrem razvoju področja raziskav kvasovke *B. bruxellensis* pa zaradi velike genetske heterogenosti med različnimi sevi ostaja še veliko vprašanj neodgovorjenih in odprtih za nadaljne raziskave.

V tem delu smo si za cilj zadali dizajnirati gensko spremenjeno vinsko kvasovko *S. cerevisiae*, ki bi bila sposobna nizkega konstitutivnega izražanja encima vinilfenol reduktaze kvasovke *B. bruxellensis*. Z nizkim konstitutivnim izražnjem encima bi dosegli, da bi bila pretvroba 4-vinilfenola v 4-etilfenol v mejah sprejemljivosti za vino.

Da bi dosegli zadani cilj, smo delo razdelili v več faz. V prvi fazi smo zbrali različne seve kvasovk *B. bruxellensis*, ki so bili na voljo v Zbirki Industrijskih Mikroorganizmov (ZIM) Biotehniške fakultete Univerze v Ljubljani, hkrati pa smo iz grozdnega mošta iz Črne gore, izolirali nov sev kvasovke *B. bruxellensis*, ki smo ga potrdili z analizo PCR-RFLP regije ITS, ga deponirali v zbirko ZIM ter ga uporabili pri nadaljnem eksperimentalnem delu.

Z namenom, da bi ugotovili, ali obstajajo razlike v hitrosti pretvorb 4-vinilfenola v 4-etilfenol, in posledično aktivnosti encima vinilfenol reduktaza različnih sevov kvasovke *B. bruxellensis*, smo se odločili izvesti poskusne fermentacije. Fermentacije so potekale v erlenamajericah v kemično definiranem gojišču z dodatkom 4-vinilfenola v miroaerobnih pogojih statične kulture. Vsaka izmed Erlenmejeric je bila inokulirana s posameznih sevom kvasovke *B. bruxellensis* oziroma kontrolnega seva *S. cerevisiae*, negativno kontrolo pa so predstavljale Erlenmejerice z gojiščem brez inokoluma. Na tak način smo preizkusili enajst različnih sevov *B. bruxllensis*. Vzorčenja so potekala ob časovnih točkah: 0, 1, 2, 4 in 6 dan. Tekom fermentacij smo s štetjem celic v raztopini metilenskega modrila pod mikroskopom spremljali koncentracijo viabilnih celic. Hkrati smo s pomočjo analize HPLC spremljali porabo 4-vinilfenola in 4-etilfenola v poskusnih fermentacijah. Analiza rezultatov je pokazala velike razlike v hitrosti rasti različnih sevov *B. bruxellensis*. Za najhitreje rastoča sta se izkazala seva ZIM 2512 in ZIM 1764, sledili so jim sevi ZIM 700, 701, 702, 703, 704, 705 in ZIM 2306; najnižjo hitrost rasti pa sta imela seva CBS 2499 in ZIM 1762. Ko smo pridobljene podatke združili z rezultati analize HPLC porabe 4-vinilfenola in nastanka 4-etilfenola z izračunom specifične hitrosti porabe 4-vinilfenola oziroma specifične hitrosti nastanka 4-etilfenola (Zupan in sod., 2013) se je izkazalo, da je imel sev ZIM 1762 najvišjo specifično hitrost nastanka 4-etilfenola, sledil mu je sev CBS 2499, preostali sevi pa so imeli nizko specifično hitrost nastanka 4-etilfenola. Rezultati te analize so potrdili, da obstajajo razlike v hitrosti pretvorb med različnimi sevi kvasovk *B. bruxellensis*, ki bi jih lahko pripisali razlikam v aktivnosti encima vinilfenol reduktaza različnih sevov *B. bruxellensis*. Kontrolne fermentacije brez inokoluma so pokazale, da se tekom fermentacije del (9 %) 4-vinilfenola izgubi zaradi hlapnosti substrata, hkrati pa so fermentacije s kontrolnim sevom *S. cerevisiae* pokazale, da se del substrata izgubi zaradi adsorpcije na celično steno (Morata in sod., 2013), kar je pojasnilo, zakaj je bila končna koncentracija 4-etilfenola nižja od začetne koncentracije 4-vinilfenola pri fermentacijah s kvasovko *B. bruxellensis*.

Sledila je primerjava najdaljših odprtih bralnih okvirjev nukleotidnega zapisa za encim vinilfenol reduktaza različnih sevov *B. bruxellensis*. Primerjali smo nukelotidne sekvence za encim vinilfenol reduktaza, ki so jih Tchobanov in sod. (2008) pridobili s primerjavo aminokislinskih zaporedij peptidnih fragmentov izoliranega encima. Primerjava najdaljših odprtih bralnih okvirjev je pokazala, da se nukleotidne sekvence za encim vinilfenol reduktaza med seboj razlikujejo, prepis sekvenc v aminokislinsko zaporedje pa je pokazal, da se razlike v nukelotidnem zapisu odražajo tudi v zapisu za aminokislinsko zaporedje, v nekaterih primerih (mutacija v start kodonu seva ZIM 705 in zgodnji stop kodoni pri sevoma ZIM 1762 in ZIM 2512) pa celo v dolžni samega encima. Na podlagi teh informacij smo sklepali, da so razlike v hitrostih pretvorbe 4-vinilfenola v 4-etilfenol posledica mutacij v nukleotidnem zapisu za encim vinilfenol reduktaza, ki se odražajo tudi v katalitični aktivnosti samega encima.

Podatke, ki smo jih pridobili s poskusnimi fermentacijami in primerjavo nukelotidnih sekvenc smo nato uporabili za konstrukcijo plazmidnih vektorjev za heterologno izražanje encima vinilfenol reduktaza v kvasovki *S. cerevisiae*. Odločili smo se za uporabo komercialnega vektorja pYES2.1 TOPO TA (Invitrogen), ki omogoča visoko inducibilno izražanje izbranega proteina v gojišču z galaktozo. Plazmidne vektorje smo osnovali na nukleotidnih zapisih za encim vinilfenol reduktaza sevov ZIM 1762 in CBS 2499 (visoka specifična hitrost nastanka 4-etilfenola) ter ZIM 701 (nizka specifična hitrost nastanka 4-etilfenola). Vse plazmidne vektorje smo nato pripravili v več verzijah in sicer z vključkom najdaljšega, drugega najdaljšega in tretjega najdaljšega odprtega bralnega okvirja brez stop kodona, ki omogoča izražanje izbranega proteina skupaj z epitopom V5, ki omogoča detekcijo izbranega proteina s specifičnimi protitelesi. Hkrati smo pripravili vse prej omenjene plazmide tudi s stop kodonom, v primeru, da bi epitop blokiral katalitično mesto encima. Poleg omenjenih vektorjev smo *in-silico* dizajnirali tudi vektorje za nizko in visoko konstitutivno izražanje encima vinilfenol reduktaza kvasovke *B. bruxellensis* v laboratorijskih in industrijskih sevov kvasovk *S. cerevisiae*.

Vektorje, osnovane na plazmidu pYES2.1 smo nato transformirali v laboratorijski sev *S. cerevisiae*, ter z njimi opravili poskusne fermentacije. Poskusne fermentacije so bile opravljene v Erlenmejericah na stresalniku v kemično definiranem sintetičnem gojišču z dodatkom 4-vinilfenola ter galaktoze za indukcijo heterolognega izražanja encima vinilfenol reduktaza kvasovke *B. bruxellensis*. Tekom dvodnevnih poskusnih fermentacij smo ob vnaprej definiranih časovnih točkah s pomočjo analize HPLC spremljali porabo 4-vinilfenola ter nastanek 4-etilfenola, ter shranili kvasno biomaso, ki smo jo nato uporabili za potrditev izražanje želenega produkta z metodo po Westernu. Pregled rezultatov analize HPLC je pokazal, da kljub porabi 4-vinilfenola v gojišču, do nastanka 4-etilfenola ni prišlo, čeprav smo z metodo po Westernu potrdili uspešno izražanje izbranega encima.

Glede na to, da smo preizkusili tri najdaljše odprte bralne okvirje vinilfenol reduktaze, ter hkrati preizkusili tudi vektorje, ki izražajo omenjeni encim brez epitopa, smo začeli dvomiti, da encim, ki so ga okarakterizirali Tchobanov in sod. (2008), poseduje vinilfenol reduktazno aktivnost. Granato in sod. (2014) so kasneje objavili članek, v katerem so okarakterizirali encim kvasovke *B. bruxellensis* z vinilfenol reduktazno aktivnostjo. Pokazali so, da naj bi encim Cu/Zn superoksid dismutaza kvasovke *B. bruxellensis* poleg svoje osnovne funkcije posedoval tudi vinilfenol reduktazno aktivnost. Odločili smo se, da

bomo preizkusili ali opisani encim res poseduje vinilfenol reduktazno aktivnost, hkrati pa smo se odločili, da bomo s ponovnim pregledom literature skušali sami najti nove kandidatne encime, ki bi v kvasovki *B. bruxellensis* imeli vinilfenol reduktazno aktivnost.

Ponoven pregled znanstvene literature je pokazal, da so tako Tchobanov in sod. (2008), kot tudi Granato in sod. (2014) v fazi s HPLC očiščene proteinske frakcije, ki je posedovala vinilfenol reduktazno aktivnost, zaznali tudi prisotnost encima trioza fosfat izomeraza, zato smo se odločili, da heterologno izrazimo tudi ta encim in preverimo, ali ima vinilfenol reduktazno aktivnost. Hkrati smo v znanstveni literaturi zasledili, da mišji in podganji urin lahko vsebujeta 4-etilfenol, kar je vzbudilo našo pozornost. Carlson in sod. (2001) so ugotovili, da se ob utišanju gena CYP2E1 oziroma CYP2F2, pretvorba 4-vinilfenola v 4-etilfenol zmanjša. Primerjava aminokislinskih zaporedij za CYP2E1 in CYP2F2 z aminokislinskimi zaporedji kvasovk *B. bruxellensis* je pokazala dva zadetka z e-vrednostjo$<10^{-4}$, in sicer predvideno citokrom p450 monooksigenazo (PCp450M) in citokrom p450 (Cp450). Odločili smo se, da v nadaljnje delo vključimo tudi ta dva encima.

Sledilo je ponovno načrtovanje in konstrukcija vektorjev, osnovanih na plazmidu pYES2.1. Plazmidni vektorji, ki so imeli vključen odprt bralni okvir gena za Cu/Zn superoksid dismutazo so bili pripravljeni v različici z in brez stop kodona, ki omogoča izražnje z oziroma brez epitopa V5, medtem ko so bili plazmidi z vključkom odprtega bralnega okvirja encimov trioza fosfat izomeraze, predvidene citokrom p450 monooksigenaze in citokroma p450 pripravljeni samo v različici s fuzijskim V5 epitopom. Hkrati smo se odločili primerjati tudi nukleotidne sekvence najdaljših odprtih bralnih okvirjev Cu/Zn superoksid dismutaze različnih sevov *B. bruxellensis*. Analiza je pokazala, da se sevi med seboj razlikujejo v nukelotidnem sekvenci za omenjeni encim in da se te spremembe odražajo tudi v aminokislinskem zaporedju, ne vplivajo pa na samo dolžino encima.

Nove vektorje, osnovane na plazmidu pYES2.1 smo transformirali v laboratorijski sev kvasovke *S. cerevisiae*, ter z njimi opravili poskusne fermentacije. Poskusne fermentacije so bile opravljene v Erlenmejericah na stresalniku v kemično definiranem sintetičnem gojišču z dodatkom 4-vinilfenola ter galaktoze za indukcijo heterolognega izražanja encima vinilfenol reduktaza kvasovke *B. bruxellensis*. Tekom dvodnevnih poskusnih fermentacij smo ob naprej definiranih časovnih točkah s pomočjo HPLC analize spremljali porabo 4-vinilfenola ter nastanek 4-etilfenola, hkrati pa smo spravljali biomaso kvasovk, ki smo jo nato uporabili za potrditev izražanje želenega produkta z metodo po Westernu. Pregled rezultatov analize HPLC je pokazal, da kljub porabi 4-vinilfenola v gojišču do nastanka 4-etilfenola ni prišlo, čeprav smo z metodo po Westernu potrdili uspešno izražanje izbranega encima.

Kljub vsem opravljenim poskusom nismo mogli povsem dokazati, da testirani kandidati ne posedujejo vinilfenol reduktazne aktivnosti, saj so se naši poskusi opirali na potrditev aktivnosti *in-vivo*, za potrditev same funkcije encima pa bi bilo potrebno še dodatno eksprimentalno delo. Obstaja verjetnost, da zaradi genetskih in fizioloških specifik izbranih gostiteljskih sevov kvasovk *S. cerevisiae* ni prišlo do pravilnega zvijanja in/ali post-transkripcijskih modifikacij encima, kar bi posledično lahko vplivalo na njegovo aktivnost. Prav tako bi lahko na pravilno zvijanje encima negativno vplivala neprimerna lokalizacija v gostiteljski celici, saj se lahko okolje posameznega celičnega organela precej

razlikuje od razmer v citosolu. Poleg že omenjenih faktorjev pa obstaja tudi verjetnost, da do pretvorbe 4-vinilfenola v 4-etilfenol ni prišlo, ker 4-vinilfenol morda ni mogel prehajati celične membrane v enaki meri, kot lahko njegov prekuzor – *p*-kumarna kislina.

Poleg same heterologne ekspresije ter *in-vitro* in *in-vivo* potrditve njegove aktivnosti je za potrditev funkcije eden izmed najbolj prepričljivih dokazov o funkciji encima uspešno utišanje izbranega gena. Žal so se v času, ko je bilo naše eksperimentalno delo izvedeno, tehnike transformacije kvasovke *B. bruxellensis* komaj razvijale (Miklenić in sod., 2013), tehnike utišanja genov v omenjeni kvasovki pa še niso bile razvite. Predvidevamo, da bo hiter razvoj molekularnih metod in znanja o kvasovki *B. bruxellensis* v bližnji prihodnosti omogočil preseči tudi te ovire, saj so bile nedavno v znanstveni literaturi objavljene potencialne avtonomne replikativne sekvence kvasovke *B. bruxellensis* (Schifferdecker in sod., 2014), kar bi lahko v prihodnosti omogočalo uporabo modernih orodij za urejanje genoma, kot je na primer sistem CRISPR/Cas9 (Jinek in sod., 2012).

Nadaljno delo v smeri identifikacije encima vinilfenol reduktaze kvasovke *B. bruxellensis* zahteva ponoven premislek o sami funkciji encima. V preteklosti so raziskovalci predvidevali, da pride do pretvorbe 4-vinilfenola v 4-etilfenol zaradi zmanjšanja toksičnosti 4-vinilfenola, kar pa so Harris in sod. (2008) ovrgli, saj so v študiji pokazali, da je 4-etilfenol bolj toksičen za *B. bruxellensis* kot 4-vinilfenol. Kasneje so Joseph in sod. (2013) v svoji študiji nakazali na možnost, da se s pretvorbo 4-vinilfenola v 4-etilfenol regenerira oksidiran kofaktor NAD^+. Hitrejšo pretvorbo 4-vinilfenol v 4-etilfenol v celičnem lizatu kvasovke *B. bruxellensis* ob prisornosti NADH oziroma NADPH so v svojih študijah pokazali že Godoy in sod. (2008), Tchobanov in sod. (2008) in Granato in sod. (2014). Procházka in sod. (2010) so v svoji študiji pokazali, da poseduje kvasovka *B. bruxellensis* več podenot encima NADH dehidrogenaza kot kvasovka *S. cerevisiae* – če ima vinilfenol reduktaza res funkcijo, ki so jo predlagali Joseph in sod. (2013), obstaja verjetnost, da se iskani encim skriva v omenjenem kompleksu, kar bi tudi pojasnilo, zakaj je bilo do sedaj tako težko potrditi njegovo aktivnost. Nadaljna možnost za odkritje encima bi bila primerjava celotnega genoma kvasovke *B. bruxellensis* z genomom bakterij iz rodu *Lactobacillus*, pri katerih je bila prav tako opisana sposobnost tvorbe etilfenolov.

Tekom eksperimentalnega dela smo bili soočeni tudi s hitro razvijajočim se področjem bioinformatike, kar je v času priprave tega dela vodilo do nedoslednosti oziroma sprememb že obstoječih podatkov. To najlažje ponazorimo na primeru vinilfenol reduktaze, ki so ga objavili Tchobanov in sod. (2008). V času, ko so Tchobanov in sod. (2008) objavili članek v katerem so okarakterizirali encim vinilfenol reduktaza kvasovke *B. bruxellensis*, je bilo javno dostopno samo 40 % genoma kvasovke *B. bruxellensis* brez anotacij (Woolfit in sod., 2007). Kmalu po tem, ko so Curtin in sod. (2012) objavil prvo celotno sekvenco genoma kvasovke *B. bruxellensis*, je avtomatska anotacija na podlagi podobnosti s karboksipeptidazo Y kvasovke *Pichia angusta* anotirala omenjeni gen kot inhibitor karboksipeptidaze Y. Ko so bile objavljene še sekvence drugih sevov kvasovke *B. bruxellensis* (Piškur in sod., 2012; Borneman in sod., 2014; Crauwels in sod., 2014; Valdes in sod., 2014) se je izkazalo, da je bil avtomatično anotiran samo drugi najdaljši bralni okvir omenjene sekvence in če opravimo iskanje dostopnih baz dandanes, lahko vidimo, da je gen, ki so ga objavili Tchobanov in sod. (2008) avtomatično anotiran kot karboksipeptidaza Y.

Vsekakor so raziskave na kvasovki *B. bruxellensis* obetavno področje, kar je razvidno že iz samega števila raziskovalnih in preglednih člankov, ki so bili objavljeni v zadnjih letih. Končnega cilja, konstrukcije gensko spremenjenega vinskega seva *S. cerevisiae* z nizko konstitutivnim izražanjem gena vinilfenol reduktaze kvasovke *B. bruxellensis*, nismo uspeli doseči. Uresničitev končnega cilja je slonela na predvidevanju, da bomo izrazili in potrdili izražanje omenjenega encima v laboratorijskem sevu kvasovke *S. cerevisiae* ter nato uravnavali njegovo izražanje v industrijskem sevu. Kljub temu smo z našim delom uspeli priti do novih dognanj o fiziologiji in genetiki kvasovke *B. bruxellensis*, hkrati pa pokazali, da bodo potrebne še dodatne raziskave, preden bomo lahko izkoristili celoten potencial encima vinilfenol reduktaze.

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I would like to thank for the financial support to Ministry of Education, Science and Sport of Republic of Slovenia (No. 1000-11-310101).

Thanks to doc. dr. Neža Čadež for sharing her knowledge and advices, and for providing *B. bruxellensis* and *S. cerevisiae* strains for our experimental work, as well as for thorough review of this thesis.

I would like to thank prof. dr. Darja Žgur Bertok and prof. dr. Matic Legiša for their professional review and for all the insightful comments that helped to raise the quality of this work. Thanks to dr. Karmen Stopar for efficient and professional review of this thesis.

Thanks to Matej Šergan for all of his help with the all of the technical challenges that were encountered and thanks to my colleagues Dragica, Lidija, Janko, Jasna, Mateja, Martina, Marko and Mia for their help and company during my stay at Biotechnical faculty. I would like to thank prof. dr. Sonja Smole-Možina for her efforts that allowed me to continue my work at Biotechnical faculty after my transfer to University of Primorska.

Thanks to company Acies Bio Ltd. for allowing me to use their equipment for some of our experiments and special thanks to Marinka Horvat, who helped me to perform those experiments.

I would also like to thank dr. ir. Jean-Marc Daran and prof. dr. Jack T. Pronk for enabling me to work at the laboratories of industrial microbiology group at Delft University of Technology as well as for donation of plasmid pRS426, which was used in our experiments. Special thanks to Pilar de la Torre Cortes for all of her help during my stay at TU Delft.

Thanks to prof. dr. Darja Barlič Maganja for warm reception at Faculty of Health Sciences and to Mitja, Nataša, Patrik, Boštjan, Majda, Andreja, Maja and all other colleagues for their help and company during my stay at Faculty of Health Sciences.

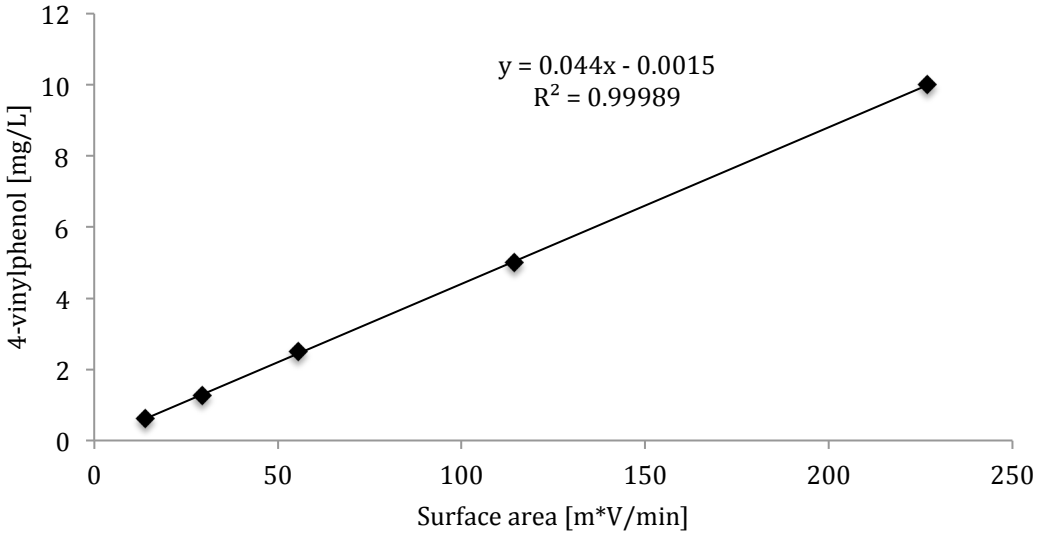
Special thanks to Jure and Miha for their help and company at the workplace as well as on other occasions.

Finaly, I would like to thank Katja for all her patience and help; to my parents, grandparents, sister Maruša, Polona and my friends for their unwavering support – this work would't be possible without them.

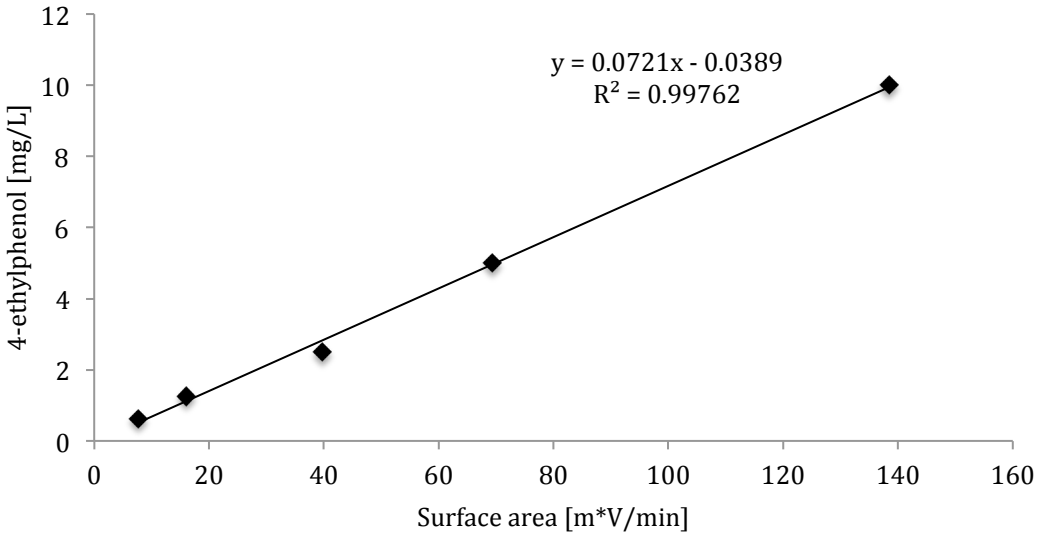
ANNEXES

ANNEX A

Sample standard curves for determination of 4-vinylphenol and 4-ethylphenol concentration



Annex A1: Standard curve for the determination of the 4-vinylphenol concentration. Values in mg/L were obtained with high-performance liquid chromatography.



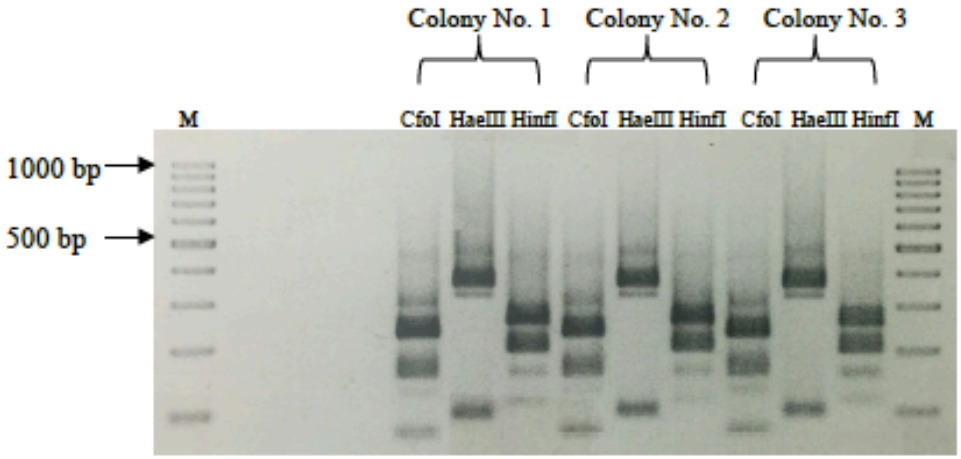
Annex A2: Standard curve for the determination of the 4-ethylphenol concentration. Values in mg/L were obtained with high-performance liquid chromatography.

ANNEX B

Sample picture of viable *B. bruxellensis* cells, stained with methylene blue solution that was used for determination of viable cell concentration with ImageJ software

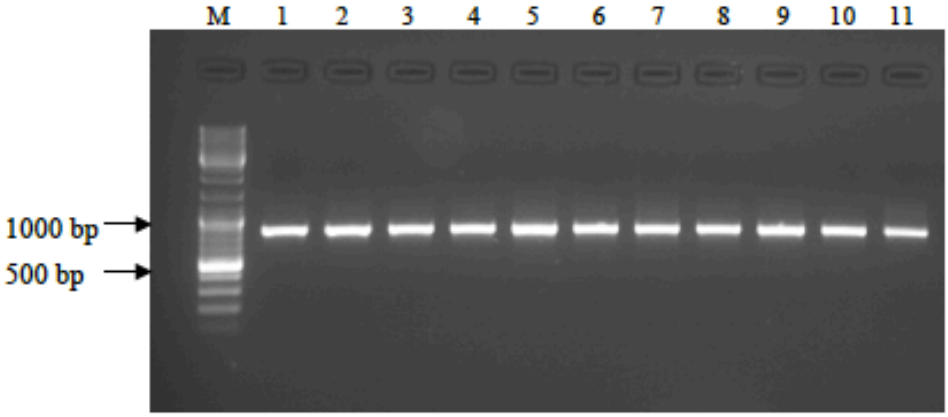
ANNEX C

Picture of agarose gel with RFLP profiles for species determination of potential *B. bruxellensis* candidate colonies

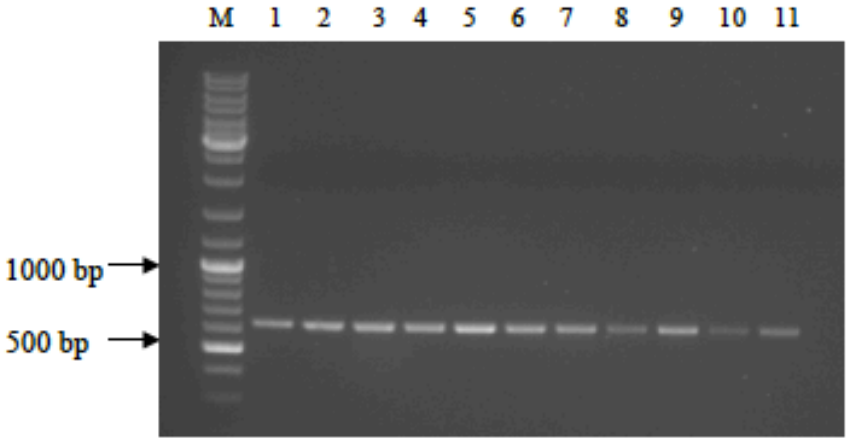


ANNEX D

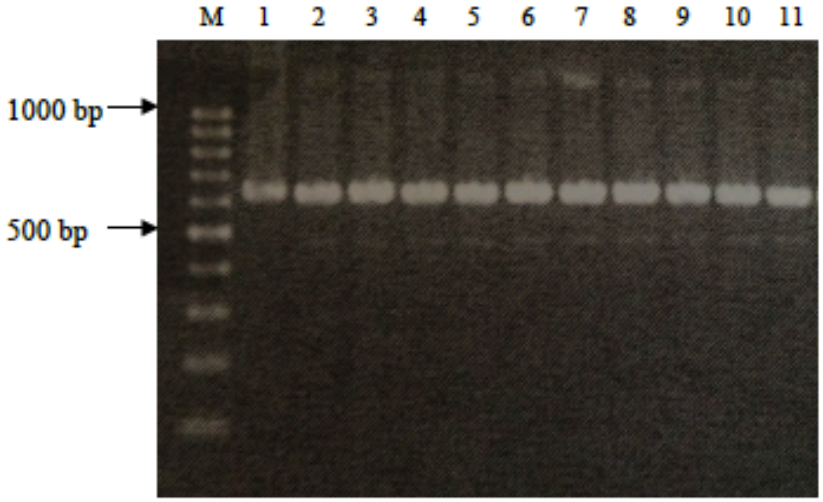
Pictures of agarose gels with PCR amplicons of the vinylphenol reductase candidate genes of *B. bruxellensis*



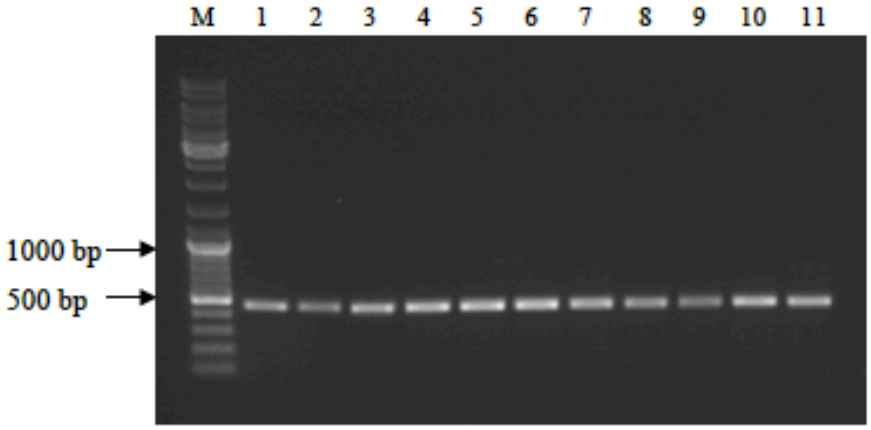
Annex D1: Picture of the agarose gel with PCR amplicons of the ORF1 of the vinylphenol reductase gene from all 11 *B. bruxellensis* strains (M – size marker)



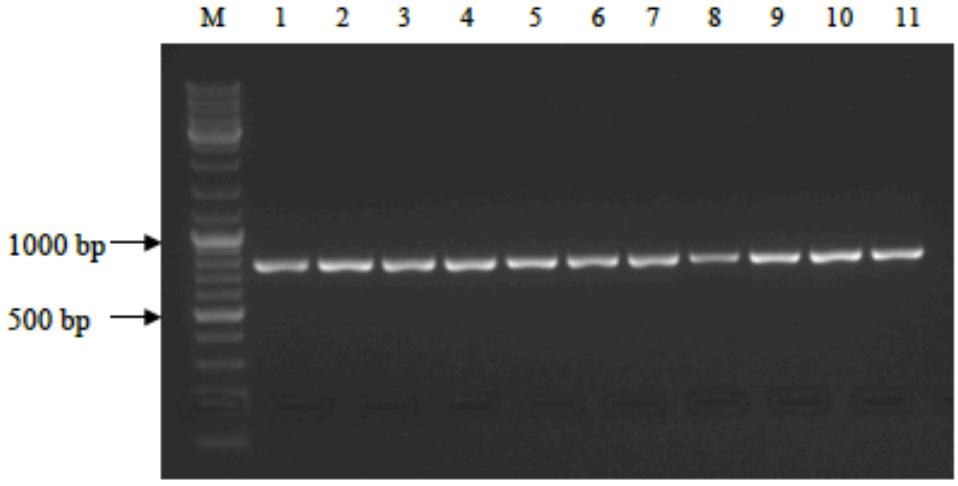
Annex D2: Picture of the agarose gel with PCR amplicons of the ORF2 of the vinylphenol reductase gene from all 11 *B. bruxellensis* strains (M – size marker)



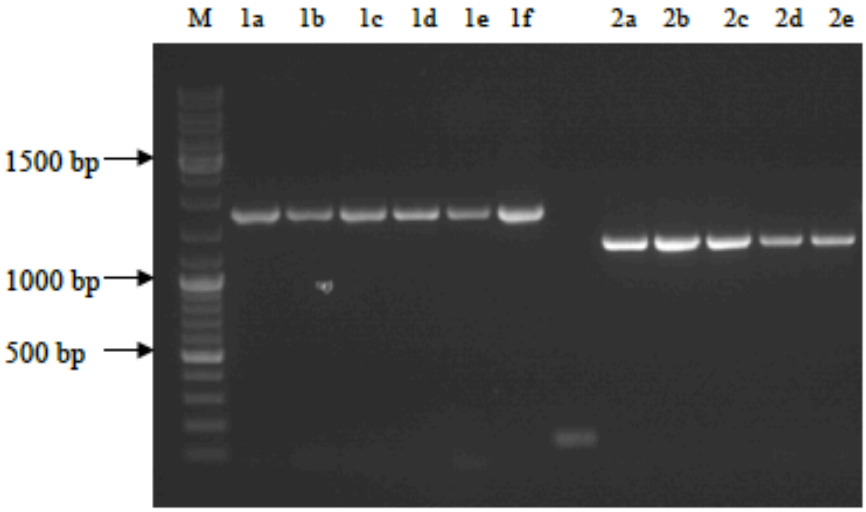
Annex D3: Picture of the agarose gel with PCR amplicons of the ORF3 of the vinylphenol reductase gene from all 11 *B. bruxellensis* strains (M – size marker)



Annex D4: Picture of the agarose gel with PCR amplicons of the ORF of the Cu/Zn superoxide dismutaze gene from all 11 *B. bruxellensis* strains (M – size marker)



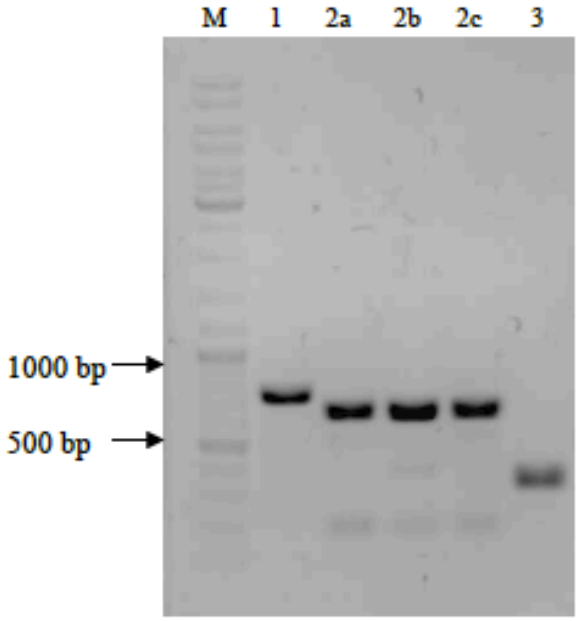
Annex D5: Picture of the agarose gel with PCR amplicons of the ORF of the triosphosphate isomerase gene from all 11 *B. bruxellensis* strains (M – size marker)



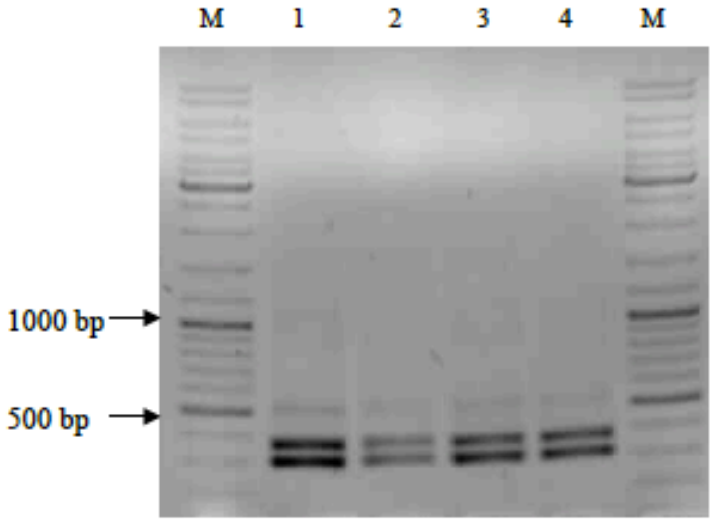
Annex D6: Picture of the agarose gel with PCR amplicons of the ORF of the cytochrome p450 monooxigenase (1a-1f) and cytochrome p450 (2a-2e) gene different *B. bruxellensis* strains (M – size marker)

ANNEX E

Sample pictures of agarose gels with PCR amplicons that were used for construction and verification of pRS426-based plasmid



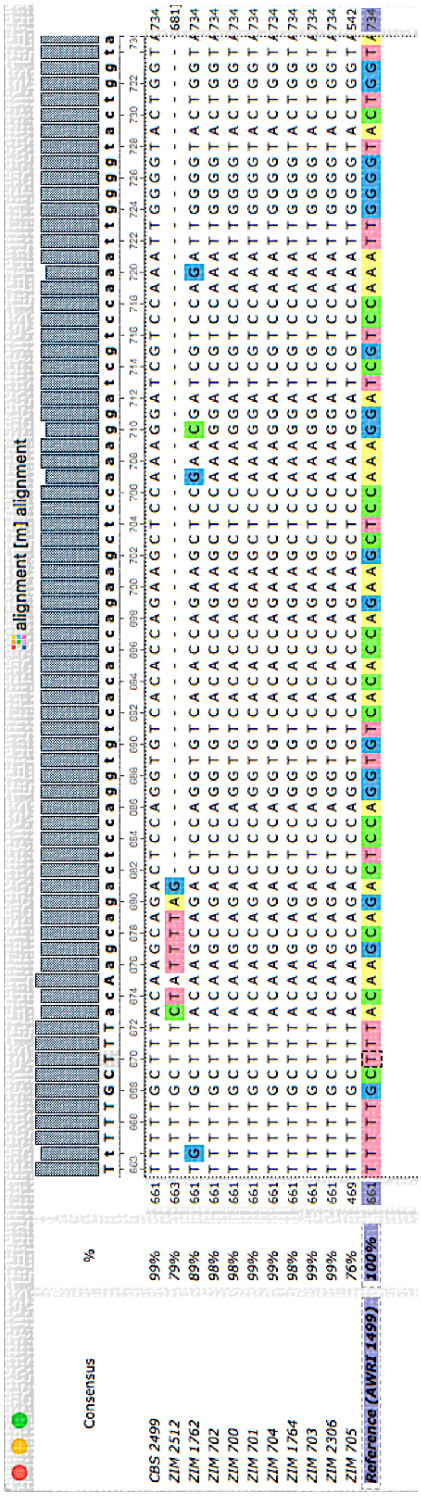
Annex E1: Picture of the agarose gel with PCR amplicons of the GPD promoter sequence (1), ORF2 of the *B. bruxellensis* vinylphenol reductase ORF2 (2a-2c) and CYC1 terminator sequence (3) (M – size marker)



Annex E2: Picture of the agarose gel with PCR amplicons of the promotor/gene (1-4; lower band) and gene/terminator (1-4; upper band) of the constructed pRS426_VPR plasmid

ANNEX F

Sample alignment region of vinylphenol reductase sequences from different *B. bruxellensis* strains aligned in Unipro UGENE program



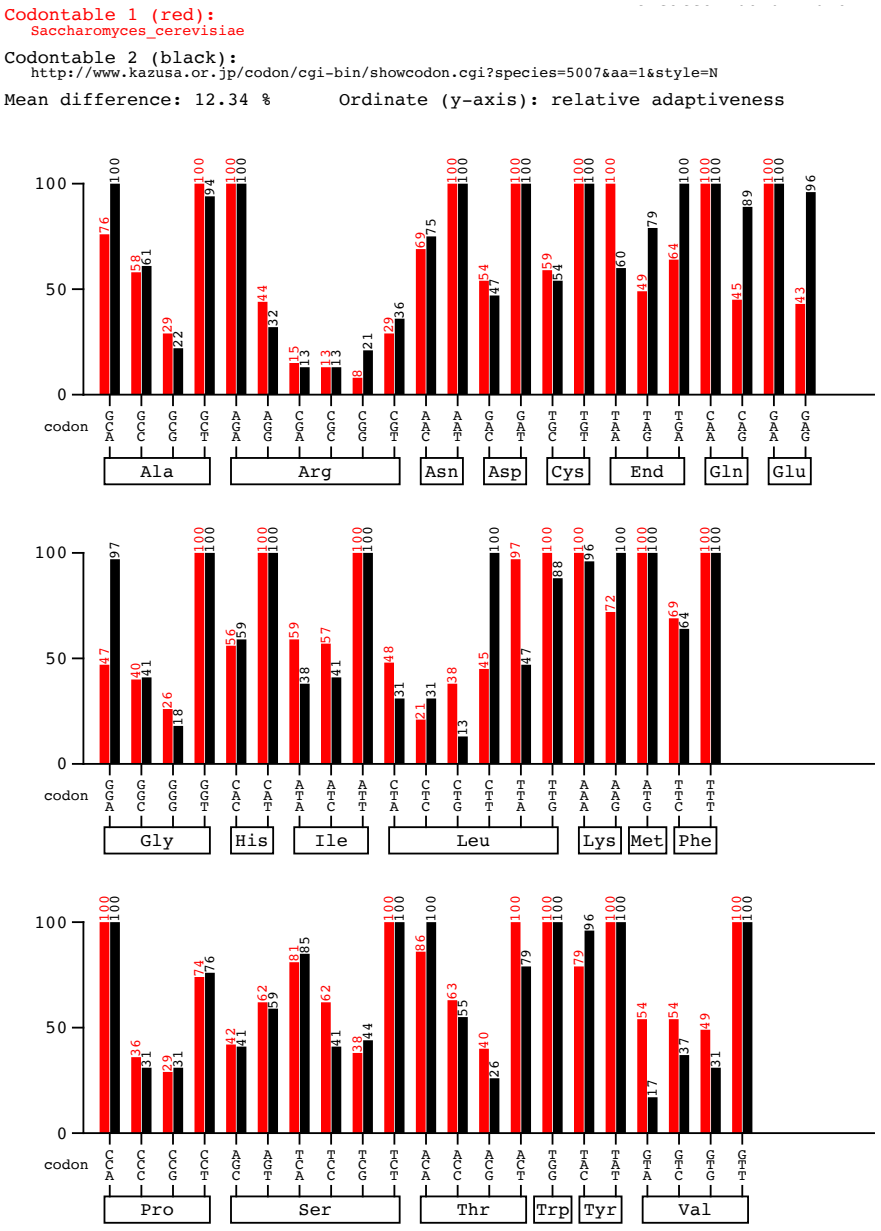
ANNEX G

Alignment of amino acid sequences of vinylphenol reductase enzyme from different *B. bruxellensis* strains performed with EMBL-EBI MUSCLE software

```
2IM_1762        MSFIAASVSIRGHIPIQALALSHITSFKQPVRAIATYLHHTQHRPSYNTYKKGFSTNSFL
2IM_2512        MSFIVASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPSYNTYKKGFSRNSFL
2IM_705         ------------------------------------------------------------
2IM_700         MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL
2IM_701         MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL
2IM_702         MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL
2IM_704         MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL
2IM_703         MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL
2IM_1764        MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL
2IM_2306        MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL
CBS_2499        MSFIAASRSIRGHIPIRALALSHSTSFKQPVRAIATYLHRTQHRPGYNTYKKGFSRNSFL

2IM_1762        LLAAMPLMTITHSVKDSLTKSEVVPTVIHDKSFLPKGFLTIQYDAGKEVGLGNNIRPADS
2IM_2512        LLAAMPLMTISDSVKDSLTKSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_705         ----MPLMTISDSVKDSLTKSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_700         LLAAMPLMTISDSVKDSLTRSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_701         LLAAMPLMTISDSVKDSLTRSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_702         LLAAMPLMTISDSVKDSLTRSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_704         LLAAMPLMTISDSVKDSLTRSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_703         LLAAMPLMTISDSVKDSLTKSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_1764        LLAAMPLMTISDSVKDSLTKSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
2IM_2306        LLAAMPLMTISDSVKDSLTKSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
CBS_2499        LLAAMPLMTISDSVKDSLTKSEVVPTVIHDKSFLPKGFLTIQYDSGKEVALGNNIRPADS
                    ******: *******.*************************:****.**********

2IM_1762        KSLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEEWSEYLHYLAIDVRLNT
2IM_2512        KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_705         KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_700         KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_701         KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_702         KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_704         KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_703         KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_1764        KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
2IM_2306        KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
CBS_2499        KNLPRIDFTLNLPSDASSTFNISKDDRFTLIVTDPDAPTRNDEKWSEYLHYLAVDVQLNT
                *.*****************************************:*********:**.***

2IM_1762        LNAENASSNDQLSTADLEGRTLYPYIGPGPPPQTGKHR---CVCLLYKQTPGVTPEAPND
2IM_2512        FNAENASSNDQLSTADLNGRTLYPYISPGPPPKNGKTQICVFCFLF--------------
2IM_705         FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
2IM_700         FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
2IM_701         FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
2IM_702         FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
2IM_704         FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
2IM_703         FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
2IM_1764        FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
2IM_2306        FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
CBS_2499        FNAENASSNDQLSTADLKGRTLYPYIGPGPPPKTGKHR---YVFLLYKQTPGVTPEAPKD
                :****************:********.*****:.** .      *:

2IM_1762        RPDWGTGIRGARAAEYAEKY----------------
2IM_2512        ------------------------------------
2IM_705         RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
2IM_700         RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
2IM_701         RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
2IM_702         RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
2IM_704         RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
2IM_703         RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
2IM_1764        RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
2IM_2306        RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
CBS_2499        RPNWGTGIRGAGAAEYAEKYKLTPYAVNFFYAQNDQQ
```

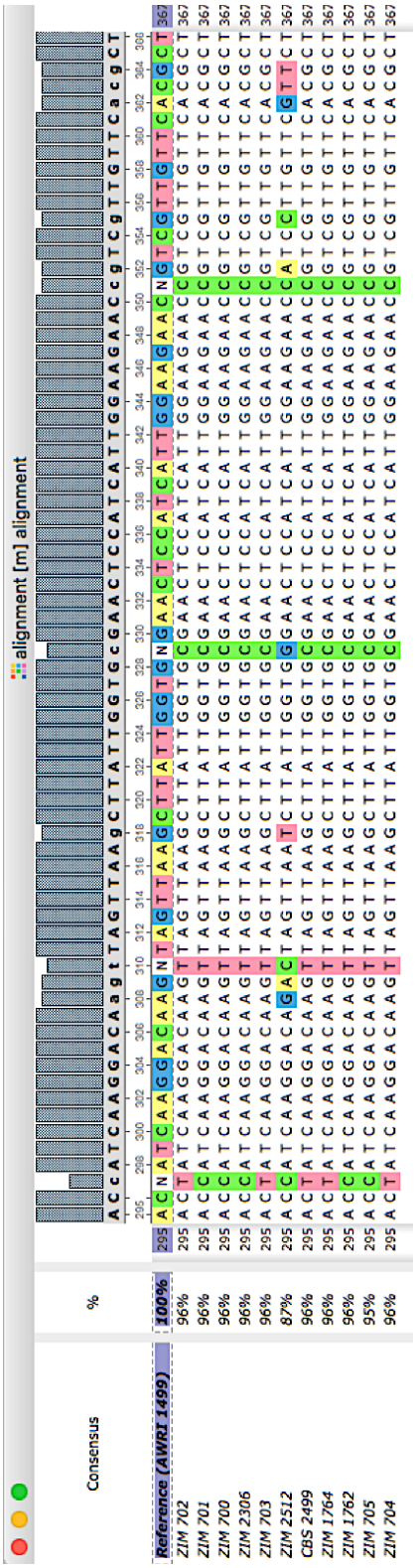
ANNEX H

Graphical representation of codon used between *S. cerevisiae* (red) and *B. bruxellensis* (black) as determined with GCUA – Graphical Codon Usage Analyzer software



ANNEX I

Sample alignment region of Cu/Zn superoxide dismutase sequences from different *B. bruxellensis* strains aligned in Unipro UGENE program



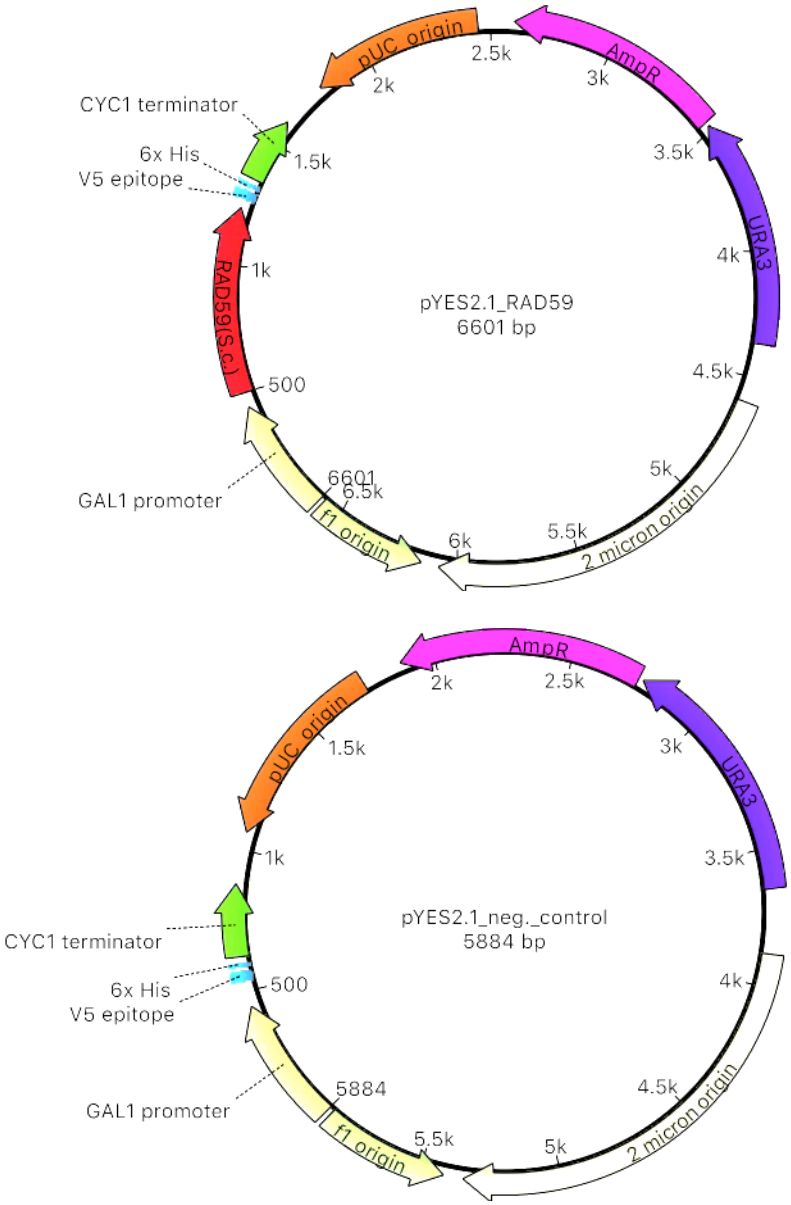
ANNEX J

Alignment of amino acid sequences of Cu/Zn superoxide dismutase enzyme from different *B. bruxellensis* strains performed with EMBL-EBI MUSCLE software

```
ZIM_2512        MVEAIAFVVRNSTVKGVGTFDQTSHREPTTINYHLQGSDPNAWIGFLIHTFGDNTNGCTS
ZIM_705         MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_700         MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_702         MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_703         MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_704         MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_1762        MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_1764        MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_2306        MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
CBS_2499        MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
ZIM_701         MVKAVAVVRGDSTVKGVVTFEQTSESEPTTINYNIEGNDPNALRGFHIHTFGDNTNGCTS
                **:*:*.*  :****** **:***  *******:::*.****  ** ************

ZIM_2512        TGPHFNPFGKTHGAPTDENRHVGDLGNINTDANGVANGTIKDRLVNLIGGNSIIGRTILV
ZIM_705         AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_700         AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_702         AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_703         AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_704         AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_1762        AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_1764        AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_2306        AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
CBS_2499        AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
ZIM_701         AGPHFNPFGKTHGAPTDENRHVGDLGNIKTDANGVAKGTIKDKLVKLIGANSIIGRTVVV
                :*************************:*******:*****.**:***.*******::*

ZIM_2512        RSGTDYLFKGGDAGSLLTDNAGGRPTCVVSGLSA
ZIM_705         HAGTDDLGKGGDAGSLQTGNAAGTPPCGVSVLSA
ZIM_700         HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
ZIM_702         HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
ZIM_703         HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
ZIM_704         HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
ZIM_1762        HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
ZIM_1764        HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
ZIM_2306        HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
CBS_2499        HAGTDDLGKGGDAGSLQTGNAGGRPACGVIGLSA
ZIM_701         HAGTDDLGKGGDAGSLQTGNAGGRPACAVIGLSA
                .:*** * ******** *.**.* *.* *  ***
```

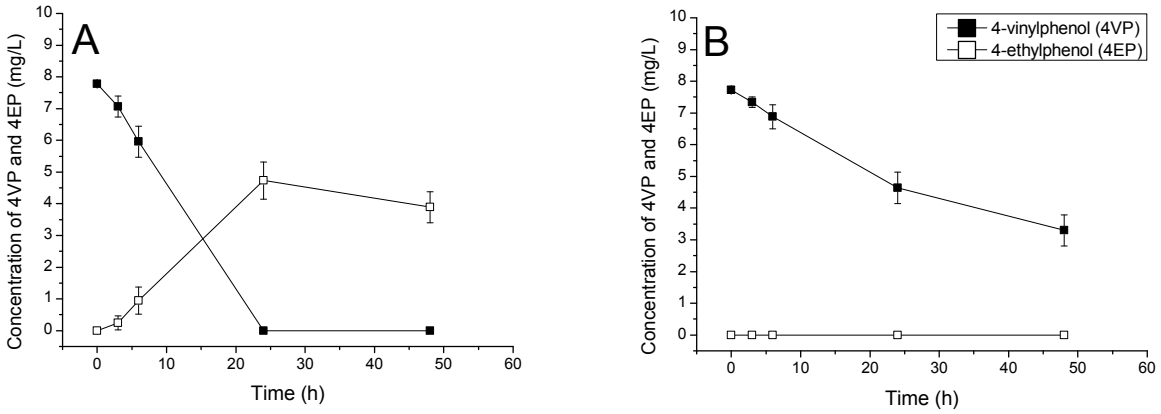
ANNEX K

Plasmid maps of plasmids for positive (pYES2.1_RAD59) and negative (pYES2.1_neg._control) of gene expression constructed with Unipro UGENE software



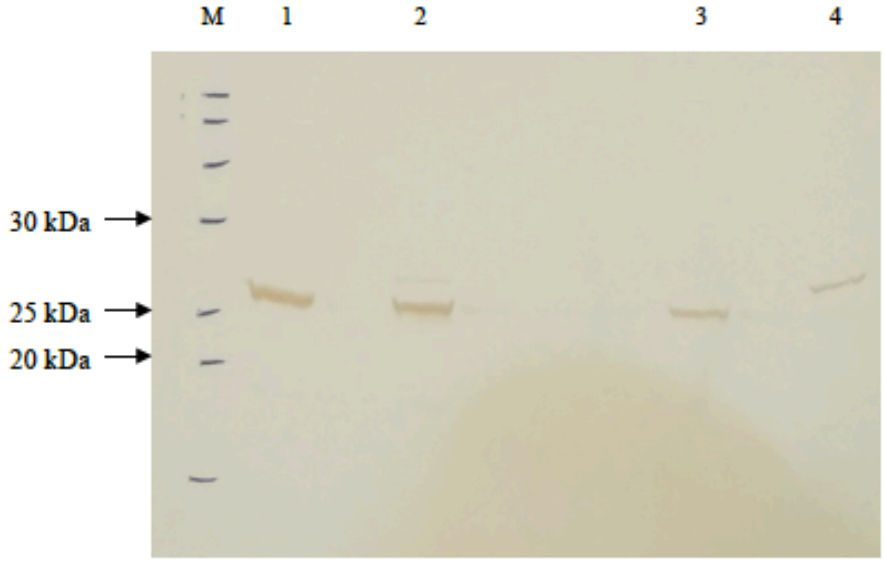
ANNEX L

Fermentation experiments in 150 mL Erlenmeyer flasks containing 100 mL of SC-URA medium for heterologous expression of *B. bruxellensis* vinylphenol reductase gene, supplemented with 20 g/L glucose and 20 g/L galactose and 10 mg/L 4-vinylphenol performed at 28°C at 160 rpm. Fermentations were performed with ZIM 2512 positive control (A) and *S. cerevisiae* T334 transformed with negative control plasmid pYES2.1_neg._control (B)

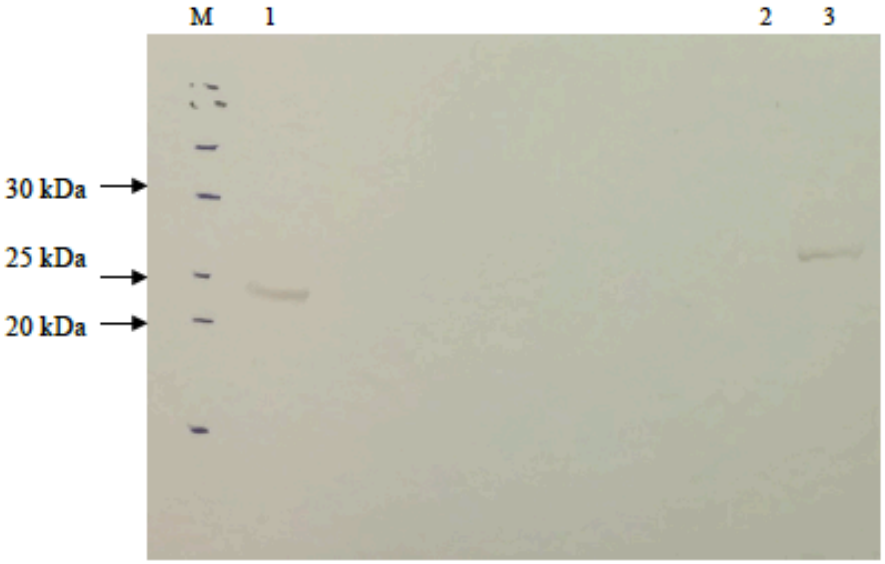


ANNEX M

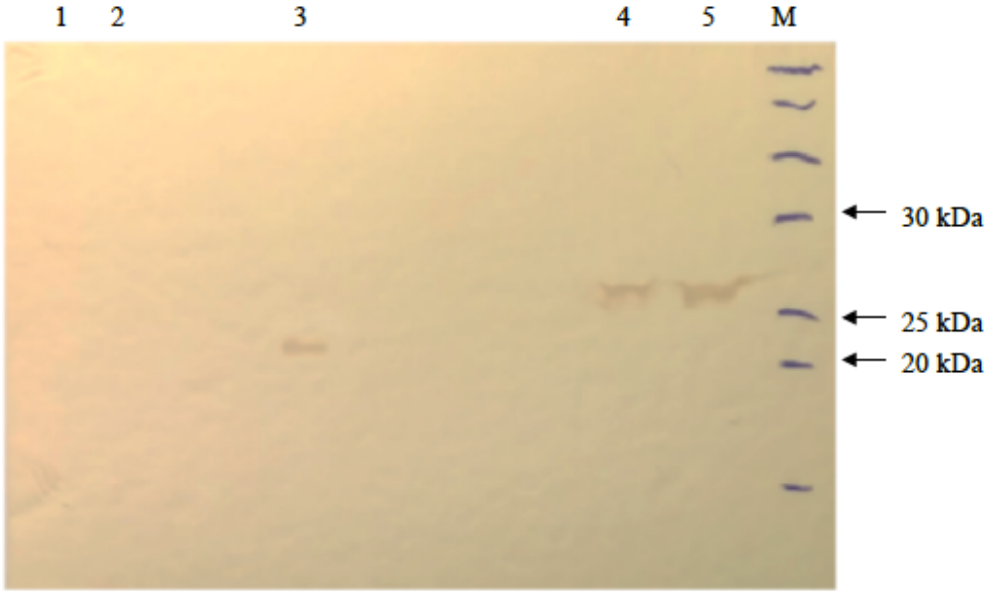
Sample pictures of nitrocellulose membrane with heterologously expressed proteins hybridized with anti-V5-HRP antibodies and visualized with chromogenic substrate



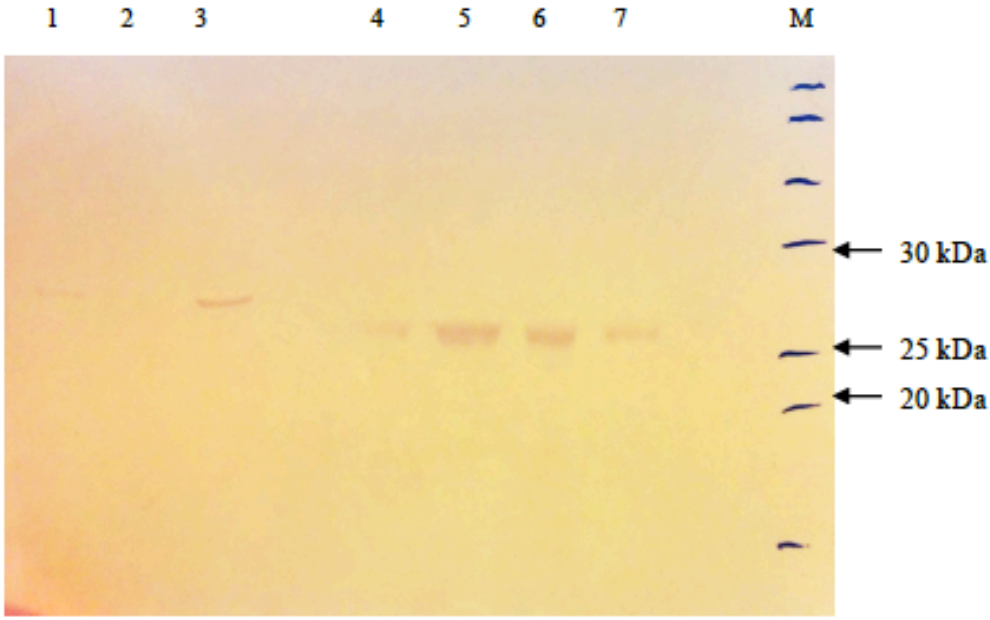
Annex M1: Picture of nitrocellulose membrane with heterologously expressed proteins, hybridized vith anti-V5-HRP antibodies and visualized with chromogenic substrate (M – size marker; 1, 2 – ORF1 of vinylphenol reductase of *B. bruxellenis*; 3 – ORF2 of vinylphenol reductase of *B. bruxellensis*; 4 – RAD59 positive control)



Annex M2: Picture of nitrocellulose membrane with heterologously expressed proteins, hybridized vith anti-V5-HRP antibodies and visualized with chromogenic substrate (M – size marker; 1 – ORF3 of vinylphenol reductase of *B. bruxellensis*; 3 – negative control; 3 – RAD59 positive control)



Annex M3: Picture of nitrocellulose membrane with heterologously expressed proteins, hybridized vith anti-V5-HRP antibodies and visualized with chromogenic substrate (1 – RAD59 positive control; 2 – negative control; 3 – Cu/Zn superoxide dismutase of *B. bruxellensis*; 4, 5 – triosephosphate isomerase of *B. bruxellensis*; M – size marker)



Annex M4: Picture of nitrocellulose membrane with heterologously expressed proteins, hybridized vith anti-V5-HRP antibodies and visualized with chromogenic substrate (1 – RAD59 positive control; 2 – negative control; 3 – cytochrome p450 of *B. bruxellensis*; 4-7 – ORF1 of vinylphenol reductase of *B. bruxellensis*; M – size marker)