TRANSFORMANT SYSTEM AND GENE EXPRESSION OF YEAST *SCHWANNIOMYCES OCCIDENTALIS*

by

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Amitabha

ABSTRACT

Schwanniomyces occidentalis (*Debaryomyces occidentalis*) is able to grow rapidly with high cell mass on cheap starch as a carbon source, produce strong amylolytic enzymes extracellularly and secrete large proteins without hyper-glycosylation and measurable extracellular proteases. *Schw. occidentalis* thus has a high potential as a useful alternative to *Saccharomyces cerevisiae* in the production of heterologous proteins. However, the molecular study of *Schw. occidentalis* has been very limited due to the insufficient transformation systems and lack of gene expression information.

A new transformation system of *Schw. occidentalis* has been developed. This system was based on vector YEp13 (*LEU2*) and a stable *leu* auxotrophic mutant, *Schw. occidentalis* DW88, obtained by treating the yeast with 1-methyl-3-nitro-1-nitrosoguanidine. The transformation efficiency of YEp13 by spheroplast-mediating method was 10^3 transformants/μg DNA. The 2-μm replicon is proposed to be responsible for YEp13 replication in *Schw. occidentalis*. The YEp13 stability in *Schw. occidentalis* was low, but it kept its structure in the yeast, suggesting that *Schw. occidentalis* DW88 does not modify foreign DNA.

After analysis of 14 cloned *Schw. occidentalis* genes and comparison of associated genes from both *Schw. occidentalis* and *S. cerevisiae*, 25 codons were arbitrarily chosen as putative preferred codons for *Schw. occidentalis*. They are similar to those of *S. cerevisiae*, except for TTA for leucine, and AAA for lysine. Codon Bias Index (CBI), a criterion to evaluate gene expression, is calculated from preferred codons. A computer program (PCBI) which reads a gene containing introns was developed to quickly calculate CBI.

ADH1 promoter of *S. cerevisiae* with a CBI 0f 0.77 (high expression level) in *Schw. occidentalis* was used to express a β-galactosidase gene of *Streptococcus thermophilus* in *Schw. occidentalis* DW88. The β-galactosidases produced by *Schw. occidentalis* and the wild strain showed similar thermostability, indicating that *Schw. occidentalis* DW88 does not change the property of heterologous proteins. The initial

expression level of *Schw. occidentalis* DW88 measured by specific activity was 3-fold as compared with that of *Escherichia coli* expressing the same gene.

Because the *ADH*1 promoter-containing vector, pVT101-L (*LEU*2-d) could not complement *Schw. occidentalis* DW88, a new expression vector, YEpE81 (6,323 bp), was constructed by substituting *S. cerevisiae LEU*2 gene for the *LEU*2-d gene carried by pVT101-L. YEpE81 similar to pVT101-L has a cassette containing the *S. cerevisiae ADH*1 promoter, multiple cloning sites, and the 3'. sequence of *ADH*1 gene. This expression vector successfully expressed the *S. thermophilus GAL* gene in *Schw. occidentalis* DW88.

*Saccharomyces diastaticus STA*3 gene encoding an extracellular glucoamylase was cloned into *Schw. occidentalis* DW88 and the transformant yielded more cell mass and glucoamylase activity than the transformant without the *STA*3 gene. The *STA*3 gene was controlled by its own promoter and the glucoamylase was directed by its own signal sequence. The pH and temperature optimum of the glucoamylases secreted by *Schw. occidentalis* and the wild type strain were similar. The major glucoamylase activity was detected from culture supernatant, but not from intracellular cell lysate, demonstrating that *Schw. occidentalis* DW88 has a strong secretion ability.

Schw. occidentalis DW88 should be a good host to produce and secrete heterologous proteins, and the putative preferred codons and program PCBI can facilitate molecular study of *Schw. occidentalis*.

RÉSUMÉ

Schwanniomyces occidentalis (*Debaryomyces occidentalis*) peut atteindre rapidement une masse cellulaire élevée en utilisant l'amidon comme source de carbone, relâche des enzymes amylolytiques puissants dans le milieu extracellulaire et sécrète de grosses protéines sans hyper-glycosylation ni activité protéasique extracellulaire détectable. *Schw. occidentalis* démontre un potentiel élevé comme alternative à l'emploi de *Saccharomyces cerevisiae* dans la production de protéines hétérologues. Toutefois, l'étude moléculaire de *Schw. occidentalis* a jusqu' à présent été limitée par le manque de systemes de transformation adéquats et par le peu de connaissances sur son expression génétique.

Un nouveau système de transformation pour *Schw. occidentalis* a été développé. Ce système est basé sur le vecteur YEp13 (*LEU2*) et un mutant *leu* auxotrophe stable, *Schw. occidentalis* DW88, obtenu par traitement de la levure avec la 1-methyl-3-nitro-1-nitrosoguanidine. L'efficacité transformationelle de YEp13 vérifiée par la méthode dite de sphéroplastes a été de 10^3 transformants/µg d'ADN. Nous proposons le réplicon de 2-µm comme étant responsable de la réplication de YEp13 chez *Schw. occidentalis*. La stabilité de YEp13 chez *Schw. occidentalis* était faible, mais l'on a observé le maintien de la structure chez cette levure, indiquant que *Schw. occidentalis* n'apporte pas de modifications à l'ADN étranger.

Suite à l'analyse de 14 gènes clonés de *Schw. occidentalis*, et la comparaison entre les gènes associés à la fois chez *Schw. occidentalis* et *S. cerevisiae*, une série de 25 codons ont été choisis arbitrairement comme codons préférentiels pour *Schw. occidentalis*. Ceux-ci sont quasi-identiques à ceux de *S. cerevisiae*, à l'exception de TTA pour la leucine, et AAA pour la lysine. Le "Codon Bias Index" (CBI), un critère utilisé lors de l'évaluation de l'expression génique, est établi par calcul des codons préférentiels. Cette tâche manuelle requiert malheureusement beaucoup de temps et d'effort. Dès lors, un logiciel informatique (PCBI) a été conçu afin d'effectuer ces calculs CBI de façon très efficace.

Le promoteur *ADH*1 de *S. cerevisiae* possédant un CBI de 0.77 (niveau d'expression élevé) chez *Schw. occidentalis* a été utilisé pour l'expression d'un gène β-galactosidase (*GAL*) de *Streptococcus thermophilus* chez *Schw. occidentalis* DW88. Les β-galactosidases produites par *Schw. occidentalis* et la souche originale de *Streptococcus thermophilus* ont montré une stabilité à la chaleur semblable, indiquant que *Schw. occidentalis* DW88 ne modifie pas les propriétes des protéines hétérologues. Le niveau d'expression initial de *Schw. occidentalis*, tel que mesuré par l'activité spécifique de l'enzyme, était environ 3 fois plus élevé que la même proteine exprimée chez *E. coli*.

Parce que le vecteur pVT101-L (*LEU*2-d) contenant le promoteur *ADH*1 ne pouvait pas complémenter *Schw. occidentalis*, un nouveau vecteur d'expression, désigné YEpE81 (6,323 pb), a été construit par substitution du gène *LEU*2-d de pVT101-L par le gène *LEU*2 de *S. cerevisiae* . Tout comme pVT101-L, YEpE81 possède une cassette contenant le promoteur *ADH*1 de *S. cerevisiae*, un site de clonage multiple, et la séquence 3' du gène *ADH*1. Ce vecteur a permis de réaliser avec succès l'expression du gène *GAL* de *S. thermophilus* chez *Schw. occidentalis*.

Le gène *STA*3 de *Saccharomyces diastaticus*, codant pour une glucoamylase extracellulaire, a été cloné dans *Schw. occidentalis* DW88, et les transformants ont montré une masse cellulaire et une activité glucoamylase plus élevée que la souche DW88 sans le gène *STA*3. Le gène *STA*3 était controlé par son propre promoteur et contenait aussi son propre signal de sécrétion extracellulaire. La glucoamylase recombinante de *S. diastaticus* sécrétée par *Schw. occidentalis* DW88 a montré un optimum de pH et de température semblable à ceux de la glucoamylase naturelle. La fraction principale d'activité glucoamylase a été détectée dans le milieu de culture liquide, très peu d'activité étant retrouvée dans les lysats cellulaires, ce qui démontre la forte capacité de sécrétion extracellulaire de *Schw. occidentalis* DW88.

Ces résultats démontrent que *Schw. occidentalis* semble être un bon candidat pour la production et la sécrétion de protéines hétérologues, et les codons préférentiels

probables, de même que le logiciel PCBI peuvent faciliter l'étude moléculaire de *Schw. occidentalis*.

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I would like to express my sincere thanks to people who helped me in this research in addition those mentioned above. Thus, I hope that I can contribute this accomplishment to all people.

FOREWORD

This thesis is submitted in the form of original papers suitable for journal publication. The first chapter is a general introduction and literature review presenting the theory and background information. The next five chapters represent the body of the thesis, each being a complete manuscript. The last chapter is a summary of the major conclusions. This thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions outlined in the Guidelines concerning Thesis Presentation, section 7 "Manuscripts & Authorship" which are as follows:

"The candidate has the option, subject to the approval of the Department, including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case, the thesis must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional material (experimental and design data as well as descriptions of equipment) must be provided in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported. The abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstracts, introductions and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted." While the inclusion of manuscripts co-authored by the candidate and others is not prohibited by McGill, the candidate is warned to make an explicit statement on who contributed to such work and to what extent, and supervisors and others will have to bear witness to the accuracy of such claims before the oral committee. It should also be noted that the task of the External Examiner is made more difficult in such cases, and it is in the candidate's interest to make authorship responsibility perfectly clear.

Although all the work reported here is the responsibility of the candidate, the project was supervised by Dr. Byong H. Lee, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATIONS

Dr. B. H. Lee, my supervisor, is co-author on all publications presented in this thesis and contributed in supervisory role. Dr. Lee fully reviewed the manuscripts. This research was supported by Gene Asia Biotech Company, Taiwan.

Professor C. F. Lee was consulted for all yeast taxonomy. He is a co-author on two publications.

Mr. W. C. Cheng carried out coding a computer program. He is a co-author on one publication.

Mr. N. Robert provided assistance in β-galactosidase gene cloning. He is a co-author on one publication.

Mr. Y. J. Choi provided assistance in β-galactosidase preparation and assay as well as glucoamylase assay. He is a co-author on two publications.

A- Part of this work has been published as follows:

Wang, T. T., and Lee, B. H. 1997. Transformation system for *Schwanniomyces occidentalis*. *Biotech. Tech.* 11: 307-309.

Wang, T. T., and Lee, B. H. 1999. Construction of an expression vector for *Schwanniomyces occidentalis*. *Appl. Microbiol. Biotech.* (Submitted)

Wang, T. T., Cheng, W. C. and Lee, B. H. 1998. A simple program to calculate codon bias index. *Mol. Biotechnol.* 10: 103-106.

Wang, T. T., Choi, Y. J., and Lee, B. H. 1999. Secretion of a *Saccharomyces diastaticus* glucoamylase by *Schwanniomyces occidentalis*. *Appl. Microbiol. Biotech.* (Submitted)

Wang, T. T., Choi, Y. J., Robert, N., and Lee, B. H. 1999. Expression of a *Streptococcus thermophilus* β-galactosidase gene in *Schwanniomyces occidentalis*. *Appl. Microbiol. Biotech.* (Submitted)

Wang, T. T., Lee, C. F., and Lee, B. H. 1999. The molecular biology of *Schwanniomyces occidentalis* Klocker. *Crit. Rev. Biotechnol.* (In press)

Wang, T. T., Lee, C. F., and Lee, B. H. 1999. Transformation systems of non-*Saccharomyces* yeast. *Crit. Rev. Biotechnol.* (In press)

B- Part of this work has been presented at Scientific Conferences:

Wang, T. T., and Lee, B. H. 1997. A transformation system for *Schwanniomyces occidentalis*. Institute of Food technologists (IFT) 54th. Annual Meeting and Food Expo, Orlando, Florida, USA. (**This work has been awarded the third place in the graduate students' paper competition, Food Biotechnology Division of the Institute of Food Technologists**).

Wang, T. T., Cheng, W. C., and Lee, B. H. 1998. A simple program to calculate codon bias index. General Meeting of American Society for Microbiology. Atlanta, Georgia, USA.

Permission to use part of this material has been obtained from the co-authors of the manuscripts listed above.

PREFACE

Claim of Original Research

1. *Schwanniomyces occidentalis* DW88 is the first putative *leu*2 auxotrophic mutant of the yeast constructed.

2. It is the first transformation system utilizing *Saccharomyces cerevisiae LEU*2 gene as genetic marker for the transformation of *Schw. occidentalis*. The *LEU*2 gene was widely used in yeast transformation, and various kinds of vectors were based on the *LEU*2 gene. Thus these developed *LEU*2-based vectors can be applied in *Schw. occidentalis* DW88.

3. It is the first investigation that *S. cerevisiae LEU*2-d gene, which was used to construct high-copy-number vectors for *S. cerevisiae*, does not function in *Schw. occidentalis*.

4. It is the first investigation to propose the putative preferred codons for *Schw. occidentalis* that facilitates evaluation of gene expression in the yeast.

5. It is the first study to calculate the codon bias index for each *Schw. occidentalis* cloned gene and heterologous genes expressing in the yeast. These results can assist in understanding gene expression in *Schw. occidentalis*.

6. PCBI is the first program reported to autonomously calculate codon bias index and saves substantial analytical time.

7. It is the first study to indicate that the *S. cerevisiae ADH*1 promoter does function in *Schw. occidentalis*.

8. YEpE81 is the first expression vector specifically constructed for *Schw. occidentalis*.

9. It is the first study to show that a heterologous β-galactosidase gene (*S. thermophilus*) can be expressed in *Schw. occidentalis*.

10. *Streptococcus thermophilus* β-galactosidase gene is the first one from food grade bacteria expressed in *Schw. occidentalis*.

11. It is the first study to show expression of a gene (*S. cerevisiae STA1*) coding for a heterologous amylolytic enzyme in *Schw. occidentalis*.

12. It is the first study to show that a heterologous signal sequence functions in *Schw. occidentalis*.

GENERAL INTRODUCTION

Yeasts have been employed as systems for academic and industrial interests to perform molecular study and heterologous protein production. These microorganisms possess a number of important advantages and characteristics:

1) simple eucaryotes that have similar biological metabolisms such as mRNA splicing mechanism, protein maturation, etc. to higher eucaryotes,
2) rapid growth in simple medium to high cell density by current fermentation techniques,
3) more advanced genetics and biochemistry than any other eucaryotes,
4) well developed and easily manipulated recombinant DNA techniques,
5) extracellular proteins which facilitates protein purification and reduces toxic shock to the yeast hosts,
6) easier mutation analysis than other higher eucaryotes, and
7) non-toxic products.

Although the major study of yeast genetics was concentrated on *Saccharomyces cerevisiae*, the yeast has certain limitations, rendering protein productions difficult due to:

1) low product yields, even with a strong promoter (Buckholz and Gleeson, 1991),
2) hyper-glycosylated proteins which might affect the protein characteristics (Lemontt et al., 1985; Moir and Dumais, 1987; Van Arsdell et al., 1987) and
3) low efficient protein secretion when the protein is larger than 30 kDa (Smith et al., 1985; Jigami et al., 1986; Moir and Dumais, 1987; De Nobel and Barnett, 1991).

Thus, other yeasts have been studied to replace *S. cerevisiae* as hosts to produce heterologous proteins. During the last 10 years, the major studies of non-*Saccharomyces* yeasts in biotechnology have been focused on *Candida* spp., *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Schizosaccharomyces pombe* (Reiser et al., 1990; Buckholz and Gleeson, 1991). Based on the knowledge and molecular techniques of *S. cerevisiae*, progress toward the molecular study of non-*Saccharomyces* yeasts has

been significant.

Yeast *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*) was found in 1909 (Klocker, 1909), and was described as a "super yeast" (Ingledew, 1987). The yeast is able to use cheap starch as a carbon source to grow with rapid growth rate and high cell mass (Moresi et al., 1983; Jamuna and Ramakrishna, 1989; Horn et al., 1992a,b), produce strong amylolytic enzymes extracellularly and secrete large proteins (Ingledew, 1987) without hyper-glycosylation and measurable extracellular proteases (Deibel et al., 1988). This yeast was also suggested as a useful alternative to *S. cerevisiae* in the production of heterologous proteins. However, the molecular study of *Schw. occidentalis* is in a nascent stage, as it has few transformation systems and lacks gene expression information.

In order to take advantage of the characteristics of *Schw. occidentalis* to produce and secrete heterologous proteins, and study the heterologous gene expression in *Schw. occidentalis*, this research was conducted to establish a new host system to express heterologous genes, to produce and secrete heterologous proteins without any modification, and to investigate codon usage of the yeast. Thus, the objectives of this research are:

1) to construct an auxotrophic mutant of *Schw. occidentalis*;
2) to develop a transformation system of *Schw. occidentalis* based on the mutant;
3) to study the codon usage of *Schw. occidentalis* and propose putative preferred codons for the yeast;
4) to develop a program for autonomously calculating codon bias index;
5) to find a strong promoter and develop an expression vector for *Schw. occidentalis*;
6) to study heterologous gene expression in *Schw. occidentalis*; and
7) to study heterologous protein secretion in *Schw. occidentalis*.

CHAPTER 1.0

LITERATURE REVIEW

1.1 TRANSFORMATION SYSTEMS OF NON-*SACCHAROMYCES* YEASTS

This chapter was summarized as the publication entitled "Transformation systems of non-*Saccharomyces* yeasts" written by Tsung-Tsan Wang and supervised by Dr. Byong H. Lee, who acted in an editorial capacity, evaluated the manuscript prior to submission to the journal. This paper was submitted to *Critical Review in Biotechnology*. This chapter serves as a part of introduction to the thesis, showing the background for the research that was done and the reasons why this project was important.

1.1.1 ABSTRACT

This review describes the transformation systems including vectors, replicons, genetic markers, transformation methods, vector stability and copy number of 13 genera and 31 species of non-*Saccharomyces* yeasts. The first non-*Saccharomyces* yeast transformation reported is for *Schizosaccharomyces pombe*, which is also found to be the most genetically studied strain among yeasts. The replicons of non-*Saccharomyces* yeast vectors are from native plasmids, chromosomal DNA and mitochondrial DNA of *Saccharomyces cerevisiae*, non-*Saccharomyces* yeasts, protozoan, plant, and animal. Vectors such as YAC, YCp, YEp, YIp, and YRp were developed for non-*Saccharomyces* yeasts. Forty-two kinds of genes from bacteria, yeasts, fungi, and plant were used as genetic markers, which could be classified into biosynthetic, dominant, and colored groups, to construct non-*Saccharomyces* yeasts vectors. The *LEU2* gene and G418 resistance gene are the two most popular markers used in the yeast transformation. So far, five methods such as spheroplast-mediating method, alkaline ion treatment method, electroporation, trans-kingdom conjugation, and biolistics have been successfully developed for non-*Saccharomyces* yeast transformation. The first three are most widely used. The highest

copy number detected from non-*Saccharomyces* yeasts is 60 copies in *Kluyveromyces lactis*. No general rule is known to illustrate the transformation efficiency, vector stability and copy number in non-*Saccharomyces* yeasts, though factors such as vector composition, host strain, transformation method, and selective pressure might influence them.

1.1.2 INTRODUCTION

Yeasts have been employed as systems for many basic questions in eucaryotic biology including gene expression and heterologous protein secretion for academic and industrial interests. Though the major study of yeast genetics has been concentrated on *Saccharomyces cerevisiae*, this yeast produces heterologous proteins with certain disadvantages:

1) low product yields, even with a strong promoter (Buckholz and Gleeson, 1991),
2) hyperglycosylated proteins which might affect the protein characteristics (Lemontt et al., 1985; Moir and Dumais, 1987; Van Arsdell et al., 1987), and
3) intracellular or periplasmic gene products when protein is larger than 30 KDa (Smith et al., 1985; Jigami et al., 1986; Moir and Dumais, 1987; De Nobel and Barnett, 1991).

In addition, as compared with other yeasts, *S. cerevisiae* is unlikely to have certain metabolic activities for alkanes, lactose, methanol, and xylose (Duvnjak et al., 1970; Sahm and Wagner, 1972; Mahoney et al., 1975; Toivola et al., 1984), productions of amino acids, amylolytic enzymes, citric acid, pigment (Abbott and Gledhill, 1971; Wilson and Ingledew, 1982), and wastewater treatment (Sato et al., 1987). Thus, the molecular study has been extended into yeasts other than *S. cerevisiae*. In other words, the genetic engineering of non-*Saccharomyces* yeasts for academic research and industrial applications becomes important. The achievements in gene cloning, gene disruption, gene expression, gene regulation and protein production in non-*Saccharomyces* yeasts have been significant in recent years. A reliable transformation system is the first requirement for these accomplishments on these yeasts. Although some literatures described non-*Saccharomyces* yeast transformation systems (Weber and Barth,

1988; Klein and Zaworski, 1990; Reiser et al., 1990), no recent progress has been reviewed and thus an attempt was made to cover up-to-date information on the transformation systems of all non-*Saccharomyces* yeasts. The nomenclature of yeasts cited in this review was based on the current taxonomy (Kurtzman and Fell, 1998). Due to up-date research, some yeasts have been re-classified. For instance, *Candida pseudotropicalis* was consolidated into *Kluyveromyces marxianus*, *Hansenula fabianii* into *Pichia fabianii*, *Hansenula polymorpha* into *Pichia angusta*, *Kluyveromyces fragilis* into *Kluyveromyces marxianus*, *Schwanniomyces occidentalis* into *Debaryomyces occidentalis* and *Yamadazyma ohmeri* into *Pichia ohmeri*.

1.1.3 TRANSFORMATION FOR NON-*SACCHAROMYCES* YEASTS

The transformation system of *S. cerevisiae* was first reported in 1978 by using a spheroplast-mediating method (Hinnen et al., 1978; Beggs, 1978). Different methods such as alkaline ion treatment to make competent cells (Ito et al., 1983), electroporation (Hashimoto et al., 1985; Karube et al., 1985), trans-kingdom conjugation (Heinemann and Sprague, 1989), biolistics (Armaleo et al., 1990), and agitation with glass beads (Costanzo and Fax, 1988) were then developed for *S. cerevisiae* transformation. From 1981, several transformation systems based on the above methodologies have been reported for at least 13 genera and 31 species of yeasts other than *Saccharomyces*.

These non-*Saccharomyces* yeasts in Table 1.1.1 include *Candida albicans* (Kurtz et al., 1986), *Candida boidinii* (Sakai et al., 1991), *Candida glabrata* (Mehra et al., 1992), *Candida maltosa* (Kunze et al., 1985), *Candida tropicalis* (Haas et al., 1990), *Candida utilis* (Kondo et al., 1995), *Cryptococcus neoformans* (Edman and Kwon-Chung, 1990), *Deb. occidentalis* (Klein and Favreau, 1988), *Kluyveromyces aestuarii* (Chen et al., 1989), *Kluyveromyces lactis* (Das and Hollenberg, 1982), *K. marxianus* (Das et al., 1984), *Kluyveromyces polysporus* (Chen et al., 1989), *Kluyveromyces thermotolerans* (Chen et al., 1989), *Kluyveromyces waltii* (Chen et al., 1989), *Kluyveromyces wickerhamii* (Chen et al., 1989), *Pachysolen tannophilus* (Wedlock and Thornton, 1989), *Phaffia rhodozyma*

(Adrio and Veiga, 1995), *P. angusta* (Gleeson et al., 1986; Roggenkamp et al., 1986; Tikhomirova et al., 1986), *P. fabianii* (Kato et al., 1997), *Pichia guilliermondii* (Kunze et al., 1985), *Pichia methanolica* (Hiep et al., 1993), *P. ohmeri* (Piredda and Gaillardin, 1994), *Pichia pastoris* (Cregg et al., 1985), *Pichia stipitis* (Ho et al., 1991), *Rhodosporidium toruloides* (Tully and Gilbert, 1985), *Schizosaccharomyces pombe* (Beach and Nurse, 1981), *Torulaspora delbrueckii* (Compagno et al., 1989), *Torulaspora pretoriensis* (Oda and Tonomura, 1995), *Trichosporon cutaneum* (Glumoff et al., 1989), *Yarrowia lipolytica* (Davidow et al., 1985; Gaillardin et al., 1985), and *Zygosaccharomyces rouxii* (Sugihara et al., 1986). As shown in Table 1.1.2, 20 transformation systems were developed in 1980s, and 11 transformation systems after 1990. *Schiz. pombe* is the most studied species. Table 1.1.3 indicates variable application of non-*Saccharomyces* yeasts for academic study, fuel and food industry, or wastewater treatment. In particular, *K. lactis*, *P. angusta*, *P. pastoris*, *Schiz. pombe*, and *Y. lipolytica* were used in the production of heterologous proteins (Reiser et al., 1990).

1.1.4 VECTORS

While some vectors used in transformation of non-*Saccharomyces* yeasts were originated from *S. cerevisiae*, the others were specifically designed for each species. Since vector amplification and DNA purification in *Escherichia coli* is much easier than those in yeasts, yeast vectors were developed as yeast/*E. coli* shuttle vectors, which can replicate in both yeast and *E. coli* host cells. These vectors contain a replicon (replication origin) and a genetic marker functional in yeast and *E. coli* host cells, respectively. Thus, these vectors can shuttle back and forth between these two organisms. One exception is YIp (yeast integrating plasmid) type vectors, which contain no functional yeast replicon but integrate into yeast host chromosome.

1.1.4.1 Replicons

The sources of replicons responsible for vector replication in non-*Saccharomyces* yeasts included 1) yeast native plasmids, and 2) autonomous replication sequences (*ARS*) from either chromosome or mitochondria (Table 1.1.1). Vectors with the former are called YEp (yeast episomal plasmid), and with the latter YRp (yeast replicating plasmid).

The yeast replicons of YEp were originated from 1) 2-μm plasmid of *S. cerevisiae* (Table 1.1.4), and 2) native plasmids of non-*Saccharomyces* yeasts (Table 1.1.5). As shown in Table 1.1.4, the 2-μm plasmid replicon functioned in 10 non-*Saccharomyces* yeasts such as *Deb. occidentalis* (Klein and Favreau, 1988), *K. lactis* (Das and Hollenberg, 1982), *P. angusta* (Gleeson et al., 1986), *P. pastoris* (Cregg et al., 1985), *P. stipitis* (Ho et al., 1991), *Pa. tannophilus* (Hayman and Bolen, 1993), *Ph. rhodozyma* (Adrio and Veiga, 1995), *Schiz. pombe* (Beach and Nurse, 1981), *T'spora. delbrueckii* (Compagno et al., 1989), and *T'spora. pretoriensis* (Oda and Tonomura, 1995), but not well in *C. albicans* (Kurtz et al., 1986), *C. glabrata* (Mehra et al., 1992), and *Rhodosp. toruloides* (Tully and Gilbert, 1985). Native plasmid pGKL1 (k1), pGKL2 (k2), and pKD1 were discovered from *K. lactis* (Gunge et al., 1981; Chen et al., 1986; Falcone et al., 1986), pKW1 from *K. waltii* (Chen et al., 1992b), pSRY1 from *T'spora delbrueckii* (Nakata and Okamura, 1996), pSB1, pSB2 and pSR1 from *Zygosaccharomyces bailii* (Toh-e et al., 1984; Araki et al., 1985; Utatsu et al., 1987), pSB3 and pSB4 from *Zygosaccharomyces bisporus* (Toh-e et al., 1984; Toh-e and Utatsu, 1985), and pSM1 from *Zygosaccharomyces fermentati* (Utatsu et al., 1987). Nonetheless, only the replicons of pGKL1 (De Louvencourt et al., 1983), pKD1 (Bianchi et al., 1987; Chen et al., 1989; Bergkamp et al., 1993), pKW1 (Chen et al., 1992b), pSR1 (Araki and Oshima, 1989) and pSRY1 (Nakata and Okamura, 1996) were used to construct yeast vectors (Table 1.1.5). These replicons not only functioned in homologous host cells, but also in heterologous host cells except pSRY1. The replicon of pGKL1 functioned in *K. lactis* (De Louvencourt et al., 1983), and *K. marxianus* (Sugisaki et al., 1985), pGKL2 in *K. marxianus* (Sugisaki et al., 1985), pKD1 in *K. aestuarii* (Chen et al., 1989), *K. lactis* (Bianchi et al., 1987), *K. marxianus* (Bergkamp et al., 1993), *K. polysporus* (Chen et al.,

1989), *K. thermotolerans* (Chen et al., 1989), *K. waltii* (Chen et al., 1989), *K. wickerhamii* (Chen et al., 1989). pKW1 functioned in *K. thermotolerans* and *K. waltii*, but not in some *Kluyveromyces* species (Chen et al., 1992b), and pSR1 expressed in *Zygosacch. rouxii* (Araki and Oshima, 1989). The replicons of pGKL1, pGKL2, pKD1, pKW1, and pSR1 also expressed in *S. cerevisiae* (Gunge and Sakaguchi, 1981; Araki et al., 1985; Fujimura et al., 1987; Araki and Oshima, 1989; Chen et al., 1989; 1992b).

In eucaryotes, few hundred replicons (*ARS* - autonomous replication sequence) are distributed throughout chromosomal and mitochondrial DNA. *ARS*s could function in vectors as DNA replication origins (Newlon, 1988; Williamson, 1985). The *ARS* sources used in non-*Saccharomyces* yeast vectors included yeasts (Tables 1.1.4 and 1.1.5), protozoan (*Tetrahymena*), plant (*Papaver somniferum*) and mouse (Roth et al., 1983). The non-*Saccharomyces* replicons were normally located within a 3-kilobase (kb) DNA fragment (Table 1.1.6). Table 1.1.4 indicates that *S. cerevisiae ARS*-based vectors functioned in 7 non-*Saccharomyces* yeasts: *C. glabrata* (Zhou et al., 1994), *C. maltosa* (Kunze et al., 1987), *Deb. occidentalis* (Dohmen et al., 1989), *Pa. tannophilus* (Wedlock and Thornton, 1989), *Ph. rhodozyma* (Faber et al., 1994), *T'spora. delbrueckii* (Compagno et al., 1989), and *T'spora. pretoriensis* (Oda and Tonomura, 1995). As shown in Table 1.1.5, non-*Saccharomyces* yeast *ARS* were from 15 species: *C. albicans* (Cannon et al., 1992), *C. boidinii* (Sakai et al., 1993), *C. glabrata* (Mehra et al., 1992), *C. maltosa* (Takagi et al., 1986), *C. tropicalis* (Sanglard and Fiechter, 1992), *C. utilis* (Tikhomirova et al., 1986), *Deb. occidentalis* (Dohmen et al., 1989), *K. lactis* (Das and Hollenberg, 1982), *P. angusta* (Roggenkamp et al., 1986; Tikhomirova et al., 1986), *P. ohmeri* (Piredda and Gaillardin, 1994), *P. pastoris* (Cregg et al., 1987; Sreekrishna et al., 1987), *P. stipitis* (Yang et al., 1994), *Schiz. pombe* (Sakaguchi and Yamamoto, 1982), *Y. lipolytica* (Meilhoc et al., 1990), and *Zygosacch. bailii* (Sugihara et al., 1986). These *ARS* functioned not only in homologous host cells but also in heterologous host cells. The *ARS*s from *C. utilis*, *K. lactis*, and *Zygosacch. bailii* could express in *P. angusta*, *K. marxianus*, and *Zygosacch. rouxii*, respectively (Das et al., 1984; Sugihara et al., 1986; Tikhomirova et al., 1986). Furthermore, the *ARS*s from *C. boidinii* (Sakai et al., 1993) and *K. lactis* (Sreekrishna et al., 1984) functioned in *S. cerevisiae*, but not all yeast *ARS*s

allowed vectors to replicate in all non-*Saccharomyces* yeasts. The *ARS* from *S. cerevisiae* did not express in *C. boidinii* (Sakai et al., 1991), *K. lactis* (Das and Hollenberg, 1982), and a *Zygosacch. bailii ARS* expressing in *S. cerevisiae* did not function in *Zygosacch. rouxii*. Another *Zygosacch. bailii ARS* expressed in *Zygosacch. rouxii* and *S. cerevisiae*, but not effectively in *Zygosacch. bailii* itself (Sugihara et al., 1986). As summarized from the above, five non-*Saccharomyces* yeasts, *Deb. occidentalis*, *Pa. tannophilus*, *Ph. rhodozyma*, *T'spora delbrueckii*, and *T'spora pretoriensis* allowed both 2-μm replicon and *S. cerevisiae ARS* to function. However, *K. lactis* permitted the replication from 2-μm, but not from *S. cerevisiae ARS*. Besides yeasts, the *ARS*s of plant and protozoan expressed in *K. lactis* (Farkasovska, 1993) and *Schiz. pombe* (Luehrsen et al., 1988), and a mouse *ARS* functioned in *Schiz. pombe* (Roth et al., 1983). These results suggest that 1) diverse *ARS*s in non-*Saccharomyces* yeasts have complex function, and 2) DNA replication mechanism appears to be similar among some eucaryotes, but expression of *ARS* is more stringent than 2-μm. Thus, selecting an *ARS* to construct vectors for non-*Saccharomyces* yeasts seems empirical.

YCp (yeast centromeric plasmid) containing a yeast centromere improves vector stability, but reduces vector copy number to about one per cell in *S. cerevisiae* (Dani and Zakian, 1983). In non-*Saccharomyces* yeasts, *S. cerevisiae CEN*6 and *CEN*4 were applied in *C. glabrata* (Zhou et al., 1994), *C. maltosa* (Kunze et al., 1987), *K. lactis* (Chen et al., 1996) and *T'spora. pretoriensis* (Oda and Tonomura, 1995), respectively. Moreover, a *C. glabrata CEN* (Kitada et al., 1996), *C. maltosa CEN*, *K. lactis CEN*2, and *Schiz. pombe CEN*3 were used in their own host cells (Murakami et al., 1995; Ohkuma et al., 1995; Zonneveld and van der Zanden, 1995; Chen et al., 1996; Nakazawa et al., 1997). Besides, yeast minichromosomes (also called as yeast artifical chromosome, YAC) of more than 270 kb, 36-110 kb and 500 kb were transferred into *Cr. neoformans* (Varma and Kwon-Chung, 1994) and *Schiz. pombe* (Allshire, 1990; Murakami et al., 1995), respectively.

Yeast vectors without functional yeast replicons are called YIp (yeast integrating plasmid), and integrate into yeast host chromosome due to the homologous recombination of target sequences existing at vectors and host chromosomal DNA.

Usually, genetic markers on vectors were used as the target sequences. Recently, in *C. utilis* (Kondo et al., 1995), *K. lactis* (Bergkamp et al., 1992; Rossolini et al., 1992), *Ph. rhodozyma* (Kim et al., 1998; Wery et al., 1997), and *Y. lipolytica* (Le Dall et al., 1994), ribosomal DNA (rDNA) was applied as target sequence to perform multiple integration of vectors into host chromosome.

Table 1.1.7 indicates that YAC, YCp, YEp, YIp and YRp vectors were applied for non-*Saccharomyces* yeast transformation, but not all of them were used for each non-*Saccharomyces* yeast. YIp vectors constructed by deleting the yeast replicons from yeast vectors were available for all non-*Saccharomyces* yeasts mentioned. YRp, YEp, YCp and YAC were utilized for 18, 17, 4 and 2 non-*Saccharomyces* yeasts, respectively. Among them, *Schiz. pombe* owned the most type of vectors for its transformation. Nonetheless, 5 non-*Saccharomyces* yeasts: *C. utilis*, *P. fabianii*, *P. guilliermondii*, *P. methanolica*, *Rhodosp. toruloides*, and *Tr. cutaneum* used YIp vectors only for transformation.

1.1.4.2 Genetic Markers

A genetic marker is one of the required elements on a vector for selection of transformants. As shown in Table 1.1.1, at least 42 kinds of genetic markers were used in non-*Saccharomyces* yeast transformation. They were from 1) *S. cerevisiae* (Table 1.1.8), 2) non-*Saccharomyces* yeasts (Table 1.1.9), and 3) organisms other than yeasts (Table 1.1.10). These genetic markers could be classified into three groups: 1) 30 biosynthetic genetic markers, 2) 11 dominant genetic markers, and 3) 1 color formation marker.

Table 1.1.8 showed that 14 biosynthetic genetic markers and 2 dominant genetic markers used for non-*Saccharomyces* yeast transformation were from *S. cerevisiae*. The biosynthetic ones including *ADE*1 (Zonneveld and van der Zanden, 1995), *ADE*2 (Hiep et al., 1993), *ARG*4 (Kunze et al., 1985), *ATP*2 (Boutry and Douglas, 1983), *CAN*1 (Ekwall and Ruusala, 1991), *CDC*9 (Barker and Johnston, 1983), *HIS*3 (Strasser et al., 1989), *HIS*4 (Cregg et al., 1985), *LEU*2 (Beach and Nurse, 1981), *LYS*2 (Gaillardin et al., 1985), *RAD*2 (McCready et al., 1989), *TRP*1 (Das and Hollenberg, 1982), *TRP*5 (Dohmen et al.,

1989), and *URA*3 (De Louvencourt et al., 1983). These genes complemented corresponding mutated alleles in at least 16 non-*Saccharomyces* yeasts (Table 1.1.8). The dominant genetic markers from *S. cerevisiae* were *SUC*2 and *HXK*2 genes, which were applied in *P. pastoris* (Sreekrishna et al., 1987), *Y. lipolytica* (Nicaud et al., 1989) and *Pa. tannophilus* (Wedlock and Thornton, 1989), respectively. *P. pastoris* and *Y. lipolytica* could not use sucrose as the sole carbon source to grow. The induction of the *SUC*2 gene rendered these two yeasts able to utilize sucrose for their growth. A glucose negative mutant of *Pa. tannophilus* survived in the presence of D-glucose or D-fructose by the introduction of a *S. cerevisiae HXK*2 gene. Among these *S. cerevisiae* genetic markers, the *LEU*2 gene was the most widely used for 13 non-*Saccharomyces* yeast transformation systems.

Up to now, at least 26 yeast genetic markers from 20 non-*Saccharomyces* yeasts were used for transformation of 21 non-*Saccharomyces* yeasts (Table 1.1.9). Among them, 21 were biosynthetic markers, 4 dominant markers and 1 color formation marker. The biosynthetic markers included *ADE*1 (Hiep et al., 1993), *ADE*2 (Kurtz et al., 1986; Klein and Favreau, 1988; Sanglard and Fiechter, 1992; Toffaletti et al., 1993; Hanic-Joyce and Joyce, 1998), *ARG*3 (*ARG*B) (Ochsner et al., 1991; Waddell and Jenkins, 1995), *ARG*5,6 (Negredo et al., 1997), *ARG*A (Reiser et al., 1994), *HIS*1 (Negredo et al., 1997), *HIS*3 (Burke and Gould, 1994; Bogdanova et al., 1995), *HIS*4 (Cregg et al., 1985), *HIS*5 (Hikiji et al., 1989), *HIS*7 (Apolinario et al., 1993), *IMH*3 (Kohler et al., 1997), *LEU*1 (Keeney and Boeke, 1994), *LEU*2 (Davidow et al., 1985; Piredda and Gaillardin, 1994; Bogdanova et al., 1995), *LYS*1 (Beach et al., 1982), *PAL* (Tully and Gilbert, 1985), *TRP*1 (Kitada et al., 1996), *TRP*3 (Bogdanova et al., 1995), *URA*1 (Sakaguchi and Yamamoto, 1982), *URA*3 (Haas et al., 1990; Gellissen et al., 1991; Sakai et al., 1991; Cannon et al., 1992; Piredda and Gaillardin, 1994; Zhou et al., 1994; Yang et al., 1994; Kato et al., 1997), *URA*4 (Grimm and Kohli, 1988; Grimm et al., 1988), and *URA*5 (Edman and Kwon-Chung, 1990). These genetic markers were from 17 non-*Saccharomyces* yeasts and applied in homologous host cell transformation by complementing corresponding auxotrophic mutants (Table 1.1.9). The 4 dominant markers were *GAL*1 (Gorman et al., 1991), *LAC*4 (Das and Hollenberg, 1982), cadmium

resistance gene (Coblenz and Wolf, 1994), and cycloheximide resistance gene (Kondo et al., 1995). They were from *C. albicans*, *C. utilis*, *K. lactis*,.*Ph. rhodozyma*, and *Schiz. pombe* and utilized in the transformation of *C. albicans*, *C. utilis*, *K. lactis*, *Ph. rhodozyma*, *Schiz. pombe*, and *T'spora. delbrueckii*. The *GAL*1 gene was used to rescue *gal*⁻ mutant of *C. albicans* on galactose (Gorman et al., 1991), while the *LAC*4 gene made *lac*4 mutant of *K. lactis* grow in lactose as the sole carbon source (Das and Hollenberg, 1982). The color formation marker was *ATX* from *Ph. rhodozyma* and used in homologous host cell transformation (Martinez et al., 1998). However, *ATX* marker provided *atx*3 and *atx*5 mutants red color development, but not a strong strategy to screen transformants. The *URA*3 gene was the most widely used genetic marker for 9 non-*Saccharomyces* yeast transformation among these genetic markers from non-*Saccharomyces* yeasts.

Furthermore, 7 kinds of genetic markers used in non-*Saccharomyces* yeast transformation were from organisms other than yeasts (Table 1.1.10). Two biosynthetic markers were *ARG*B gene from *Aspergillus nidulans* (Ochsner et al., 1991) and *PPI* gene from plant, *Arabidopsis thaliana* (Nitschke et al., 1992). Five dominant markers were aureobasidin resistance gene from *Aureobasidium pullulans* (Hashida-Okado et al., 1998), blasticidin S resistance gene from *Aspergillus terreus* (Kimura et al., 1994), G418 resistance gene from *E. coli* (Das and Hollenberg, 1982), hygromycin resistance gene from *E. coli* (Cordero and Gaillardin, 1996) and phleomycin-resistant gene (Gaillardin and Ribet, 1987) from *E. coli* as well as *Streptoalloteichus hindustanus* (Glumoff et al., 1989). These genetic markers were controlled by either their own promoters or eucaryotic promoters and used for transformation of 16 non-*Saccharomyces* yeasts. G418 resistance gene expressed in non-*Saccharomyces* yeasts by its own promoter except in *P. angusta* and *Schiz. pombe*. In these two yeasts, *S. cerevisiae ADH*1 promoter (Lang-Hinrichs et al., 1990; Janowicz et al., 1991; Merckelbach et al., 1993) or a cauliflower mosaic virus promoter (Gmunder and Kohli, 1989) controlled G418 resistant gene. The SV40 early promoter controlled the *Aspergillus blasticidin* S resistant gene functioning in *Schiz. pombe* (Kimura et al., 1994). The promoter and terminator of *Y. lipolytica XPR*2 gene expressed the hygromycin-resistance gene in *Y. lipolytica* (Cordero and Gaillardin, 1996).

The *Y. lipolytica LEU2* promoter controlled the expression of *E. coli* phleomycin-resistant gene in *Y. lipolytica* (Gaillardin and Ribet, 1987). Moreover, in *Tr. cutaneum*, the *E. coli* hygromycin resistance gene and *Streptoalloteichus hindustamus* phleomycin resistance gene were controlled by the *GPD* promoter and the *TRPC* terminator of *Aspergillus nidulans* (Glumoff et al., 1989). Among these dominant markers, G418 resistance gene was the most common for 13 non-*Saccharomyces* yeast transformation.

For using biosynthetic genetic markers, corresponding auxotrophic mutants were required and constructed by mutation that is time-consuming. On the other hand, it is relatively simple to utilize dominant genetic markers in transformation, but not all yeasts could be the hosts for dominant markers. *C. albicans* and *T'spora. delbrueckii* were naturally resistant to G418, and thus G418 resistant gene can not be used for these yeast transformation (Kurtz et al., 1986; Nakata and Okamura, 1996). Table 1.1.7 indicates that only 13 non-*Saccharomyces* yeasts used both biosynthetic and dominant markers for their transformation, and the other 18 non-*Saccharomyces* yeasts utilized only one kind of genetic marker for transformation. Among non-*Saccharomyces* yeasts, *Schiz. pombe* used the most genetic markers. Neither biosynthetic nor dominant markers were used for all non-*Saccharomyces* yeast. Biosynthetic and dominant markers functioned in 25 and 19 non-*Saccharomyces* yeasts, respectively, and color formation marker was used only in *Ph. rhodozyme*.

1.1.5 TRANSFORMATION METHODS

Transformation methods of *S. cerevisiae* contain spheroplast-mediating method, competent cell method, electroporation, conjugation, biolistics, and agitation by glass beads. Table 1.1.11 shows that the first 5 methods were successfully applied for non-*Saccharomyces* yeast transformation, but no publication found mentioned on the agitation by glass beads which was used in non-*Saccharomyces* yeast also. None of non-*Saccharomyces* yeasts possessed all these transformation methods, while no particular kind of the transformation methods was prevalent for all non-*Saccharomyces* yeasts.

Conjugation and biolistics methods were used for 4 and 1 non-*Saccharomyces* yeast, respectively. Competent cell, electroporation, and spheroplast were the main methods applied for 20, 17, and 21 non-*Saccharomyces* yeasts, respectively. While 11 non-*Saccharomyces* yeasts used all of three methods, 14 non-*Saccharomyces* yeasts utilized only one of them for transformation. The basic principles and procedures of these methods for non-*Saccharomyces* yeasts are similar to those for *S. cerevisiae*. Electroporation method normally resulted in the highest transformation efficiency up to 10^7 transformants per μg DNA (Sanchez et al., 1993) than other methods. One exception was in *P. fabianii* transformation, which showed about 4-fold higher efficiency in competent cell method than in electroporation (Kato et al., 1997). Although electroporation was the most efficient method, and competent cell was a simple way to perform, the spheroplast-mediating method seemed to be the most widely used in non-*Saccharomyces* transformation. It is probably due to an easy and reliable technique to prepare and regenerate spheroplasts (protoplast) and transfer vector DNA into spheroplasts (protoplast). Compared with spheroplast-mediating method, competent cell method saved considerable time and gave nearly comparable transformation *efficiency*. The trans-kingdom conjugation method has been used for *K. lactis*, *P. angusta*, and *Pa. tannophilus* (Hayman and Bolen, 1993), and *Schiz. pombe* (Sikorski et al., 1990). Vector YEp13 was transferred from *E. coli* into these yeasts with a mobilizing vector pDPT51 carried by *E. coli*. A big YAC was successfully introduced into *S. cerevisiae* by trans-kingdom conjugation (Mahmood et al., 1996), but no report indicated that YAC could be introduced into non-*Saccharomyces* yeasts by this method. The biolistics only occurred in *Cr. neoformans* (Toffaletti et al., 1993). Some transformation methods of non-*Saccharomyces* yeasts have been improved continuously to be easier, more simple and efficient than before, especially for *Schiz. pombe*. A method keeping *Schiz. pombe* spheroplasts in freezing condition at –70 °C provided transformation efficiency of 10^3 transformants per μg DNA as usual after 1-year storage (Jimenez, 1991; Zhao et al., 1993). Additionally, the transformation *efficiency* of *Deb. occidentalis*, *K. lactis*, *P. angusta*, and *Schiz. pombe* after long-term storage of competent cells at -70 °C for several months did not significantly decrease (Dohmen et al., 1991). One-step transformation, an improved competent cell method, was developed for *Y. lipolytica* and

Schiz. pombe with transformation efficiency of 10^5 and 10^4 transformants per μg DNA, respectively (Chen et al., 1992a; 1997). A similar simple method was also developed for *P. angusta* with transformation efficiency of 10^5 transformants per μg DNA (Berardi and Thomas, 1990).To increase transformation efficiency, liposome and polylysine were used for the transformation of *Schiz. pombe* with a big minichromosome (Allshire, 1990) and *T'spora. delbrueckii* (Nakata and Okamura, 1996), respectively.

1.1.6 TRANSFORMATION EFFICIENCY

No general rule can be applied to summarize transformation efficiency of non-*Saccharomyces* yeasts, as the transformation results were mostly obtained from different experiments with different transformation methods, vectors, and host cells. Therefore, this review discusses only few typical phenomena, but not cases covering all non-*Saccharomyces* transformation systems. Transformation efficiency was found to be affected by factors including vector DNA, host strain, spheroplast (protoplast) regeneration, and transformation method.

1.1.6.1 Vector DNA

In general, non-*Saccharomyces* yeasts showed less transformation efficiency with YIp-type vectors than with YCp-, YEp-, and YRp-type vectors that is similar to that of *S. cerevisiae*. Although heterologous *ARS*s functioned in non-*Saccharomyces* yeasts, homologous *ARS*s seemed to offer higher transformation efficiency than heterologous *ARS*s. This phenomenon was observed from *P. angusta* (Sierkstra et al., 1991) and *Deb. occidentalis* (Dohmen et al., 1989). In *P. angusta*, YEp (with 2-μm replicon), YRp (*P. angusta ARS*) and YIp vector rendered *P. angusta* transformation efficiency of 2, 50, and 15 transformants per μg DNA, respectively. This suggests that 2-μm replicon has a negative effect on transformation efficiency for *P. angusta* (Sierkstra et al., 1991). In another case, *P. angusta ARS*-containing vector offered 10-fold higher transformation efficiency than *S. cerevisiae ARS*-containing vector (Roggenkamp et al., 1986). In *Deb.*

occidentalis, *S. cerevisiae* 2-μm replicon and *ARS*-containing vectors gave transformation efficiency of 10^2 and 10^1 transformants per μg DNA, respectively. However, the transformation efficiency was increased up to 10^3 transformants per μg DNA when a *Deb. occidentalis ARS*-containing vector was used (Dohmen et al., 1989). Furthermore, different *ARS*s provided different effects on transformation *efficiency*. Vectors containing different *ARS*s from *K. lactis* provided *K. lactis* transformation *efficiency* in difference of 10 folds (Das and Hollenberg, 1982; Sreekrishna et al., 1984; Chen et al., 1992b; 1996). Different *P. angusta ARS* showed diverse transformation efficiency from 10^1 to 10^3 in *P. angusta* (Sohn et al., 1996). The sources of genetic marker might also affect transformation efficiency. Vector pYM3 with *S. cerevisiae HIS*4 gave *P. pastoris* 100-time more transformation efficiency than pYM4 with *P. pastoris HIS*4 (Cregg et al., 1985). The transformation efficiency of YIp was increased by using linearized form of vectors, and examples included in *C. utilis* (Kondo et al., 1995), *C. glabrata* (Hanic-Joyce and Joyce, 1998), *P. angusta* (15 folds higher) (Faber et al., 1992), *P. fabianii* (8 folds higher) (Kato et al., 1997), *P. stipitis* (1.5 folds higher) (Yang et al., 1994) and *P. ohmeri* (4 folds higher) (Piredda and Gaillardin, 1994).

1.1.6.2 Spheroplast (protoplast)

Spheroplast (protoplast) regeneration was supposed to affect transformation efficiency. Vector PTY75 (YEp type) had lower transformation efficiency in *K. lactis* than in *S. cerevisiae*: many *K. lactis* showed 10-times lower efficiency of protoplast regeneration than *S. cerevisiae* (Das and Hollenberg, 1982). In *Ph. rhodozyma*, the optimal incubation time for spheroplast regeneration in non-selective medium was about 36 hours. When incubation time was more or less 36 hours, the transformation efficiency reduced (Adrio and Veiga, 1995). The highest transformation efficiency of *K. lactis* was obtained when the protoplast was incubated in non-selective medium for 24 hr before the addition of G418 into medium. This indicates that *K. lactis* protoplast regeneration might not be strong enough to resist G418 attack before 24-hour incubation (Bianchi et al., 1987).

1.1.6.3 Host Strains

Some transformation efficiency was strain-dependent. With the same method, the transformation efficiency of pYe (*ARG*4)411 in *P. guilliermondii* was higher than in *S. cerevisiae* and in *C. maltosa* (Kunze et al., 1985). This effect also occurred among *P. angusta* (Faber et al., 1994), *K. lactis* (Chen et al., 1989), and *Ph. rhodozyma* (Adrio and Veiga, 1995). The transformation *efficiency* of vectors, YEp24 (with 2-μm replicon) and YRpH1 (with *S. cerevisiae ARS*) in *T'spora. pretoriensis* were similar, but less than those in *S. cerevisiae* (Oda and Tonomura, 1995).

1.1.6.4 Transformation Methods

Although long term cryo-storage of *Schiz. pombe* spheroplasts at –70°C did not significantly affect the transformation efficiency (Jimenez, 1991; Zhao et al., 1993), the storage of *P. angusta* competent cells at –80 °C reduced transformation efficiency by 2-5 folds (Faber et al., 1994). This indicates that –80 °C is not suitable for maintenance of yeast competent cells. Liposome and polylysine also enhanced transformation efficiency. Liposome helped to introduce a minichromosome larger than 500 kb into *Schiz. pombe* with a 50-times higher efficiency than before (Allshire, 1990). With polylysine treatment of protoplast, the transformation efficiency of *T'spora. delbrueckii* increased by 3,000 folds (Nakata and Okamura, 1996).

1.1.7 VECTOR STABILITY

Vector or plasmid stability is defined as a ratio of the number of cells remaining a transformed phenotype among an amount of cells after growing in non-selective conditions for certain generations. Instability of vector in the absence of selection pressure is a universal problem. Like transformation efficiency, there is no general rule to explain vector stability in non-*Saccharomyces* yeasts. Most vector stability obtained was from different experiments with diverse vectors, host cells, and determining methods.

However, in some cases, vector stability in non-*Saccharomyces* yeasts was influenced by factors including additional gene, *ARS*, DNA form and size,.genetic marker, host, vector composition, and vector type.

1.1.7.1 Vector Type

Integrating vectors showed higher stability than replicating vectors in non-*Saccharomyces* yeasts. Moreover, centromeres made .vectors very stable in yeast host cells. For example, the stability of YCp vectors reached 93 % after 10 generations in *C. glabrata* (Kitada et al., 1996). In *C. maltosa* and *T'spora. pretoriensis*, the stability of both YEp- and YRp-type vectors was less than that of YCp-type vectors (Oda and Tonomura, 1995; Ohkuma et al., 1995).

1.1.7.2 Vector Composition

Vectors based on *S. cerevisiae* 2-μm plasmid have different stability in different non-*Saccharomyces* yeasts. The 2-μm plasmid contains a replicon and three regions, *REP*1, *REP*2 and *STB* (*REP*3) genes required for plasmid amplification and stable maintenance. The replicon and STB gene are cis-acting, but *REP*1 and *REP*2 genes trans-acting. The 2-μm plasmid-containing vectors usually possess its replicon and the *STB* gene, but not *REP*1 and *REP*2 genes. Thus, a *S. cerevisiae* with 2-μm is essential to stably maintain 2-μm-based vectors. Otherwise, in a host cell without the 2-μm plasmid, the vectors have only normal amplification, but poor partition resulting in low stability (Soidla and Nevzgliadova, 1987). The stability of 2-μm-based vectors in non-*Saccharomyces* yeasts tended to be either high or low. After 10 generations, the stability of pTY75-*LAC*4 was 50 % in *K. lactis* (Das and Hollenberg, 1982), YEp13 was 68 % in *Pa. tannophilus* (Hayman and Bolen, 1993), pYA2 and pYA4 was 10 % and 29 %, respectively, in *P. pastoris* (Cregg et al., 1985). On the other hand, pYAS1, pLK1, and YEp24 had less than 10 % of stability in *Deb. occidentalis* and *T'spora. delbrueckii*, respectively (Compagno et al., 1989; Dohmen et al., 1989; Oda and Tonomura, 1995). These results imply that *K. lactis* and *Pa. tannophilus* provided *REP*1- and *REP*2-like

functions to stabilize the 2-μm based vectors, but *Deb. occidentalis*, *P. pastoris*, and *T'spora. delbrueckii* lacked the *REP*1- and *REP*2-like functions.

The trans-acting complement also appeared in some non-*Saccharomyces* yeast plasmids, such as pKD1 and pKW1. Vectors pCXJ-Kan1 and KEp6 contained whole sequence and only the replicon of pKD1 (Chen et al., 1989). Similarly, pKWC11 and pXXK3 possessed entire sequence and only the replicon of pKW1 (Chen et al., 1992b). These vectors were introduced into *K. lactis* without pKD1 and *K. waltii* without pKW1, respectively. After 20 generations, pCXJ-Kan1 and pKWC11 had almost 100 % of stability, but the stabilities of KEp6 and pXXK3 were about 10 % and 1 %, respectively. Some domains of these two native plasmids seem to encode trans-acting proteins required for vector amplification (Chen et al., 1989; 1992b). However, the trans-acting complement was not clear from other non-*Saccharomyces* plasmid pSR1 (Araki et al., 1985). Moreover, the plasmid pGKL1 replication requires the presence of pGKL2 (Gunge et al., 1982), and thus a host carrying pGKL2 was required for pGKL1-based vectors.

1.1.7.3 *ARS*

Different *ARS* performed various effects on vector stability in some non-*Saccharomyces* yeasts. The stability of *S. cerevisiae ARS*-containing vectors in non-*Saccharomyces* yeasts was not high. In *C. glabrata*, after 10 generations, the stability of pMIR4 (with *S. cerevisiae* mitochondrial *ARS*) was 33%, while no cells maintained pRS316 (with *S. cerevisiae ARS*) (Hanic-Joyce and Joyce, 1998). Moreover, YRp7*TRP*5, pUK7, and YRpH1 had low stability in *Deb. occidentalis* (1 %), in *T'spora. delbrueckii* (10 %), and in *T'spora. pretoriensis* (10 %) after 8 or 15 generations, respectively (Compagno et al., 1989; Dohmen et al., 1989; Oda and Tonomura, 1995). However, not all *S. cerevisiae ARS* functioned in all non-*Saccharomyces* yeasts. Vector YRp7 only behaved as a YIp-type vector integrating into *K. lactis* chromosome (Das and Hollenberg, 1982). The results suggest that the DNA replication mechanisms of *S. cerevisiae* are different from that of non-*Saccharomyces* yeasts, and thus *S. cerevisiae ARS* did not

function well in some non-*Saccharomyces* yeasts. In *K. lactis*, the stability of vectors with divers *K. lactis ARS* ranged from 0 to 29 % after 10 generations (Das and Hollenberg, 1982), and from 0. 3 to 5 % after 20 generations (Sreekrishna et al., 1984). On the other hand, vector stability not affected by different *ARS* was found in *P. angusta* (Roggenkamp et al., 1986), *P. pastoris* (Cregg et al., 1985), and *T'spora. pretoriensis* (Oda and Tonomura, 1995). Homologous *ARS* seemed to have higher vector stability than heterologous one. For instance, vectors with *Deb. occidentalis ARS* had the highest stability (10 - 20 %) than ones containing *S. cerevisiae* 2-μm replicon (5 - 10 %) or *S. cerevisiae ARS* (1 %) in *Deb. occidentalis* after 15 generations. Besides, the vector with *Deb. occidentalis ARS* also had higher transformation efficiency and higher copy number than ones with *S. cerevisiae ARS* or 2-μm replicon (Dohmen et al., 1989). It means that the vector stability is associated with transformation and copy number. Additionally, after 10 generations, in *P. pastoris*, vectors with *P. pastoris ARS* showed higher stability than ones with 2-μm replicon (Cregg et al., 1985). These observations could be considered as further evidence to elucidate that 1) *ARS* expressing in non-*Saccharomyces* yeasts is complex, 2) among eucaryotes, there is a similar DNA replication mechanism, and 3) it is empirical to choose an *ARS* for constructing non-*Saccharomyces* yeast vectors.

1.1.7.4 Hosts and other factors

In some cases, vector stability depended on non-*Saccharomyces* yeast host cells. Vector pCXJ-kan1 showed different stability from 21 to 100 % after 10 generations in different *Kluyveromyces* strains such as *K. aestuarii*, *K. marxianus*, *K. thermotolerans*, *K. waltii*, and *K. wickerhamii* (Chen et al., 1989; 1992b). After 12 generations, the stabilities of pYe(*ARG*4)441 in *S. cerevisiae*, *C. maltosa*, and *P. guilliermondii* were 89 %, 79 %, and 48 %, respectively (Kunze et al., 1985). The results suggest that these yeasts provided components required for DNA replication with different efficiency for specific replicons.

The source of genetic marker can also be one of the factors affecting vector stability. Vector pYA2 (with *S. cerevisiae HIS*4) and pYA4 (with *P. pastoris HIS*4)

possessed 10 % and 29 % of stability, respectively, after 10 generations (Cregg et al., 1985). It was proposed that homologous genetic markers are better than heterologous ones for vector stability.

In *Schiz. pombe*, circular minichromosome had less stability than linear minichromosome, and became unstable with increasing in size (Murakami et al., 1995). The circular or large minichromosome may not be suitable for spindle attachment and segregation during mitosis.

With assistance of additional genes, vector stability was improved in two cases. In *Schiz. pombe*, to stably maintain a minichromosome required high expression of a *TOP2* gene encoding type II DNA topoisomerase (Murakami et al., 1995). In addition, a DNA fragment including a *STB* locus and its adjoining region gained higher vector stability in *P. angusta* by 70 times (Bogdanova et al., 1998).

1.1.8 VECTOR COPY NUMBER

Vector copy number is responsible for gene dosage carried by the vector that, in turn, relates to the yield of protein production. High gene dosage is expected by industry when the high gene expression does not damage to host cells. Table 1.1.12 lists some vector copy number from 12 non-*Saccharomyces* yeasts. The vector copy number ranged from 1 to 142 (Chen et al., 1996), which is less than 200 found in *S. cerevisiae* (Lopes et al., 1991). Although it is difficult to make a general rule on vector copy number in non-*Saccharomyces* yeasts due to few results collected, it appears that the presence of rDNA and selective pressure could increase vector copy number in non-*Saccharomyces* yeasts.

Vectors integrating or replicating had no clear effect on vector copy number in non-*Saccharomyces* yeasts, because it depends on the integration number and the replicon property. In *C. maltosa*, YRp vector had higher copy number than YCp vector (Ohkuma et al., 1995). Nonetheless, in *P. pastoris*, YEp and YRp vectors contained less

copy number than YIp vector, which offered multiple integration (Cregg et al., 1985; Clare et al., 1991a,b).

S. cerevisiae ARS played ambiguous effects on vector copy number in non-*Saccharomyces* yeasts. In *C. albicans*, vectors with *S. cerevisiae ARS* had higher copy number than ones with homologous *ARS* (Hanic-Joyce and Joyce, 1998; Cannon et al., 1992; Mehra et al., 1992; Zhou et al., 1994). However, in *Deb. occidentalis* and *P. pastoris*, *S. cerevisiae ARS* negatively influenced on vector copy number (Cregg et al., 1985; Dohmen et al., 1989). In *Deb. occidentalis*, vectors with *Deb. occidentalis ARS* had the highest copy number (10) than those containing *S. cerevisiae* 2-μm replicon (3) or *S. cerevisiae ARS* (1) (Dohmen et al., 1989). Moreover, in *P. pastoris*, 2-μm-based vector had 6-copy number, but *P. pastoris ARS* increased the copy number up to 13 (Cregg et al., 1985).

rDNA and selective pressure gave positive effect on vector copy number. Regular integration rendered lower copy number of 1-19 (Clare et al., 1991a,b; Sierkstra et al., 1991; Keeney and Boeke, 1994) than multiple integration assisted by rDNA (4-60) (Bergkamp et al., 1992; Rossolini et al., 1992; Le Dall et al., 1994; Van Dijken and Pronk, 1995; Wery et al., 1997). Moreover, vector copy number in non-*Saccharomyces* yeasts was also affected by selective pressure from environment, which was also found in *S. cerevisiae* (Hashida-Okado et al., 1998). Containing a vector with a G418 resistance gene, *K. lactis*; *P. pastoris*, and *Schiz. pombe* enhanced vector copy number with increased amount of G418 in medium (Scorer et al., 1994; Tohda et al., 1994; Chen et al., 1996). In *K. lactis*, the copy number of pCXJ3 was 97 under 50 μg/ml of G418, but increased up to 142 in the presence of 2 mg/ml of G418 (Chen et al., 1996).

1.1.9 CONCLUSIONS AND PERSPECTIVES

Transformation system including vector and DNA transfer technique is prerequisite for genetic study of organisms, and the first attempt on transformation in

yeasts was achieved with *S. cerevisiae*. Since the yeast has some disadvantages such as hyper-glycosylation of secreted proteins, and the study of other yeasts is required to acquire the knowledge, transformation studies were extended into non-*Saccharomyces* yeasts. Employing the well-developed knowledge and the genetic techniques of *S. cerevisiae*, the progress of establishing transformation systems for non-*Saccharomyces* yeasts was very remarkable. One hundred genera presenting over 700 species of yeasts appear on the current yeast taxonomy (Kurtzman and Fell, 1998), and the transformation systems of more than one-tenth yeasts (13 genera and 31 species of non-*Saccharomyces*) have been constructed. The research, however, has mainly been concentrated on *K. lactis*, *P. pastoris*, *P. angusta* and especially on *Schiz. pombe*, which are important strains for academic study and industry. Five of six methods originally developed for *S. cerevisiae* were used in transformation of non-*Saccharomyces* yeast that could provide flexibility to select a suitable one for a specific strain which may have a hindrance in transformation. Non-*Saccharomyces* yeasts should be able to express both homologous and heterologous replicons for vector amplification and genes as selective markers with different efficiency in some cases. This suggests that there is compatibility in genetics between the yeasts and other organisms. This also offers a powerful tool to carry out basic research such as DNA replication, gene expression and regulation. Although G418 resistance gene was the most widely used dominant marker in non-*Saccharomyces* yeasts, some yeasts are naturally resistant to G418, thereby, limiting the vast application of this marker in yeast genetics. Meanwhile, biosynthetic markers such as *LEU2* and *URA3* genes are commonly universal markers for most yeasts. Generally, YEp and YRp vectors had higher copy numbers than YIp and YCp in yeasts, but they encounter stability problem. Thus, to make sure of both stability and high copy number is recommended.

Transformation system have played a major role in yeast genetics, and will continue to do so more extensively in the foreseeable future for other non-*Saccharomyces* yeasts. A possible strategy to develop new transformation systems for the yeasts is as follows. First, spheroplast (protoplast)-mediating method and vectors with *S. cerevisiae* 2-μm replicon or *ARS* are recommended. It, thus, requires optimizing the condition for generation and regeneration of the target yeast spheroplast (protoplast). As no

auxotrophic mutants are obtained, one must choose drug resistance markers for yeasts sensitive to aureobasidin, blasticidin, cadmium, cycloheximide resistance gene, G418, hygromycin, or phleomycin. When drug-sensitive is observed, the current corresponding drug resistance gene, controlled by a yeast promoter, could be selected to do transformation. If not successful, an alternative is to make auxotrophic mutants with the phenotypes that can be complemented by the biosynthetic markers mentioned above. When this trial also fails, other strategy based on auxotrophic mutants can be designed:

1) Construct a genomic library of the target yeast with a low copy number *E. coli* vector such as pBR322 or its derivatives;
2) Transfer the library into the target yeast by spheroplast (protoplast)-mediating method;
3) Screen transformants;
4) Isolate total genomic DNA from the transformants;
5) Digest the genome DNA with different suitable restriction enzymes, isolate suitable-sized DNA fragments, ligate, transfer the recombinant DNA into *E. coli* and screen transformants.

YIp vectors containing the *E. coli* vector plus the cloned gene might exist in the *E. coli* transformants. These vectors may be used as backbones to isolate *ARS* and carry out other research of the target yeast. Thus, this can avoid the argument of using drug in transformation.

Table 1.1.1. The vectors used in non-*Saccharomyces* yeast transformation systems. (The information listed as an epitome was the first one appeared in publication collected.)

Vector	Size^	Type	Genetic Marker & Source	Replicon & Source	Reference
C. albicans					
-	-	YIp	*ARG5,6* of *C. albicans*		Negredo et al., 1997
-	-	YIp	*HIS1* of *C. albicans*		Negredo et al., 1997
pGKIII	-	-	*IMH3* of *C. albicans*		Kohler et al., 1997
pMK3	15.9	YIp	*ADE2* of *C. albicans*		Kurtz et al., 1986
pRC2312	-	YRp	*URA3* of *C. albicans*	*ARS* of *C. albicans*	Cannon et al., 1992
pWC25	-	YIp	*GAL1* of *C. albicans*		Gorman et al., 1991
C. boidinii					
pBARCU1	7.4	YRp	*URA3* of *C. boidinii*	*ARS* of *C. boidinii*	Sakai et al., 1993
pRCU350	8.0	YIp	*URA3* of *C. boidinii*		Sakai et al., 1991
C. glabrata					
p112-8XM	-	YCp	*URA3* of *S. cerevisiae*	*ARS* of *C. glabrata*	Kitada et al., 1996
pCgACH-3	-	YCp	*HIS* of *C. glabrata*	*ARS* of *C. glabrata*	Kitada et al., 1996
pCgACT-14	-	YCp	*TRP1* of *C. glabrata*	*ARS* of *C. glabrata*	Kitada et al., 1996
pMIR4	10.0	YRp	*ADE2* of *C. glabrata*	*ARS* of *S. cerevisiae*	Hanic-Joyce and Joyce, 1998
pRS316	-	YCp	*URA3* of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Zhou et al., 1994
pYM6	6.9	YRp	*LEU2* of *S. cerevisiae*	*ARS* of *C. glabrata*	Mehra et al., 1992
pUAUR1-C	6.7	YIp	Aureobasidin resistance gene of *A. pullulans*		Hashida-Okado et al., 1998
U1	-	YIp	*URA3* of *C. glabrata*		Zhou et al., 1994
C. maltosa					
pBTH10B	10.5	YRp	*HIS5* of *C. maltosa*	*ARS* of *C. maltosa*	Hikiji et al., 1989
pDP12	10.6	YRp	*LYS2* of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Kunze et al., 1987
pDP13	15.4	YCp	*LYS2* of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Kunze et al., 1987
pRJ1	-	YRp	*ADE1* of C. maltosa	*ARS* of *C. maltosa*	Sasnauskas et al., 1992
pTRA1	11.0	YRp	*LEU2* of *S. cerevisiae*	*ARS* of *C. maltosa*	Takagi et al., 1986
pUAHHH1	-	YCp	*HIS5* of *C. maltosa*	*ARS* of *C. maltosa*	Nakazawa et al., 1997
pYe (*ARG4*)411	7.4	YIp	*ARG4* of *S. cerevisiae*		Kunze et al., 1985
C. tropicalis					
pCU3	4.9	YIp	*URA3* of *C. tropicalis*		Haas et al., 1990
pMK16	-	YRp	*ADE2* of *C. tropicalis*	*ARS* of *C. tropicalis*	Sanglard and Fiechter, 1992
C. utilis					
pCLRE2	-	YIp	Cycloheximide resistance gene of *C. utilis*		Kondo et al., 1995
Cr. neoformans					
-	270	YAC	-	-	Varma and Kwon-Chung1994
pCnade2c	4.8	YIp	*ADE2* of *Cr. neoformans*		Toffaletti et al., 1993
p*URA5*g2	7.5	YIp	*URA5* of *Cr. neoformans*		Edman and Kwon-Chung, 1990
Deb. occidentalis					
pADE	11.1	YEp	*ADE2* of *Deb. occidentalis*	2-μm of *S. cerevisiae*	Klein and Favreau, 1988
pYAS1	15.6	YEp	*TRP5* of *S. cerevisiae*	2-μm of *S. cerevisiae*	Dohmen et al., 1989

Table 1.1.1. (Cont.) The vectors used in non-*Saccharomyces* yeast transformation systems.

Vector	Size^	Type	Genetic Marker & Source	Replicon & Source	Reference
pYES2.0		YEp	*URA*3 of *S. cerevisiae*	2-μm of *S. cerevisiae*	Dave and Chattoo, 1997
YRp7*TRP*5	9.0	YRp	*TRP*5 of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Dohmen et al., 1989
YRpJD2	8.7	YRp	*TRP*5 of *S. cerevisiae*	*ARS* of *Deb. occidentalis*	Dohmen et al., 1989
K. aestuarii					
pCXJ-kan1	9.6	YEp	G418 resistance gene of *E. coli*	pKD1 of *K. lactis*	Chen et al., 1989
K. lactis					
-	-	YCp	*ADE*1 of *S. cerevisiae*	*ARS* of K. lacti	Zonneveld and van der Zanden, 1995
-	-	YCp	*ADE*2 of *S. cerevisiae*	*ARS* of K. lacti	Zonneveld and van der Zanden, 1995
KEp6	6.5	YEp	*URA*3 of *S. cerevisiae*	pKD1 of *K. lactis*	Wesolowski-Louvel et al., 1988
pABm126-1	15.8	YEp	G418 resistance gene of *E. coli*	pGKL1 of *K. lactis*	Yamakawa et al., 1985
pCXJ-kan1	9.6	YEp	G418 resistance gene of *E. coli*	pKD1 of *K. lactis*	Bianchi et al., 1987
pCXJ12	6.5	YEp	*LEU*2 of *S. cerevisiae*	pKD1 of *K. lactis*	Chen et al., 1996
pCXJ20	7.6	YCp	*LEU*2 of *S. cerevisiae*	pKD1 of *K. lactis*	Chen et al., 1996
pCXJ24	7.3	YCp	*LEU*2 of *S. cerevisiae*	pKD1 of *K. lactis*	Chen et al., 1996
pKARS12	10.8	YRp	*TRP*1 of *S. cerevisiae*	*ARS* of K. lacti	Das and Hollenberg, 1982
pKR7	8.2	YRp	G418 resistance gene of *E. coli*	*ARS* of *K. lactis*	Sreekrishna et al., 1984
pL3	7.3	YEp	*URA*3 of *S. cerevisiae*	pGKL1 of K. lacti	De Louvencourt et al., 1983
pMK4	-	YRp	-	*ARS* of *P. somniferum*	Farkasovska, 1993.
pTY75-*LAC*4	26	YEp	*LAC*4 of *K. lactis* G418 resistance gene of *E. coli*	2-μm of *S. cerevisiae*	Das and Hollenberg, 1982
pUAUR1-C	6.7	YIp	Aureobasidin resistance gene of gene *A. pullulans*		Hashida-Okado et al., 1998
YIprd1-LYS	7.9	YIp	*HIS*3 of *S. cerevisiae*		Rossolini et al., 1992
YRp7[b]	5.8	YRp	*TRP*1 of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Das and Hollenberg, 1982
K. marxianus					
pGL2	6.9	YRp	*TRP*1 of *S. cerevisiae* G418 resistance gene of *E. coli*	*ARS* of *K. lactis*	Das et al., 1984
pKL2	?	YRp	*URA*3 of *S. cerevisiae*	*ARS* of *K. lactis*	Iborra, 1993
pKMGAL	-	YEp	*LEU*2 of *S. cerevisiae*	pKD1 of *K. lactis*	Bergkamp et al., 1993
pKRHIS	10.1	YRp	*HIS*3 of *S. cerevisiae* G418 resistance gene of *E. coli*	*ARS* of *K. lactis*	Basabe et al., 1996
pUAUR1-C	6.7	YIp	Aureobasidin resistance gene of *A. pullulans*		Hashida-Okado et al., 1998
K. polysporus					
pCXJ-kan1	9.6	YEp	G418 resistance gene of *E. coli*	pKD1 of *K. lactis*	Chen et al., 1989

Table 1.1.1. (Cont.) The vectors used in non-*Saccharomyces* yeast transformation systems.

Vector	Size^	Type	Genetic Marker & Source	Replicon & Source	Reference
K. thermotolerans					
pCXJ-kan1	9.6	YEp	G418 resistance gene of *E. coli*	pKD1 of *K. lactis*	Chen et al., 1989
pKWC11		YEp	G418 resistance gene of *E. coli*	pKW1 of *K. waltii*	Chen et al., 1992b
K. waltii					
pCXJ-kan1	9.6	YEp	G418 resistance gene of *E. coli*	pKD1 of *K. lactis*	Chen et al., 1989
pKWC11		YEp	G418 resistance gene of *E. coli*	pKW1 of *K. waltii*	Chen et al., 1992b
K. wickerhamii					
pCXJ-kan1	9.6	YEp	G418 resistance gene of *E. coli*	pKD1 of *K. lactis*	Chen et al., 1989
Pa. tannophilus					
YEp13	10.7	YEp	*LEU2* of *S. cerevisiae*	2-μm of *S. cerevisiae*	Hayman and Bolen, 1993
YRp/*HXK2*-8	-	YRp	*HXK2* of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Wedlock and Thornton, 1989
Ph. rhodozyma					
pGH-1	11.4	YEp	*URA3* of *S. cerevisiae* G418 resistance gene of *E. coli*	2-μm of *S. cerevisiae*	Adrio and Veiga, 1995
pR142H2	5.6	YIp	*ATX* of *Ph. rhodozyma*		Martinez et al., 1998
pTRLR1	4.4	YIp	Cycloheximide resistance gene of *Ph. rhodozyma*		Kim et al., 1998
P. angusta					
p18B1	5.3	YIp	*LEU2* of *P. angusta*		Bogdanova et al., 1995
pFMD-22a	6.0	YRp	*URA3* of *P. angusta*	*ARS* of *P. angusta*	Gellissen et al., 1991
pHARS	-	YRp	*URA3* of *S. cerevisiae*	*ARS* of *P. angusta*	Roggenkamp et al., 1986
pHIP6	5.8	YRp	*LEU2* & *URA3* of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Faber et al., 1994
pL2	-	YRp	*LEU2* of *S. cerevisiae*	*ARS* of *C. utilis*	Tikhomirova et al., 1986
pMH	-	YRp	*LEU2* of *S. cerevisiae*	*ARS* of *P. angusta*	Tikhomirova et al., 1986
pRBS	-	YRp	*URA3* of *S. cerevisiae* G418 resistance gene of *E. coli*	*ARS* of *P. angusta*	Janowicz et al., 1991
pTZ18R-ADE	-	YIp	*ADE2* of *S. cerevisiae*		Bogdanova et al., 1995
pTZ18R-HIS	-	YIp	*HIS3* of *P. angusta*		Bogdanova et al., 1995
PtZ18R-TRP	-	YIp	*TRP3* of *P. angusta*		Bogdanova et al., 1995
YEp13	10.6	YEp	*LEU2* of *S. cerevisiae*	2-μm of *S. cerevisiae*	Gleeson et al., 1986
P. fabianii					
pHFura3	4.1	YIp	*URA3* of *P. fabianii*		Kato et al., 1997
P. guilliermondii					
pYe (*ARG4*)411	7.4	YIp	*ARG4* of *S. cerevisiae*		Kunze et al., 1985
P. methanolica					
-	-	YIp	*ADE1* of p. methanolica		Hiep et al., 1993

Table 1.1.1. (Cont.) The vectors used in non-*Saccharomyces* yeast transformation systems.

Vector	Size^	Type	Genetic Marker & Source	Replicon & Source	Reference
-	-	YIp	*ADE2* of *S. cerevisiae*		Hiep et al., 1993
-	-	YIp	*LEU2* of *S. cerevisiae*		Hiep et al., 1993
P. ohmeri					
poLEU02	9.6	YIp	*LEU2* of *P. ohmeri*		Piredda and Gaillardin, 1994
poURA03	6.8	YIp	*URA3* of *P. ohmeri*		Piredda and Gaillardin, 1994
YoLEU02	-	YRp	*LEU2* of *P. ohmeri*	*ARS* of *P. ohmeri*	Piredda and Gaillardin, 1994
YoURA03	-	YRp	*URA3* of *P. ohmeri*	*ARS* of *P. ohmeri*	Piredda and Gaillardin, 1994
P. pastoris					
pPICK9	9.3	YIp	G418 resistance gene of *E. coli*		Scorer et al., 1994
pSG927	5.6	YRp	*HIS4* of *P. pastoris*	*ARS* of *P. pastoris*	Cregg et al., 1987
pTSU1	12.0	YRp	*SUC2* of *S. cerevisiae*	*ARS* of *P. pastoris*	Sreekrishna et al., 1987
pYA2	15.4	YEp	*HIS4* of *S. cerevisiae*	2-μm of *S. cerevisiae*	Cregg et al., 1985
pYA4	16.5	YEp	*HIS4* of *P. pastoris*	2-μm of *S. cerevisiae*	Cregg et al., 1985
pYMI14	9.4	YIp	*ARG4* of *S. cerevisiae*		Cregg and Madden, 1989
P. stipitis					
pJHS	7.2	YRp	*HIS3* of ?	*ARS* of ?	Morosoli et al., 1993
pUCKm8	7.6	YEp	*LEU2* of *S. cerevisiae* G418 resistance gene of *E. coli*	2-μm of *S. cerevisiae*	Ho et al., 1991
pVY	-	YRp	*URA3* of *P. stipitis*	*ARS* of *P. stipitis*	Yang et al., 1994
Rhodosp. toruloides					
pHG8	16.0	YIp	*PAL* of *Rhodosp. toruloides*		Tully and Gilbert, 1985
Schiz. pombe					
-	36-110	YAC	-	-	Murakami et al., 1995
-	500	YAC	-	-	Allshire, 1990
-	-	-	G418 resistance gene of *E. coli*		Gmunder and Kohli, 1989
-	-	YEp	*LEU2* of *S. cerevisiae* *LYS1* of *Schiz. pombe*	2-μm of *S. cerevisiae*	Beach et al., 1982
paR3	5.7	YRp	*ARG3* of *Schiz. pombe*	*ARS* of *Schiz. pombe*	Waddell and Jenkins, 1995
pART1	7.2	YRp	*PPI* of plant *LEU2* of *S. cerevisiae*	*ARS* of ?	Nitschke et al., 1992
pBG1	-	YRp	*HIS3* of *Schiz. pombe*	*ARS* of ?	Burke and Gould, 1994
pcDSP21-BSD	-	YRp	*URA3* of *S. cerevisiae* Blasticidin S resistance gene of *A. terreus*	*ARS* of *Schiz. pombe*	Kimura et al., 1994
pCG1	4.5	YIp	*URA4* of *Schiz. pombe*		Grimm et al., 1988
pEA500	8.3	YRp	*HIS7* of *Schiz. pombe*	*ARS* of *Schiz. pombe*	Apolinario et al., 1993
pFL20-01	16.6	YIp	*URA3* of *S. cerevisiae* Cadmium resistance gene of *Schiz. pombe*		Coblenz and Wolf, 1994
pFYM225	4.6	YRp	*URA1* of *Schiz. pombe*	*ARS* of *Schiz. pombe*	Sakaguchi and Yamamoto, 1982

Table 1.1.1. (Cont.) The vectors used in non-*Saccharomyces* yeast transformation systems.

Vector	Size^	Type	Genetic Marker & Source	Replicon & Source	Reference
pIRT2-*CAN*1	-	-	*LEU*2 of *S. cerevisiae* *CAN*1 of *S. cerevisiae*	-	Ekwall and Ruusala, 1991
pJ14	-	YEp	*ATP*2 of *S. cerevisiae*	2-μm of *S. cerevisiae*	Boutry and Douglas, 1983
pJDB248	-	YEp	*LEU*2 of *S. cerevisiae*	2-μm of *S. cerevisiae*	Beach and Nurse, 1981
pJK148	5.3	YIp	*LEU*1 of *Schiz. pombe*		Keeney and Boeke, 1994
pJK210	5.0	YIp	*URA*4 of *Schiz. pombe*		Keeney and Boeke, 1994
pR12Sclig2	13.2	YIp	*CDC*9 of *S. cerevisiae* *LEU*2 of *S. cerevisiae*		Barker and Johnston, 1983
pSP4	9.3	YRp	*LYS*2 of *S. cerevisiae*	*ARS* of *Schiz. pombe*	Cottarel, 1995
pUR18	-	YRp	*URA*4 of *Schiz. pombe*	*ARS* of *Schiz. pombe*	Barbet et al., 1992
pYC1-α	15	YEp	*HIS*3 of *S. cerevisiae*	2-μm of *S. cerevisiae*	Strasser et al., 1989
pYTARS	-	YRp	*URA*3 of *S. cerevisiae*	*ARS* of *Tetrahymena*	Luehrsen et al., 1988
*URA*4+pCG1	-	YIp	*URA*4 of *Schiz. pombe*		Grimm and Kohli, 1988
YEp13-RAD	-	YEp	*LEU*2 of *S. cerevisiae* *RAD*2 of *S. cerevisiae*	2-μm of *S. cerevisiae*	McCready et al., 1989
YEp24	7.8	YEp	*URA*3 of *S. cerevisiae*	2-μm of *S. cerevisiae*	Sanchez et al., 1988
T'spora. delbrueckii					
pLK1	13.0	YEp	G418 resistance gene of *E. coli*	2-μm of *S. cerevisiae*	Compagno et al., 1989
pSRY21	10.1	YEp	Cycloheximide resistance gene of ?	pSRY1 of *T'spora. delbrueckii*	Nakata and Okamura, 1996
pUK7	7.4	YRp	G418 resistance gene of *E. coli*	*ARS* of *S. cerevisiae*	Compagno et al., 1989
YEp24	7.8	YEp	*URA*3 of *S. cerevisiae*	2-μm of *S. cerevisiae*	Watanabe et al., 1996
T'spora. pretoriensis					
YCp50	8.0	YCp	*URA*3 of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Oda and Tonomura, 1995
YEp24	7.8	YEp	*URA*3 of *S. cerevisiae*	2-μm of *S. cerevisiae*	Oda and Tonomura, 1995
YRpH1	6.5	YRp	*URA*3 of *S. cerevisiae*	*ARS* of *S. cerevisiae*	Oda and Tonomura, 1995
Tr. cutaneum					
pAN7-1	-	YIp	Hygromycin resistance gene of *E. coli*		Glumoff et al., 1989
pAN8-1	-	YIp	Phleomycin resistance gene of *S. hindustanus*		Glumoff et al., 1989
pAN923-41	-	YIp	*ARG*B of A. nidulans		Ochsner et al., 1991
-	-	YIp	*ARG*B of *Tr. cutaneum*		Ochsner et al., 1991
-	-	YIp	*ARG*A of *Tr. cutaneum*		Reiser et al., 1994
Y. lipolytica					
pIMR100	-	YIp	*URA*3 of ?		Park et al., 1997
pINA92	3.7	YIp	Phleomycin resistance gene of *E. coli*		Gaillardin and Ribet, 1987
pINA46S	12.5	YIp	*LYS*2 of *S. cerevisiae*		Gaillardin et al., 1985
pINA764	9.6	YIp	*URA*3d of *Y. lipolytica*		Le Dall et al., 1994
pINA169	12.7	YIp	*LEU*2 of *S. cerevisiae* *SUC*2 of *S. cerevisiae*		Nicaud et al., 1989

Table 1.1.1. (Cont.) The vectors used in non-*Saccharomyces* yeast transformation systems.

Vector	Size[a]	Type	Genetic Marker & Source	Replicon & Source	Reference
pINA176	-	YRp	*LEU2* of *S. cerevisiae*	*ARS* of *Y. lipolytica*	Meilhoc et al., 1990
pINA1025	10.7	YRp	Hygromycin resistance gene of *E. coli*	*ARS* of *Y. lipolytica*	Cordero and Gaillardin, 1996
YIp333		YIp	*LYS2* of *S. cerevisiae*		Gaillardin et al., 1985
pLD40	6.7	YIp	*LEU2* of *Y. lipolytica*		Davidow et al., 1985
Zygosacch. rouxii					
pKS8	16.7	YRp	*LEU2* of *S. cerevisiae*	*ARS* of *Zygosacch. bailii*	Sugihara et al., 1986
YIp32::TB	6.5	YEp	*LEU2* of *S. cerevisiae*	pSR1 of *Zygosacch. bailii*	Araki and Oshima, 1989

[a] The size of vector is given by kb.

[b] YRp vector not replicating in *K. lactis* but integrating into host chromosome.

Table 1.1.2. The chronicle of transformation systems for non-*Saccharomyces* yeasts.

Year	Yeast	Reference
1978	*S. cerevisiae*	Hinnen et al., 1978; Beggs, 1978
1981	*Schiz. pombe*	Beach and Nurse, 1981
1982	*K. lactis*	Das and Hollenberg, 1982
1984	*K. marxianus*	Das et al., 1984
1985	*C. maltosa*	Kunze et al., 1985
	P. guilliermondii	Kunze et al., 1985
	P. pastoris	Cregg et al., 1985
	Rhodosp. toruloides	Tully and Gilbert, 1985
	Y. lipolytica	Davidow et al., 1985; Gaillardin et al., 1985
1986	*C. albicans*	Kurtz et al., 1986
	P. angusta	Gleeson et al., 1986; Roggenkamp et al., 1986; Tikhomirova et al., 1986
	Zygosacch. rouxii	Sugihara et al., 1986
1988	*Deb. occidentalis*	Klein and Favreau, 1988
1989	*K. aestuarii*	Chen et al., 1989
	K. polysporus	Chen et al., 1989
	K. thermotolerans	Chen et al., 1989
	K. waltii	Chen et al., 1989
	K. wickerhamii	Chen et al., 1989
	Pa. tannophilus	Wedlock and Thornton, 1989
	T'spora. delbrueckii	Compagno et al., 1989
	Tr. cutaneum	Glumoff et al., 1989
1990	*C. tropicalis*	Haas et al., 1990
	Cr. neoformans	Edman and Kwon-Chung, 1990
1991	*C. boidinii*	Sakai et al., 1991
	P. stipitis	Ho et al., 1991
1992	*C. glabrata*	Mehra et al., 1992
1993	*P. methanolica*	Hiep et al., 1993
1994	*P. ohmeri*	Piredda and Gaillardin, 1994
1995	*C. utilis*	Kondo et al., 1995
	Ph. rhodozyma	Adrio and Veiga, 1995
	T'spora. pretoriensis	Oda and Tonomura, 1995
1997	*P. fabianii*	Kato et al., 1997

Table 1.1.3. The characteristics and applications of some non-*Saccharomyces* yeasts.

Yeast	Characteristics	Reference
C. albicans	Human pathogen	Kozinn and Taschdjian, 1966
C. boidinii	Methanol metabolism	Sahm and Wagner, 1972
C. glabrata	Human pathogen	Odds, 1988
C. maltosa	Alkane metabolism	Meyer et al., 1975
C. tropicalis	Human pathogen Alkane and fatty acid metabolisms	Blyth, 1964; Duvnjak et al., 1970
C. utilis	Amino acids and single cell protein productions	Abbott and Gledhill, 1971
Cr. neoformans	Human pathogen	Kozel and Cazin, 1971
Deb. occidentalis	Amylolytic enzyme production	Wilson and Ingledew, 1982
K. lactis	Lactose metabolism Heterologous protein production	Dickson et al., 1979 Reiser et al., 1990
K. marxianus	Lactose metabolism	Mahoney et al., 1975
K. wickerhamii	Kluver effect	Castrillo et al., 1996
Pa. tannophilus	Xylose metabolism	Wedlock and Thornton, 1989
Ph. rhodozyma	Astaxanthin production	An et al., 1991
P. angusta	Methanol metabolism Heterologous protein production	Van Dijken et al., 1975 Reiser et al., 1990
P. fabianii	Wastewater treatment	Sato et al., 1987
P. guilliermondii	Riboflavin metabolism study	Logvinenko et al., 1987
P. pastoris	Methanol metabolism Heterologous protein production	Reiser et al., 1990
P. stipitis	Xylose metabolism	Toivola et al., 1984
Schiz. pombe	Eucaryotic study Heterologous protein production	Nasim et al., 1989 Reiser et al., 1990
T'spora delbrueckii	Ethanol production	Nakata and Okamura, 1996
Y. lipolytica	Citric acid and single cell protein productions Heterologous protein production	Abbott and Gledhill, 1971 Reiser et al., 1990
Zygosacch. rouxii	Soy sauce fermentation	Hamada et al., 1989

Table 1.1.4. The non-*Saccharomyces* host ranges of *S. cerevisiae* replicons.

Yeast	Reference
2-μm plasmid	
Deb. occidentalis	Klein and Favreau, 1988
K. lactis	Das and Hollenberg, 1982
P. angusta	Gleeson et al., 1986
P. pastoris	Cregg et al., 1985
P. stipitis	Ho et al., 1991
Pa. tannophilus	Hayman and Bolen, 1993
Ph. rhodozyma	Adrio and Veiga, 1995
Schiz. pombe	Beach and Nurse, 1981
T'spora. delbrueckii	Compagno et al., 1989
T'spora. pretoriensis	Oda and Tonomura, 1995
Autonomous replicating sequence	
C. glabrata	Zhou et al., 1994
C. maltosa	Kunze et al., 1987
Deb. occidentalis	Dohmen et al., 1989
Pa. tannophilus	Wedlock and Thornton, 1989
Ph. rhodozyma	Faber et al., 1994
T'spora. delbrueckii	Compagno et al., 1989
T'spora. pretoriensis	Oda and Tonomura, 1995

Table 1.1.5. The non-*Saccharomyces* replicons used in yeast transformation.

Replicon & source	Host range	Reference
Replicon of plasmid		
pGKL1 of *K. lactis*	*K. lactis*	De Louvencourt et al., 1983
	K. marxianus	Sugisaki et al., 1985
	S. cerevisiae	Gunge and Sakaguchi, 1981
pGKL2 of *K. lactis*	*K. marxianus*	Sugisaki et al., 1985
	S. cerevisiae	Gunge and Sakaguchi, 1981
pKD1 of *K. lactis*	*K. aestuarii*	Chen et al., 1989
	K. lactis	Bianchi et al., 1987
	K. marxianus	Bergkamp et al., 1993
	K. polysporus	Chen et al., 1989
	K. thermotolerans	Chen et al., 1989
	K. waltii	Chen et al., 1989
	K. wickerhamii	Chen et al., 1989
	S. cerevisiae	Chen et al., 1989
pKW1 of *K. waltii*	*K. thermotolerans*	Chen et al., 1992b
	K. waltii	Chen et al., 1992b
	S. cerevisiae	Chen et al., 1992b
pSR1 of *Zygosacch. bailii*	*Zygosacch. rouxii*	Araki and Oshima, 1989
	S. cerevisiae	Araki et al., 1985
pSRY1 of *T'spora. delbrueckii*	*T'spora. delbrueckii*	Nakata and Okamura, 1996
Autonomous replicating sequence (*ARS*)		
ARS of *C. albicans*	*C. albicans*	Cannon et al., 1992
ARS of *C. boidinii*	*C. boidinii*	Sakai et al., 1993
	S. cerevisiae	Sakai et al., 1993
ARS of *C. glabrata*	*C. glabrata*	Mehra et al., 1992
ARS of *C. maltosa*	*C. maltosa*	Takagi et al., 1986
ARS of *C. tropicalis*	*C. tropicalis*	Sanglard and Fiechter, 1992
ARS of *C. utilis*	*P. angusta*	Tikhomirova et al., 1986
ARS of *Deb. occidentalis*	*Deb. occidentalis*	Dohmen et al., 1989
ARS of *K. lactis*	*K. lactis*	Das and Hollenberg, 1982
	K. marxianus	Das et al., 1984
	S. cerevisiae	Sreekrishna et al., 1984

Table 1.1.5. (Cont.) The non-*Saccharomyces* replicons used in yeast transformation.

Replicon & source	Host range	Reference
ARS of *P. angusta*	*P. angusta*	Roggenkamp et al., 1986; Tikhomirova et al., 1986
ARS of *P. ohmeri*	*P. ohmeri*	Piredda and Gaillardin, 1994
ARS of *P. pastoris*	*P. pastoris*	Cregg et al., 1987; Sreekrishna et al., 1987
ARS of *P. stipitis*	*P. stipitis*	Yang et al., 1994
ARS of *Schiz. pombe*	*Schiz. pombe*	Sakaguchi and Yamamoto, 1982
ARS of *Y. lipolytica*	*Y. lipolytica*	Meilhoc et al., 1990
ARS of *Zygosacch. bailii*	*Zygosacch. rouxii*	Sugihara et al., 1986

Table 1.1.6. The sizes of some non-*Saccharomyces ARS*.

Source	Size (kb)	Reference
Chromosomal DNA		
C. albicans	1.2	Cannon et al., 1990
Cr. neoformans	1.1	Varma and Kwon-Chung, 1998
C. glabrata	0.5	Mehra et al., 1992
C. maltosa	0.2	Ohkuma et al., 1995
	1.3	Sasnauskas et al., 1992
	3.8	Takagi et al., 1986
K. lactis	1.2	Das and Hollenberg, 1982
	1.6	Das and Hollenberg, 1982
	1.8	Fabiani et al., 1990
	1.8	Sreekrishna et al., 1984
	2.0	Das and Hollenberg, 1982
	2.2	Das and Hollenberg, 1982
	2.2	Das and Hollenberg, 1982
	2.3	Das and Hollenberg, 1982
	2.4	Sreekrishna et al., 1984
	2.8	Sreekrishna et al., 1984
	3.2	Das and Hollenberg, 1982
	3.6	Sreekrishna et al., 1984
	3.7	Sreekrishna et al., 1984
	3.8	Sreekrishna et al., 1984
	4.0	Sreekrishna et al., 1984
	4.2	Sreekrishna et al., 1984
	5.0	Das and Hollenberg, 1982
P. angusta	1.3	Tikhomirova et al., 1986
	1.3	Tikhomirova et al., 1986
	1.5	Sohn et al., 1996
	1.5	Tikhomirova et al., 1986
	1.9	Tikhomirova et al., 1986
	2.1	Tikhomirova et al., 1986
Schiz. pombe	0.6	Brun et al., 1995
	2.8	Caddle and Calos, 1994
	3.8	Johnston and Barker, 1987

Table 1.1.6. (Cont.) The sizes of non-*Saccharomyces ARS*.

Source	Size (kb)	Reference
Y. lipolytica	1.0	Fournier et al., 1993
	1.3	Fournier et al., 1993
	1.0	Matsuoka et al., 1993
	1.6	Matsuoka et al., 1993
Zygosacch. bailii	0.3	Sugihara et al., 1986
Mitochondrial DNA		
C. utilis	1.2	Tikhomirova et al., 1986
	3.4	Tikhomirova et al., 1986
P. angusta	0.6	Tikhomirova et al., 1986
	2.0	Tikhomirova et al., 1986
	2.0	Tikhomirova et al., 1986
	2.2	Tikhomirova et al., 1986

Table 1.1.7. A summary of non-*Saccharomyces* yeast transformation systems from Table 1.1.1..

Yeast	Vector					Genetic Marker frequency[a]		
	YAC	YCp	YEp	YIp	YRp	Biosynthetic	Dominant	Colored
C. albicans				+	+	5	1	
C. boidinii				+	+	1		
C. glabrata		+		+	+	5	1	
C. maltosa		+		+	+	5		
C. tropicalis				+	+	2		
C. utilis				+			1	
Cr. neoformans	+			+		2		
Deb. occidentalis			+	*	+	4		
K. aestuarii			+	*			1	
K. lactis		+	+	+	+	6	3	
K. marxianus			+	+	+	4	2	
K. polysporus			+	*			1	
K. thermotolerans			+	*			1	
K. waltii			+	*			1	
K. wickerhamii			+	*			1	
Pa. tannophilus			+	*	+	1	1	
Ph. rhodozyma			+	+		1	2	1
P. angusta			+	+	+	5	1	
P. fabianii				+		1		
P. guilliermondii				+		1		
P. methanolica				+		3		
P. ohmeri				+	+	2		
P. pastoris			+	+	+	2	2	
P. stipitis			+	*	+	3	1	
Rhodosp. toruloides				+		1		
Schiz. pombe	+		+	+	+	15	3	
T'spora. delbrueckii			+	*	+	1	2	
T'spora. pretoriensis		+	+	*	+	1		
Tr. cutaneum				+		2	2	
Y. lipolytica				+	+	3	3	
Zygosacch. rouxii			+	*	+	1		

+ The type of vector was used in the non-*Saccharomyces* yeast transformation.

* YIp vectors could be constructed by deleting yeast replicons from YEp or YRp vectors.

[a] The number appearing in Table 1.1 indicates the quantity of genetic markers used in the yeast transformation.

Table 1.1.8. Genetic markers of *S. cerevisiae* used in non-*Saccharomyces* yeast transformation.

Marker	Gene product	Yeast species	Reference
Biosynthetic genetic markers			
*ADE*1	N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase	*K. lactis*	Zonneveld and van der Zanden, 1995
*ADE*2	Phosphoribosylaminoimidazole carboxylase	*K. lactis*	Zonneveld and van der Zanden, 1995
		P. angusta	Bogdanova et al., 1995
		P. methanolica	Hiep et al., 1993
*ARG*4	Argininosuccinate lyase	*C. maltosa*	Kunze et al., 1985
		P. guilliermondii	Kunze et al., 1985
		P. pastoris	Cregg and Madden, 1989
*ATP*2	F1-ATPase β-subunit	*Schiz. pombe*	Boutry and Douglas, 1983
*CAN*1	Arginine permease	*Schiz. pombe*	Ekwall and Ruusala, 1991
*CDC*9	DNA ligase	*Schiz. pombe*	Barker and Johnston, 1983
*HIS*3	IGP dehydratase	*K. lactis*	Rossolini et al., 1992
		K. marxianus	Basabe et al., 1996
		Schiz. pombe	Strasser et al., 1989
*HIS*4	*HIS*4 polypeptide	*P. pastoris*	Cregg et al., 1985
*LEU*2	β-Isopropylmalate dehydrogenase	*P. stipitis* *Pa. tannophilus*	Ho et al., 1991 Hayman and Bolen, 1993
		C. glabrata	Mehra et al., 1992
		C. maltosa	Takagi et al., 1986
		K. lactis	Chen et al., 1996
		K. marxianus	Bergkamp et al., 1993
		P. angusta	Tikhomirova et al., 1986
		P. angusta	Gleeson et al., 1986
		P. methanolica	Hiep et al., 1993
		Schiz. pombe	Beach and Nurse, 1981

Table 1.1.8. (Cont.) Genetic markers of *S. cerevisiae* used in non-*Saccharomyces* yeast transformation.

Marker	Gene product	Yeast species	Reference
		Y. lipolytica	Nicaud et al., 1989
		Zygosacch. rouxii	Sugihara et al., 1986
LYS2	α-Aminoadipate reductase	*C. maltosa* *Schiz. pombe*	Kunze et al., 1987 Cottarel, 1995
		Y. lipolytica	Gaillardin et al., 1985
RAD2	Excision repair protein	*Schiz. pombe*	McCready et al., 1989
TRP1	Phophoribosyl anthranilate isomerase	*K. lactis* *K. marxianus*	Das and Hollenberg, 1982 Das et al., 1984
TRP5	Tryptophan synthase	*Deb. occidentalis*	Dohmen et al., 1989
		Deb. occidentalis	Dohmen et al., 1989
URA3	Orotidone-5'-phosphate decarboxylase	*Schiz. pombe*	Luehrsen et al., 1988
		Schiz. pombe	Sanchez et al., 1988
		T'spora. delbrueckii	Watanabe et al., 1996
		C. glabrata	Zhou et al., 1994
		Deb. occidentalis	Dave and Chattoo, 1997
		K. lactis	De Louvencourt et al., 1983
		K. marxianus	Iborra, 1993
		P. angusta	Roggenkamp et al., 1986
		Ph. rhodozyma	Adrio and Veiga, 1995
		T'spora. pretoriensis	Oda and Tonomura, 1995
		T'spora. pretoriensis	Oda and Tonomura, 1995
Dominant genetic marker			
HXK2	Hexokinase PII	*Pa. tannophilus*	Wedlock and Thornton, 1989
SUC2	Invertase	*P. pastoris*	Sreekrishna et al., 1987
		Y. lipolytica	Nicaud et al., 1989

Table 1.1.9. Genetic markers of non-*Saccharomyces* yeasts used in non-*Saccharomyces* yeast transformation.

Genetic Maker	Gene Product	Source	Host range	Reference
*ADE*1	N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase	*P. methanolica*	*P. methanolica*	Hiep et al., 1993
*ADE*2	Phosphoribosylamino imidazole carboxylase	*C. albicans* *C. glabrata*	*C. albicans* *C. glabrata*	Kurtz et al., 1986 Hanic-Joyce and Joyce, 1998
		C. tropicalis	*C. tropicalis*	Sanglard and Fiechter, 1992
		Cr. neoformans	*Cr. neoformans*	Toffaletti et al., 1993
		Deb. occidentalis	*Deb. occidentalis*	Klein and Favreau, 1988
*ARG*3 (*ARG*B)	Ornithine carbamoyltransferase	*Schiz. pombe*	*Schiz. pombe*	Waddell and Jenkins, 1995 Ochsner et al., 1991
		Tr. cutaneum	*Tr. cutaneum*	
*ARG*5,6	Acetylglutamate kinase, acetlyglutamyl-Phosphate reductase	*C. albicans*	*C. albicans*	Negredo et al., 1997
*ARG*A	Carbamoyl phosphate synthetase	*Tr. cutaneum*	*Tr. cutaneum*	Reiser et al., 1994
HIS	-	*C. glabrata*	*C. glabrata*	Kitada et al., 1996
*HIS*1	ATP phosphoribosyltransferase	*C. albicans*	*C. albicans*	Negredo et al., 1997
*HIS*3	IGP dehydratase	*P. angusta*	*P. angusta*	Bogdanova et al., 1995
		Schiz. pombe	*Schiz. pombe*	Burke and Gould, 1994
*HIS*4	*HIS*4 polypeptide	*P. pastoris*	*P. pastoris*	Cregg et al., 1985
*HIS*5	Histidinol-phosphate aminotransferase	*C. maltosa*	*C. maltosa*	Hikiji et al., 1989
*HIS*7	Glutamine amido transferase	*Schiz. pombe*	*Schiz. pombe*	Apolinario et al., 1993
*IMH*3	Inosine-5'-monophosphate dehydrogenase	*C. albicans*	*C. albicans*	Kohler et al., 1997
*LEU*1	β-Isopropylmalate dehydrogenase	*Schiz. pombe*	*Schiz. pombe*	Keeney and Boeke, 1994

Table 1.1.9. (Cont.) Genetic markers of non-*Saccharomyces* yeasts used in non-*Saccharomyces* yeast transformation.

Genetic Maker	Gene Product	Source	Host range	Reference
LEU2	β-Isopropylmalate dehydrogenase	*P. angusta*	*P. angusta*	Bogdanova et al., 1995
		P. ohmeri	*P. ohmeri*	Piredda and Gaillardin, 1994
		P. ohmeri	*P. ohmeri*	Piredda and Gaillardin, 1994
		Y. lipolytica	*Y. lipolytica*	Davidow et al., 1985
LYS1	α-Aminoadipate reductase	*Schiz. pombe*	*Schiz. pombe*	Beach et al., 1982
PAL	Phenylalanine ammonia-lyase	*Rhodosp. toruloides*	*Rhodosp. toruloides*	Tully and Gilbert, 1985
TRP1	Phophoribosyl anthranilate isomerase	*C. glabrata*	*C. glabrata*	Kitada et al., 1996
TRP3	Indole-3-glycerol phospate synthase	*P. angusta*	*P. angusta*	Bogdanova et al., 1995
URA1	Aspartate transcarbamylase	*Schiz. pombe*	*Schiz. pombe*	Sakaguchi and Yamamoto, 1982
URA3	Orotidone-5'-phosphate decarboxylase	*C. albicans*	*C. albicans*	Cannon et al., 1992
		C. boidinii	*C. boidinii*	Sakai et al., 1991
		C. glabrata	*C. glabrata*	Zhou et al., 1994
		C. tropicalis	*C. tropicalis*	Haas et al., 1990
		P. angusta	*P. angusta*	Gellissen et al., 1991
		P. fabianii	*P. fabianii*	Kato et al., 1997
		P. ohmeri	*P. ohmeri*	Piredda and Gaillardin, 1994
		P. ohmeri	*P. ohmeri*	Piredda and Gaillardin, 1994
		P. stipitis	*P. stipitis*	Yang et al., 1994
URA4	Dihydroorotase	*Schiz. pombe*	*Schiz. pombe*	Grimm et al., 1988
		Schiz. pombe	*Schiz. pombe*	Grimm and Kohli, 1988
URA5	Orotidine monophosphate pyrophosphorylase	*Cr. neoformans*	*Cr. neoformans*	Edman and Kwon-Chung, 1990
II. Dominant marker				
GAL1	Galactokinase	*C. albicans*	*C. albicans*	Gorman et al., 1991
LAC4	beta-Galactosidase	*K. lactis*	*K. lactis*	Das and Hollenberg, 1982

Table 1.1.9. (Cont.) Genetic markers of non-*Saccharomyces* yeasts used in non-*Saccharomyces* yeast transformation.

Genetic Maker	Gene Product	Source	Host range	Reference
Cadmium resistance gene	Phytochelatin	*Schiz. pombe*	*Schiz. pombe*	Coblenz and Wolf, 1994
Cycloheximide resistance gene	Ribosomal protein L41	-	*T'spora. delbrueckii*	Nakata and Okamura, 1996
		C. utilis	*C. utilis*	Kondo et al., 1995
		Ph. rhodozyma	*Ph. rhodozyma*	Kim et al., 1998
III. Color formation				
ATX	Astaxanthin	*Ph. rhodozyma*	*Ph. rhodozyma*	Martinez et al., 1998

Table 1.1.10. Non-yeast genetic markers used in non-*Saccharomyces* yeast transformation.

Genetic Maker	Gene Product	Source	Host range	Reference
I. Biosynthetic marker				
*ARG*B	Ornithine carbamoyltransferase	*A. nidulans*	*Tr. cutaneum*	Ochsner et al., 1991
PPI	Protein phosphatase	Plant	*Schiz. pombe*	Nitschke et al., 1992
II. Dominant marker				
Aureobasidin resistance gene		*A. pullulans*	*C. glabrata* *K. lactis* *K. marxianus*	Hashida-Okado et al., 1998
Blasticidin S resistance gene	Blasticidin S deaminase	*A. terreus*	*Schiz. pombe*	Kimura et al., 1994
G418 resistance gene	Aminoglycoside phosphotransferase-3' (I)	*E. coli*	*K. aestuarii* *K. lactis*	Chen et al., 1989 Das and Hollenberg, 1982
			K. marxianus	Das et al., 1984
			K. polysporus	Chen et al.., 1989
			K. thermotolerans	Chen et al.., 1989
			K. waltii	Chen et al., 1989
			K. wickerhamii	Chen et al., 1989
			P. angusta	Janowicz et al., 1991
			P. pastoris	Scorer et al., 1994
			P. stipitis	Ho et al., 1991
			Ph. rhodozyma	Adrio and Veiga, 1995
			Schiz. pombe	Gmunder and Kohli, 1989
			T'spora. delbrueckii	Compagno et al., 1989
Hygromycin resistance gene	Hygromycin B phosphotransferase	*E. coli*	*Tr. cutaneum*	Glumoff et al., 1989
		E. coli	*Y. lipolytica*	Cordero and Gaillardin, 1996
Phleomycin resistance gene		*E. coli* *S. hindustanus*	*Y. lipolytica* *Tr. cutaneum*	Gaillardin and Ribet, 1987 Glumoff et al., 1989

Table 1.1.11. Transformation methods used in non-*Saccharomyces* yeast transformations.

Yeast	Competent cell	Electroporation	Spheroplast	Trans-kingdom conjugation	Biolistics
C. albicans	Sakai et al., 1994	Brown et. al., 1996	Kurtz et al., 1986		
C. boidinii	Sakai et al., 1991		Sakai et al., 1991		
C. glabrata		Macreadie et al., 1994			
C. maltosa	Kunze et al., 1985	Takagi et al., 1986	Takagi et al., 1986		
C. tropicalis	Haas et al., 1990	Rohrer and Picataggio, 1992	Haas et al., 1990		
C. utilis		Kondo et al., 1995			
Cr. neoformans		Edman and Kwon-Chung, 1990			Toffaletti et al., 1993
Deb. occidentalis	Dohmen et al., 1989	Costaglioli et al., 1994	Klein and Favreau, 1988		
K. aestuarii			Chen et al., 1989		
K. lactis	Yamakawa et al., 1985	Sanchez et al., 1993 Bolen and McCutchan, 1992	Das and Hollenberg, 1982	Hayman and Bolen, 1993	
K. marxianus	Das et al., 1984	Bergkamp et al., 1993 Iborra, 1993	Das et al., 1984		
K. polysporus			Chen et al., 1989		
K. thermotolerans			Chen et al., 1989		
K. waltii			Chen et al., 1989		
K. wickerhamii			Chen et al., 1989		
Ph. rhodozyma	Wery et al., 1997	Rubinstein et al., 1996	Adrio and Veiga, 1995		
Pa. tannophilus	Wedlock and Thornton, 1989			Hayman and Bolen, 1993	

Table 1.1.11. (Cont.) Transformation methods used in non-*Saccharomyces* yeast transformations.

Yeast	Competent cell	Electroporation	Spheroplast	Trans-kingdom conjugation	Biolistics
P. angusta	Gleeson et al., 1986 Roggenkamp et al., 1986	Faber et al., 1994 Van der Klei et al., 1993 Martinez et al., 1993	Gleeson et al., 1986; Tikhomirova et al., 1986	Hayman and Bolen, 1993	
P. fabianii	Kato et al., 1997	Kato et al., 1997			
P. guilliermondii	Kunze et al., 1985				
P. methanolica	Hiep et al., 1993				
P. ohmeri		Piredda and Gaillardin, 1994			
P. pastoris	Paifer et al., 1994	Crane and Gould, 1994 Martinez et al., 1993	Cregg et al., 1985		
P. stipitis	Morosoli et al., 1993	Yang et al., 1994			
Rhodosp. toruloides			Tully and Gilbert, 1985		
Schiz. pombe	Gaillardin et al., 1983	Hood and Stachow, 1990	Beach and Nurse, 1981	Sikorski et al., 1990	
T'spora. delbrueckii	Watanabe et al., 1996		Compagno et al., 1989		
T'spora. pretoriensis	Oda and Tonomura, 1995				
Tr. cutaneum			Glumoff et al., 1989		
Y. lipolytica	Davidow et al., 1985	Meilhoc et al., 1990	Gaillardin et al., 1985		
Zygosacch. rouxii	Sugihara et al., 1986		Sugihara et al., 1986		

Table 1.1.12. Vector copy number in some non-*Saccharomyces* yeasts.

Yeast	Vector	Copy number	Reference
C. albicans	YRp, *ARS* of *C. albicans*	2-3	Cannon et al., 1992
	YRp, *ARS* of *C. glabrata*	5	Mehra et al., 1992
	YRp, *ARS* of *S. cerevisiae*	10-31	Zhou et al., 1994
	YRp, *ARS* of *S. cerevisiae*	10	Hanic-Joyce and Joyce, 1998
C. maltosa	YCp	1-2	Ohkuma et al., 1995
	YRp, *ARS* of *C. maltosa*	> 20	Ohkuma et al., 1995
Deb. occidentalis	YEp, replicon of 2-μm	2-3	Dohmen et al., 1989
	YRp, *ARS* of *Deb. occidentalis*	3.8-6.6	Klein and Favreau, 1991
	YRp, *ARS* of *Deb. occidentalis*	5-10	Dohmen et al., 1989
	YRp, *ARS* of *S. cerevisiae*	1	Dohmen et al., 1989
K. lactis	YIp + rDNA	4-40	Rossolini et al., 1992
	YIp + rDNA	60	Bergkamp et al., 1992
	YIp + rDNA	15	Van Dijken and Pronk, 1995.
	YEp, replicon of pKD1	142	Chen et al., 1996
K. marxianus	YEp, replicon of pKD1	25	Bergkamp et al., 1993
P. angusta	YIp	3	Sierkstra et al., 1991
	YRp, *ARS* of *P. angusta*	6	Faber et al., 1992
	YRp, *ARS* of *P. angusta*	15-20	Bogdanova et al., 1998
P. ohmeri	YRp, *ARS* of *P ohmeri*	40	Piredda and Gaillardin, 1994
P. pastoris	YIp	19	Clare et al., 1991a
	YIp	13	Clare et al., 1991b
	YEp, replicon of 2-μm	6	Cregg et al., 1985
	YRp, *ARS* of *P. pastoris*	13	Cregg et al., 1985
P. stipitis	YRp, *ARS* of *P stipitis*	10	Yang et al., 1994
Ph. rhodozyma	YIp + rDNA	> 50	Wery et al., 1997
Schiz. pombe	YIp	≧1	Keeney and Boeke, 1994
	YRp, *ARS* of *Schiz. pombe*	6	Brun et al., 1995
Y. lipolytica	YIp + rDNA	60	Le Dall et al., 1994
	YRp, *ARS* of *Y. lipolytica*	1-2	Matsuoka et al., 1993

LITERATURE REVIEW

1.2 THE MOLECULAR BIOLOGY OF *SCHWANNIOMYCES OCCIDENTALIS* KLOCKER

This chapter was summarized as a major part of the publication entitled "The Molecular Biology of *Schwanniomyces occidentalis* Klocker" written by Tsung-Tsan Wang and supervised by Dr. Byong H. Lee, who acted in an editorial capacity, evaluating the manuscript prior to submitting it to the journal. This paper has been accepted by *Critical Review in Biotechnology*. This chapter also serves as a part of introduction to the thesis, showing the background for the research that was done and the reasons why this project was significant.

1.2.1 ABSTRACT

This review describes the molecular studies of *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*) concerning transformation, genome, gene cloning, gene structure, gene expression and its characteristics to application. *Schw. occidentalis* appeared to have at least 5 or 7 chromosomes and no native plasmid from the yeast was reported. Three transformation systems based on complementation of *Schw. occidentalis* auxotrophic mutants were established. Vectors with the replicon of 2-μm plasmid and autonomous replication sequences (*ARS*) of *Saccharomyces cerevisiae* and *Schw. occidentalis ARS* replicated extrachromosomally in *Schw. occidentalis* transformants, without modification of the transformed vector DNA. So far, at least twenty-one *Schw. occidentalis* genes encoding 14 different proteins have been cloned. Most of the *Schw. occidentalis* genes have shown similarity (45 - 91 %) with the corresponding genes of other organisms, especially of *S. cerevisiae*. However, some *Schw. occidentalis* genes possess other unique structures for their operators, promoters, transcription initiation sites and terminators. Some foreign genes were expressed in *Schw. occidentalis*, while *Schw. occidentalis* genes functioned in other

yeasts and bacteria, *Escherichia coli*, and *Streptomyces lividans*. Due to a strong ability of secretion and low level of glycosylation, *Schw. occidentalis* might be a promising host to produce heterologous proteins.

1.2.2 INTRODUCTION

Yeasts have been employed as systems for examining many basic questions in eucaryotic biology including gene expression and secretion of heterologous proteins for academic and industrial interests. These microorganisms possess a number of important advantages and characteristics:

1) simple eucaryotes that have similar biological metabolisms such as mRNA splicing mechanism, protein maturation, etc. to higher eucaryotes,
2) rapid growth in simple medium to high cell density by current fermentation techniques,
3) more advanced genetics and biochemistry than any other eucaryotes,
4) well developed and easily manipulated recombinant DNA techniques,
5) extracellular proteins which facilitates protein purification and reduces toxic shock to the yeast hosts,
6) easier mutation analysis than other higher eucaryotes, and
7) non-toxic products.

Although the major study of yeast genetics was concentrated on *Saccharomyces cerevisiae*, the yeast has certain limitations, rendering production of heterologous proteins difficult due to:

1) low product yields, even with a strong promoter (Buckholz and Gleeson, 1991),
2) hyperglycosylated proteins which might affect the protein characteristics (Lemontt et al., 1985; Moir and Dumais, 1987; Van Arsdell et al., 1987) and
3) intracellular or periplasmic gene products when protein is larger than 30 kDa (Smith et al., 1985; Jigami et al., 1986; Moir and Dumais, 1987; De Nobel and Barnett, 1991).

Thus, other yeasts have been studied to replace *S. cerevisiae* as hosts to produce heterologous proteins. During the last 10 years, the major studies of non-*Saccharomyces* yeasts in biotechnology have been focused in *Candida* spp., *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Schizosaccharomyces pombe* (Reiser et al., 1990; Buckholz and Gleeson, 1991). Based on the knowledge and molecular techniques of *S. cerevisiae*, progress toward the molecular study of non-*Saccharomyces* yeasts has been significant.

Since *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*) has been described as a "super yeast" (Ingledew, 1987), this yeast was also suggested as a useful alternative to *S. cerevisiae* in the production of heterologous proteins. The molecular study of *Schw. occidentalis*, however, is very limited, although some progress has been made. This review summarizes the recent achievements on the molecular aspects of *Schw. occidentalis* and the characteristics of this yeast that makes it particularly attractive to produce heterologous proteins.

1.2.3 TAXONOMY OF *SCHW. OCCIDENTALIS*

Since the yeast *Schw. occidentalis* was found from the soil of the island of St. Thomas in the West Indies (Klocker, 1909), *Schwanniomyces castellii* (Capriotti, 1957), *Schwanniomyces alluvius* (Phaff et al., 1960), *Schwanniomyces persoonii* (Van der Walt, 1962) and *Schwanniomyces ukranicus* (Kvasnikov et al., 1979) of the genus which assimilate diverse carbon sources (Phaff and Miller, 1984) were isolated. The DNA/DNA reassociation experiments showed that DNA from *Schw. occidentalis* had high DNA homology (97 % or more) with *Schw. alluvius* and *Schw. castellii*, but less homology (80%) with the DNA from *Schw. occidentalis* (Price, 1978). Taxonomically, *Schw. alluvius*, *Schw. castellii*, and *Schw. persoonii* were actually consolidated into *Schw. occidentalis* (Phaff and Miller, 1984; Nakase et al., 1998). This incorporation was supported by electrophoretic karyotypes (Janderova and Sanca, 1992). All of these

Schwanniomyces spp. showed 6 or 7 chromosomal DNA bands and similar profiles after electrophoresis. However, no significant difference was found in the sequences of the 18S and 25S of ribosomal RNA between *Schwanniomyces* and *Debaryomyces* (Kurtzman and Robnett, 1991). The current taxonomic classification of *Schw. occidentalis* has thus been changed into *Debaryomyces occidentalis* (Klocker) (Kurtzman and Robnett, 1991; Nakase et al., 1998). Nonetheless, the original name of *Schwanniomyces* will be used in this review, because the research results were mainly derived from the *Schwanniomyces* spp.

1.2.4 PRODUCTION OF ENZYMES

Schw. occidentalis produces diverse extracellular enzymes such as α-galactosidase (Ulezlo et al., 1991), β-glucosidase (Klein et al., 1989a), inulinase (Guiraud et al., 1982), invertase (Klein et al., 1989a), phytase (Lambrechts et al., 1992; 1993; Segueilha et al., 1992; 1993) and amylolytic enzymes, α-amylase and glucoamylase (Wilson and Ingledew, 1982; Simoes-Mendes, 1984; Lusena et al., 1985; Clementi and Rossi, 1986; Moranelli et al., 1987; Howard et al., 1988; Klein et al., 1989a). *Schw. occidentalis* is one of few yeasts that can completely hydrolyze soluble starch (Touzi et al., 1982; Horn et al., 1988). Its strong amylolytic enzymes are capable of hydrolyzing cheap starches e.g. cassava (Jamuna and Ramakrishna, 1989; Hongpattarakere and H-Kittikun, 1995), corn starch (Raspor, 1987), potato (Moresi et al., 1983; Kombila et al., 1985; Moresi and Medici, 1989), sorghum (Horn et al., 1992a&b) and wheat (Zaire et al., 1988). *Schw. occidentalis* was thus used to produce ethanol (Calleja et al., 1982; Wilson et al., 1982; Zaire et al., 1988; Mok and Duvnjak, 1992; Ryu et al., 1992; Saucedo-Castaneda et al., 1992a&b&b&d) and single cell protein (Wilson et al., 1982; Levy- Rick et al., 1982; Jamuna and Ramakrishna, 1989; Horn et al., 1992b; Hongpattarakere and H-Kittikun, 1995) directly from fermenting starches. Moreover, *Schw. occidentalis* could also produce unknown enzymes to degrade the wood hydrolysate of lignocellulosics with high content of levoglucosan (1, 6-anhydro-β-D-glucopyranose). The wood pyrolysate was produced from Waterloo Fast Pyrolysis Process (WFPP) and the pyrolysate

hydrolysis was used in ethanol production (Prosen et al., 1993). Moreover, some techniques have been developed to improve growth rate, biomass and ethanol yields of the yeast, and to monitor starch hydrolysis by the yeast (Moresi et al., 1983; Zaire et al., 1988; Moresi and Medici, 1989; Horn et al., 1992a&b; Saucedo-Castaneda et al., 1992a&b&c&d; Saucedo-Castaneda et al, 1994; Ciesarova et al., 1995).

1.2.5 TRANSFORMATION SYSTEMS OF *SCHW. OCCIDENTALIS*

Until now, three transformation systems were developed for *Schw. occidentalis* (Klein and Favreau, 1988; Dohmen et al., 1989; Dave and Chattoo, 1997). As shown in Table 1.2.1, all of them were based on the complementation of auxotrophic mutants with corresponding genes. *ADE2*, *LEU2*, *TRP5*, and *URA3* genes complemented *ade2*, *leu*, *trp5*, and *ura3* (or *ura5*) auxotrophic mutants of *Schw. occidentalis*, respectively. The *ADE2* gene was cloned from *Schw. occidentalis*, but the *LEU2*, *TRP5* and *URA3* genes were derived from *S. cerevisiae*. The transformations were carried out by either spheroplast-based method (Klein and Favreau, 1988), competent cell method (Dohmen et al., 1989; Dave and Chattoo 1997), or electroporation method (Costaglioli et al., 1994) with transformation efficiency of 10^3, $1\text{-}10^4$, 10^5 transformants per μg of DNA, respectively. *Schw. occidentalis* had lower transformation efficiency than *S. cerevisiae* with the same vector and transformation method (Dohmen et al., 1989). The linear and supercoiled forms of vector DNA resulted in equal transformation efficiency, suggesting that *Schw. occidentalis* adopted vector DNA in these two forms without difference. When recovered from *Schw. occidentalis* transformants, both linear and supercoiled forms of vector DNA showed similar structures. This indicates that *Schw. occidentalis* efficiently converts vector DNA from linear into circular form (Klein and Favreau, 1988). The vectors used in *Schw. occidentalis* transformations included the types of yeast episomal plasmid (YEp), and yeast replication plasmid (YRp). Nonetheless, no report described *Schw. occidentalis* transformation using yeast centromere plasmid (YCp) and yeast artifical chromosome (YAC). The YEp vectors contained the replicon of *S. cerevisiae* 2-μm plasmid. The YRp vectors possessed autonomous replication sequences (*ARS*) from

either *S. cerevisiae* or *Schw. occidentalis*, indicating that the *ARS* from other yeasts could be used to construct *Schw. occidentalis* vectors. However, vectors with different types of replicons had diverse transformation efficiencies and mitotic stabilities. In non-selective medium, after 15 generations, the stabilities of vectors containing *Schw. occidentalis ARS*, 2-μm plasmid replicon and *S. cerevisiae ARS* were 10-20 %, 5-10 % and 1 %, respectively (Dohmen et al., 1989). Vectors with *Schw. occidentalis ARS* had higher copy number (5-10) than vectors with *S. cerevisiae* replicons, 2-μm plasmid replicon and *ARS* (1-3) (Dohmen et al., 1989). It hints that the copy number is related to vector stabilities. Vector DNA was recovered from *Schw. occidentalis* transformants without change in their structures. These results suggest that *Schw. occidentalis* duplicates vector DNA without any modification. Thus, it appears that a promising cloning system can be developed (Dave and Chattoo 1997).

1.2.6 GENOME AND GENES

1.2.6.1 Endogenous Plasmid

Although native plasmids pGKL1 (k1), pGKL2 (k2), and pKD1 were found from *K. lactis* (Gunge et al., 1981; Chen et al., 1986; Falcone et al., 1986), pKW1 from *Kluyveromyces waltii* (Chen et al., 1992), 2-μm from *S. cerevisiae* (Guerineau et al., 1976; Hollenberg et al., 1976), pSRY1 from *Torulaspora delbrueckii* (Nakata and Okamura, 1996), pSB1, pSB2 and pSR1 from *Zygosaccharomyces bailii* (Toh-e et al., 1984; Araki et al., 1985; Utatsu et al., 1987), pSB3 and pSB4 from *Zygosaccharomyces bisporus* (Toh-e et al., 1984; Toh-e and Utatsu, 1985), and pSM1 from *Zygosaccharomyces fermentati* (Utatsu et al., 1987), endogenous plasmid of *Schw. occidentalis* has not been reported.

1.2.6.2 Chromosome

Field inversion gel electrophoresis (FIGE) of genomic DNA revealed that *Schw. occidentalis* contained at least five chromosomes, each larger than 1,000 kilobase (kb) (Johnston et al., 1988; Janderova and Sanca, 1992). Contour-clamped homogenous electric field (CHEF) also showed that *Schw. occidentalis* possesses at least 7 chromosomes (Del Pozo et al., 1993). The G+C content of total *Schw. occidentalis* DNA was 35 % (Price et al., 1978). The structures and genetic maps of *Schw. occidentalis* chromosomes are still unknown, but more data are forthcoming by recent studies. For example, *SCR*1 and *SCR*2 genes are located in chromosome II and V, respectively, of *Schw. occidentalis* ATCC 26077 (Del Pozo et al., 1993). *LEU*2 gene is present in chromosome IV of strain ATCC 26074 and chromosome V of strain R91 (Iserentant and Verachtert, 1995). *URA*3 and *TUB*2-like DNA sequences exist on different chromosomes (Johnston et al., 1988). There are two loci for *EG*1-like sequence (Prakash and Seligy, 1988), and at least two genes encoding α-amylase (Claros et al., 1993) and glucoamylase (Dohmen et al., 1990) in the *Schw. occidentalis* genome.

1.2.6.3 Gene Cloning

Before transformation systems are developed for *Schw. occidentalis*, the strategy to clone *Schw. occidentalis* genes was performed in other host cells, *Escherichia coli* and *S. cerevisiae*. At least 21 *Schw. occidentalis* genes encoding 14 kinds of enzymes or proteins have been cloned (Table 1.2.2). By colony or plaque hybridization in *E. coli* with cDNA or oligonucleotide DNA as a probe, *EG*1, *ENA*2, *GAM*1, *HXK*, *INV* and *GDH* genes of *Schw. occidentalis* were cloned (Prakash and Seligy, 1988; Klein et al., 1989b; Dohmen et al., 1988; 1990; De Zoysa et al., 1991; Rose, 1995; Banuelos and Rodríguez-Navarro, 1998). In *S. cerevisiae*, the *Schw. occidentalis* genes were cloned either by complementing corresponding auxotrophic mutants or by directly observing their expression phenotypes. *ADE*2, *ENA*1, $CYC1_0$, *HAK*1, *LEU*2 and *ODC* genes of *Schw. occidentalis* were screened by complementing corresponding *S. cerevisiae* auxotrophic mutants of *ade*2, *ena*1, cytochrome c-less, *trk*1, *leu*2 and *ura*3, respectively (Klein and Favreau, 1988; Klein and Roof, 1988; Amegadzie et al., 1990; Banuelos et al., 1995; Iserentant and Verachtert, 1995; Banuelos and Rodríguez-Navarro, 1998). On the other

hand, cycloheximide resistance genes (*SCR*1 and *SCR*2) were obtained by selecting dominant transformants under the presence of cycloheximide (Del Pozo et al., 1993). α-Amylase (*AMY* and *SWA*) genes were selected by the transformants that showed a clear halo against background on starch containing plates (Abarca et al., 1988; Strasser et al., 1989; Wang et al., 1989; Abarca et al., 1991; Park et al., 1992; Puta et al., 1994). The majority of the *Schw. occidentalis* genes cloned were α-amylase determinants. *Schw. occidentalis* ATCC 26076 and 26077 were the main strains of gene sources. Among the cloned genes, the *ENA*2 was the biggest (3,246 base pair (bp)), and *SCR*2 smallest (321 bp). Their G+C contents ranged from 31.83 % to 40.51 %, which are similar to those of *Schw. occidentalis* (35 %) (Price et al., 1978) and *S. cerevisiae* (39 – 40 %) (Sharp and Cowe, 1991). However, no detailed studies were reported on cloning of the β-glucosidase gene.

1.2.6.4 Gene Structures

Among the 21 *Schw. occidentalis* genes cloned, 16 of them have been sequenced. Because three *AMY* genes share a very high identity, only one of the *AMY* genes plus the other 13 sequenced genes will be discussed. Because insufficient data are currently available, the gene structures including 5'- untranslated region, open reading frame and 3'- untranslated region were deduced directly from their own DNA sequences.

1.2.6.4.1 5'- Untranslated Region

The alignment of the 5' regions of 12 *Schw. occidentalis* genes revealed some consensus elements: 5' – operator – promoter – transcription initiation site – 3' (Table 1.2.3) whose structures are similar to those reported in *S. cerevisiae* genes, but are not in a common feature. Not all *Schw. occidentalis* genes showed distinct structures of operator and transcription initiation site. The intervals between the same two elements varied among these 12 *Schw. occidentalis* genes.

The putative operator of the *Schw. occidentalis* genes appeared to contain at least 2 types of binding sites: a proposed activator binding site (GNNNNTNNCG) (Figure 1.2.1) (Guarente, 1992), and a possible regulatory protein binding site (TGACTC) (Hinnebusch, 1988; Daignan-Fornier and Fink, 1992). The *CYC*1_0 and *HAK*1 genes possessed these two binding sites. The remaining *Schw. occidentalis* genes studied contained at least one of them found at different locations within the specific genes. Exceptions were the *LEU*2, *GDH*, *HXK*, and *SWA*2 genes. The exceptions might be due to the lack of known sequence in their 5' flanking regions to find out the binding sites, or the use of other operator sequences for regulation of gene expression. Figure 1.2.1 shows that the activator binding sites are present from 2 to 521 bp upstream from TATA boxes. The *AMY*1 and *SCR*2 genes even contain 2 and 3 tandem copies of the activator binding site, respectively, with intervals more than 160 bp. When the region of –533 to –305 containing the TGACTC site was deleted, no *CYC*1_0 mRNA was detected (Amegadzie et al., 1990). This suggests that the TGACTC should be a regulation site associated with the *CYC*1_0 gene expression. Table 1.2.3 indicates that the activator binding sites are farther apart from promoters than the regulatory protein binding sites, which usually locate before promoters. However, exceptions are that the operators of the *ADE*2 and *GAM*1 genes are supposed to exist between the CAAT and TATA boxes. Furthermore, in the *HAK*1 gene, the TGACTC was located behind the TATA box. The question of whether these two types of binding sites or other sequences control gene expression, and whether the different arrangements of these two sites affect gene expressions must be further investigated. In addition, their regulation mechanisms and why there exist tandem copies in an *AMY* gene also require further study. Besides, in the 5' flanking regions of the *AMY*1 genes, a poly (dA-dT) structure of $_{-155}$AAAATTTATTAAAATTTTA$_{137}$ found from some of *S. cerevisiae* genes appears to function as an upstream-promoter element for the gene expression (Struhl, 1985).

The *Schw. occidentalis* promoters were proposed to contain the structures of CAAT and TATA box (Table 1.2.3). The TATA box is responsible for transcription initiation, whereas the CAAT is supposed to increase the transcription rate. In some of *Schw. occidentalis* genes listed in Table 1.2.3, the CAAT is located at 22 to 292 bp

upstream from TATA boxes, which is present at 67 to 235 bp from the translation start codon ATG. The structures of putative TATA boxes of the *Schw. occidentalis* genes consist of at least 5 types: TATAAA, TATAAT, TATACT, TATATA and TATGAT (Table 1.2.4), which are similar to those of *S. cerevisiae* (Sentenac and Hall, 1982). The TATAAA appeared to be the most common type in *Schw. occidentalis* genes.

From the *S. cerevisiae* genes studied, a common organization of TATA box --- (CT)n --- PyAAPu --- ATG (Dobson et al., 1982; Burke et al., 1983) considered as transcription initiation site was also deduced from *Schw. occidentalis* genes (Figure 1.2.2). The CT block with diverse lengths (from 4 to 21 bp) positioned behind the TATA boxes with distinct spans (from 0 to 134 bp). PyAAPu placed after the CT block (from 1 to 111 bp) and before the ATG codon (from 0 to 29 bp) with different spans. Besides, all the *Schw. occidentalis* genes listed in Figure 1.2.2 contained different copies (from 1 to 5) of PyAAPu spaced by different nucleotides (from 0 to 35 bp) except the *CYC*1_0 gene. Among the various structures of PyAAPu, TAAA appeared to be the most preferred sequence in the *Schw. occidentalis* genes. By S1-nuclease mapping, the transcription initiation sites were expected for the *AMY*, *EG*1, *GAM*1, *INV*, *SCR*2 and *SWA*2 genes with 37-42, 65-70, 8-11, 26-28, 10 and 25 bp before ATG codon, respectively. These sites included a residue of "A" which was explained as the transcription initiation point for the *SCR*2 and *SWA*2 genes. However, the PyAAPu could not be found from the 5' flanking region of the *CYC*1_0 gene suggesting that *Schw. occidentalis* genes use other structures for transcription initiation.

1.2.6.4.2 Open Reading Frame

In Figure 1.2.3, the alignment of sequences flanking the translation initiation codon ATG of 12 *Schw. occidentalis* genes showed the conserved 5'- (A/G) NN ATG (A/G) N (A/T) – 3'. A purine residue usually is located at the position of –3 and +4, and adenine or thymine residue present at +6 position, which is in accordance with the structure found from eucaryotic genes (Kozak, 1981). This conserved region is supposed

to affect the recognition of the ATG codon by eucaryotic ribosomes or other components associated with translation initiation (Kozak, 1981; 1984).

1.2.6.4.3 3'- Untranslated Region

In many *S. cerevisiae* genes, there are two consensus sequences, 5'- TAG --- TAGT (or TATGT) --- TTT – 3', and AATAAA, important for transcription termination and polyadenylation (Proudfoot and Brownlee, 1976; Zaret and Sherman, 1982). The latter was found only from the *SCR*2 and *SWA*2 genes, but the former was deduced from all *Schw. occidentalis* genes studied, except for the *CYC*1$_0$, *GDH* and *LEU*2 genes (Table11). Only part of the consensus sequence could be found from the *CYC*1$_0$ and *LEU*2 genes. Besides AATAAA, two similar sequences of AAGAAA and AATATA seem to be common in the 3' – untranslated regions of the *Schw. occidentalis* genes, except *ADE*2 and *AMY*1 genes (Table 1.2.5). The *CYC*1$_0$ and *LEU*2 genes do not contain the typical sequence of 5' - TAG --- TAGT --- TTT – 3', but possessed one AAGAAA segment. It suggests that the sequences of AAGAAA and AATATA also play a role in transcription termination and/or polyadenylation as AATAAA sequence does. Figure 1.2.8 also shows that most of the *Schw. occidentalis* genes contained at least one of the specific sequences. However, whether *Schw. occidentalis* mRNA transcription is terminated by these kinds of specific sequences or by other kinds is not well understood.

1.2.6.5 Gene Homology and Protein Structures

When the nucleotide or putative amino acid sequences were compared, the *Schw. occidentalis* genes (or proteins) generally showed high homologies (45 - 91 %) with the corresponding genes (or proteins) from *S. cerevisiae* and other organisms, except for the *EG*1, *GAM*1 and *LEU*2 genes (Prakash and Seligy, 1988; Dohmen et al., 1990; Iserentant and Verachtert, 1995). The *EG*1 gene seemed to encode a novel protein, because its gene product had little homology with other known yeast structural proteins. Although the *Schw. occidentalis LEU*2 gene complemented a *leu*2 auxotrophic mutant of *S. cerevisiae* (Iserentant and Verachtert, 1995), these *LEU*2 genes from these two yeasts share a low

homology. The *Schw. occidentalis GAM*1 gene has no homology with other known *GAM* genes (Dohmen et al., 1990; Naim et al., 1991; Bui et al., 1996). The sequence comparison performed by computer revealed that the *Schw. occidentalis* glucoamylase possesses significant similarities with human lysosomal α-glucosidase, human isomaltase, rabbit isomaltase, rabbit sucrase, and rat sucrase. This indicates that these 6 enzyme-encoding genes might be derived from a common ancestral gene (Naim et al., 1991). One of the advantages of comparing the sequence homology is to infer DNA and protein structures of interest based on other genes or proteins. From the analysis of the putative amino acid sequence, the gene product encoded by *EG*1 should not be a DNA binding protein, because it did not have the characteristic structure of the protein (Prakash and Seligy, 1988). The possible catalytic sites (Asp^{152} - His^{157}, Gly^{237} – His^{245} and Phe^{328} – Asp^{333}) of an α-amylase and an active site ($M^{112}GGGKGG^{118}$) of NADP-dependent glutamate dehydrogenase (De Zoysa et al., 1991; Park et al., 1992) were also deduced by sequence comparison. However, site-directed mutagenesis can further provide direct evidences to show some essential sites of enzymes or proteins. This method showed that: 1) Gln^{56} was responsible for cycloheximide resistance offered by the *SCR*2 gene (Del Pozo et al., 1993); 2) Asn^{229} was the single site for glycosylation essential for the activity, stability and secretion of an α-amylase encoded by a *SWA*2 gene (Yanez et al., 1998); 3) Asp^{470} was required for *Schw. occidentalis* glucoamylase activity; and 4) Trp^{468} was involved in the α-1, 6 activity but not α-1, 4 activity of *Schw. occidentalis* glucoamylase (Hulseweh et al., 1997). Furthermore, comparison of enzymes from wild type and mutant type of *Schw. occidentalis* showed that the yeast hexose-ATP-kinase contained two catalytic sites for hexokinase-like and glucokinase-like activity, respectively (McCann et al., 1987).

1.2.6.6 Splicing

As *Schw. occidentalis* is a eucaryotic organism, its genes might contain intron structures, but only the *SCR*2 gene from the yeast genes discussed showed a putative intron (Del Pozo et al., 1993). A consensus structure of intron derived from *S. cerevisiae* gene could be found on a 452 bp segment (+5 to +456 from ATG codon) of the *SCR*2

gene. This putative intron has a structure of 5'- GTATGT – $(N)_{425}$ - ATACTAACA – NNN – TTCTATATAG – 3' which is required for mRNA splicing (Woolford, 1989; Ruby and Abelson, 1991; Rymond and Rosbash, 1992).

1.2.7 GENE EXPRESSION

1.2.7.1 Expression Regulation

The expressions of the *AMY*, *CYC*1_0, *ENA*1, *ENA*2, GAM1, *HAK*1, *INV* and *SWA* genes of *Schw. occidentalis* were regulated (Table 1.2.6) (De Mot and Verachtert, 1986; Abarca et al., 1989, 1991; Dohmen et al., 1989, 1990; Dowhanick et al., 1987; Klein et al., 1989b; Wang et al., 1989; Amegadzie et al., 1990; Dowhanick et al., 1990; Banuelos et al., 1995). All these genes are associated with either carbohydrate hydrolysis (*AMY*, *GAM*1, *INV*, and *SWA*) or respiration (*CYC*1_0) to produce energy, except the *ENA*1, *ENA*2, and *HAK*1 genes related to K^+ and Na^+ transport with energy consumption. Generally, these gene expressions were activated by substrates, but inhibited by products. Glucose, an end product from carbohydrate hydrolysis, is a main energy source for most organisms. While glucose thus seems to play a key product to inhibit the *AMY*, *CYC*1_0, *GAM*1, *INV*, and *SWA* gene expressions, it induces the expression of the *HAK*1 gene, which was inhibited by antibiotic neomycin. The *GAM*1 gene was repressed by heat shock as well. Moreover, when *Schw. occidentalis* was exposed to glucose, glucose negatively affected the glucoamylase activity not only by repressing the *GAM*1 gene expression, but also by activating unknown mechanism to degrade the intracellular and extracellular glucoamylase (Dowhanick et al., 1990). The glucose repression is also abundantly found in *S. cerevisiae* (Trumbly 1992; del Castillo Agudo and Gozalbo 1994; Gozalbo and del Castillo Agudo 1994; Gancedo 1998). On the other hand, lactose and sucrose induced the *INV* gene expression. Maltose, starch and another strong inducer β-cyclodextrin (De Mot and Verachtert, 1986) activated both *AMY* and *GAM*1 gene expressions, but the inductions could be overridden by glucose repression (Wang et al., 1989; Dowhanick et al., 1990). The *ENA*2 gene was activated by a high external pH.

Besides the high external pH, the presence of Na^+ was also required for the expression of *ENA*1 (Banuelos and Rodríguez-Navarro, 1998). The *CYC*1_0 gene was activated by oxygen. Other interesting finding is that glucose repression of the *CYC*1_0 gene did not occur in *Schw. occidentalis*, but did in *S. cerevisiae* for an unknown reason. The *EG*1 only expressed in the early stage of cell growth, and thus its expression regulation might be controlled by an unknown mechanism during cell cycle (Prakash and Seligy, 1988). In addition, The regulations of the *AMY*, *CYC*1_0 and *GAM* gene expressions were demonstrated at the mRNA level (Abarca et al., 1989, 1991; Amegadzie et al., 1990; Dowhanick et al., 1990).

1.2.7.2 Heterologous Gene Expression

1.2.7.2.1 *Schw. occidentalis* DNA (genes) in Other Organisms

Two *Schw. occidentalis ARS* functioned in their own hosts as well as in *S. cerevisiae* (Dohmen et al., 1989, 1990; Piontek et al., 1990). The mitotic stability and copy number of a vector with *Schw. occidentalis ARS* were same in *S. cerevisiae* and *Schw. occidentalis* (Dohmen et al., 1989). This means that the components required for *S. cerevisiae* DNA replication initiation could normally work on *Schw. occidentalis* replicons. However, no further investigation reveals the structures of *Schw. occidentalis ARS* and whether the *ARS* could function in other yeasts or not.

Some *Schw. occidentalis* genes expressed in other yeasts such as *S. cerevisiae*, *K. lactis*, *S. pombe* and in bacteria, *E. coli*, and *Streptomyces lividans*. The genes expressed in *S. cerevisiae* included *ADE*2 (Klein and Favreau, 1988), *AMY* (Abarca et al., 1988; 1989; Strasser et al., 1989; Wang et al., 1989; Abarca et al., 1991; Park et al., 1992; Puta et al., 1994), *CYC*1_0 (Amegadzie et al., 1990), *GDH* (De Zoysa et al., 1991), *HAK*1 (Banuelos et al., 1995), *HXK* (Rose, 1995), *LEU*2 (Iserentant and Verachtert, 1995), *ODC* (Klein and Roof, 1988), *SCR*1, *SCR*2 (Del Pozo et al., 1993), *SWA*1 (Abarca et al., 1988), and *SWA*2 (Abarca et al., 1991; Puta et al., 1994). Moreover, a *Schw. occidentalis* α-amylase was produced and secreted by *K. lactis*, *S. cerevisiae*, and *S. pombe* (Strasser et

al., 1989). Furthermore, the *Schw. occidentalis LEU*2 gene complemented *E. coli leu*B mutant (Iserentant and Verachtert, 1995), and *S. lividans* produced and secreted *Schw. occidentalis* α-amylase when the *AMY* gene was cloned into *S. lividans* (Wang et al., 1990, unpublished). These gene expressions show that the promoters, signal sequences and regulatory systems of the *Schw. occidentalis* genes functioned in other yeasts and in procaryotes. However, genes expressed in *S. cerevisiae* and *S. pombe* with different transcription initiation sites (Russell, 1983). Whether this phenomenon also occurs on other *Schw. occidentalis* genes expressing in other organisms or not is unknown. Not all *Schw. occidentalis* promoters could function in other host cells. The *Schw. occidentalis GAM*1 gene did not express in *S. cerevisiae* and *H. polymorpha*, unless the *GAM*1 promoter was replaced with *S. cerevisiae* promoters of *GAL*1, *PDC*1 and *ADH*1 and *H. polymorpha* formate dehydrogenase promoter (Dohmen et al., 1989, 1990; Gellissen et al., 1990; 1991). In this case, the signal sequence of the glucoamylase was maintained during the DNA recombination and directed the secretion of glucoamylase into medium. The signal sequence of the *GAM*1 gene was also applied successfully to secrete a leech hirudin from *H. polymorpha* (Weydemann et al., 1995). Thus, at least some *Schw. occidentalis* genes offer the potential to heterologously express in other microorganisms.

1.2.7.2.2 Heterologous DNA (Genes) in *Schw. occidentalis*

The replicon of 2-μm plasmid and an *ARS* of *S. cerevisiae* functioned in *Schw. occidentalis* (Klein and Favreau, 1988; Dohmen et al., 1989). This indicates that the replication initiation components of *Schw. occidentalis* can recognize these foreign replicons. However, a vector with a *S. cerevisiae ARS* showed less transformation efficiency, mitotic stability and copy number than vectors containing the 2-μm plasmid replicon or a *Schw. occidentalis ARS*. It suggests that the replication initiation of *ARS* may be more complex than that of 2-μm plasmid replicon, and *Schw. occidentalis* stabilizes its *ARS* more easily than a heterologous *ARS* (Dohmen et al., 1989). Choosing an *ARS* to construct vectors for *Schw. occidentalis* should thus be empirical. It has been reported that the *LEU*2, *TRP*5 and *URA*3 genes of *S. cerevisiae* (Dohmen et al., 1989; Dave and Chattoo, 1997; Wang and Lee, 1997) and a *Clostridium thermocellum* cellulase

gene were heterologously expressed in *Schw. occidentalis* (Piontek et al., 1990). The *LEU2*, *TRP5* and *URA3* genes of *S. cerevisiae* complemented the corresponding auxotrophic mutants of *Schw. occidentalis*. These results imply that genes other than the *LEU2*, *TRP5* and *URA3* genes from *S. cerevisiae* or other yeasts might function as genetic markers in corresponding auxotrophic mutants of *Schw. occidentalis*. Additionally, *C. thermocellum* cellulase was expressed and secreted by *Schw. occidentalis*, when transformants carried a recombinant DNA in which the cellulase gene was fused with a *Schw. occidentalis GAM1* gene (Piontek et al., 1990). The fate of foreign DNA in *Schw. occidentalis* has also been studied. Vectors pYES2.0 and YEp13 recovered from *Schw. occidentalis* transformants did not change in their sizes and structures (Dave and Chattoo 1997), revealing that *Schw. occidentalis* does not modify foreign DNA and could be used as a promising host to accommodate heterologous genes for protein productions.

1.2.8 SECRETION

1.2.8.1 Secretion Ability and Glycosylation

Schw. occidentalis not only produces extracellular enzymes such as α-amylase, glucoamylase, β-glucosidase, invertase, but also secretes very efficiently large protein (glucoamylase) with molecular weight up to 155 kDa into medium (Wilson and Ingledew, 1982; Dowhanick et al., 1987; 1990; Howard et al., 1988; Boze et al., 1989b; Klein et al., 1989a). At least 95 % of α-amylase could be excreted (Strasser et al., 1992). The *Schw. occidentalis* invertase was found in the culture medium, but the *S. cerevisiae* invertase was detected in the periplasmic space (Klein et al., 1989b). Moreover, *Schw. occidentalis* has less glycosylation on secreted proteins than *S. cerevisiae*. Amylolytic enzymes produced by *Schw. occidentalis* contained only 5-15 % of carbohydrate moiety (Deibel et al., 1988; Boze et al., 1989b; Strasser et al., 1989; Park et al., 1992). *Schw. occidentalis* invertase contained only 17 % glycosylation, compared to 60 % from *S. cerevisiae* (Klein et al., 1989a&b). Besides, *Schw. occidentalis* did not produce measurable amount of

extracellular proteases (Klein et al., 1989a). Thus, *Schw. occidentalis* should be a better host system than *S. cerevisiae* to produce heterologous proteins.

1.2.8.2 Signal Sequences

The *AMY*, *GAM*1, *INV* and *SWA*2 genes encode extracellular enzymes and the *SCR*2 gene was proposed to encode a nuclear protein. Both extracellular and nuclear proteins are directed to their specific target locations by a signal peptide, called signal sequence and nuclear location sequence, respectively (Figure 1.2.4). The *SCR*2 gene encoded a putative ribosomal protein, which contained a common nuclear localization sequence of Pro-Lys-Thr-Arg-Lys required to transport the ribosome protein through nuclear membrane (Del Pozo et al., 1993). The signal sequences are located on the N-terminus of the proteins with different length and amino acid composition. The α-amylases, glucoamylase and invertase of *Schw. occidentalis* were supposed to possess signal sequences in length from 22 to 33 residues of amino acids. Generally, the signal sequences have a tripartite structure: an N-terminus with positive-charged amino acids, a central region with high hydrophobic property and a C-terminus with a polar amino acid as cleavage site (von Heijne, 1985). Except for the invertase, both the α-amylases and the glucoamylase had signal sequences with a positive-charged amino acid (either Arg or Lys) at their N-terminus whose role was proposed to be important for properly directing signal sequence toward endoplasmic reticulum (Sakaguchi et al., 1992). However, the invertase might either use a polar residue of glutamine (Q) substituting the positive-charged amino acids or use a different mechanism to orient the signal sequence to endoplasmic reticulum. The spans of the hydrophobic region of the signal sequences are from 4 to 8 amino acid residues. Compared with other secreted proteins (von Heijne, 1986), the proposed cleavage sites of these secreted *Schw. occidentalis* enzymes theoretically occurred after the sequence of VAS or VSA (except QAA from glucoamylase) at the C-terminus following the hydrophobic central region. Another way to directly certify the cleavage sites is to determine the N-terminal sequences of the purified matured proteins. By this way, the cleavage site of the glucoamylase was known to be after the sequence of QAA

(Dohmen et al., 1990). However, these two methods might give different cleavage sites. The cleavage sites of the signal sequences of the *Schw. occidentalis* α-amylases were predicted to be between Ala^{25} and Gln^{26}. However, the N-terminal amino acids of the purified matured α-amylases from different host cells indicated that their cleavage sites should be between Phe^{30} and Asp^{31} or between Arg^{33} and Asp^{34} (Strasser et al., 1989; Puta et al., 1994). A proteolytic processing might be required for the α-amylase maturation, but it is not clear why the endo-cleavage site of the same enzyme is different. Further work is required to resolve these phenomena.

1.2.9 APPLICATIONS AND STRAIN IMPROVEMENT

It is postulated that the cloned *Schw. occidentalis* genes might be employed for studying the physiology, biochemistry and genetics of this microorganism. As the *EG*1 gene only expressed in the early stages of growth, it could be used to study the mechanisms controlling cell division at the molecular level (Prakash and Seligy, 1988). The $CYC1_0$ gene might be used to enhance our understanding of the respiratory system of *Schw. occidentalis* and possibly as a new index of yeast classification (Amegadzie et al., 1990). The *ADE*2, *GDH*, *LEU*2 and *ODC* genes could be utilized to study the biosynthesis of purine, pyrimidine and amino acids. The *HXK* gene is associated with phosphorylation in carbohydrate metabolism (Rose, 1995). *Schw. occidentalis* has a low ethanol tolerance (Wilson et al., 1982), and thus it might require the high expression of $CYC1_0$ to prevent ethanol accumulation when the cells grow in a high sugar medium. The *ENA*1, *ENA*2, and *HAK*1 genes could be used to investigate the K^+ and Na^+ transport systems in fungi (Banuelos et al., 1995; Banuelos and Rodríguez-Navarro, 1998). Codon usage has been considered as one of the factors affecting gene expression (Sharp and Cowe, 1991). However, the effect of codon usage in *Schw. occidentalis* gene expression requires more work. The cloned genes could also be used to investigate regulation of gene expression and the relationships between proteins and DNA. Due to a high homology, some cloned genes from other organisms such as *S. cerevisiae* might be used as probes to map the chromosomes of *Schw. occidentalis*.

For vector constructions, the *ADE2*, *AMY*, *GDH*, *GAM*1, *LEU2*, *ODC*, *SCR*1 and *SCR*2 genes could be used as genetic markers. The *ADE2*, *GDH*, *LEU2* and *ODC* genes could complement corresponding auxotrophic mutants. The *SCR*1 and *SCR*2 genes offer their host cells ability to resist cycloheximide (Abarca et al., 1990; Del Pozo et al., 1991; 1993). As well, the G418-resistant determinant from a G418-resistant *Schw. occidentalis* (Panchal et al., 1984) is another potential genetic marker which has been used in other yeasts such as *H. polymorpha* (Janowicz et al., 1991), *Kluyveromyces* spp. (Das and Hollenberg, 1982; Das et al., 1984; Chen et al., 1989; Chen et al., 1992b), *Phaffia rhodozyma* (Adrio and Veiga, 1995), *Pichia* spp. (Ho et al., 1991; Scorer et al., 1994), *S. cerevisiae* (Jimenez and Davies, 1980), *S. pombe* (Gmunder and Kohli, 1989), and *Torulaspora delbrueckii* (Compagno et al., 1989). The *AMY* and *GAM*1 genes might be used together as a genetic marker to select yeast transformants with the ability to grow on medium containing starch as a sole carbon source. Furthermore, the *Schw. occidentalis* promoters can be used to construct expression vectors. A real instance is an introduction of some useful restriction enzyme cutting sites into the region between the promoter and terminator of *GAM*1 by *in-vitro* mutagenesis to construct an expression vector (Piontek et al., 1990).

Schw. occidentalis should be an excellent host cell to produce heterologous proteins. In industrial scale for enzyme production, secretion has always been emphasized. Secretion not only facilitates protein purification, but also offers secretory pathway for protein folding and post-translational modification environment. Additionally, secretion can prevent host cells from damage if toxic heterologous proteins are present intracellularly. These highlight why the progress towards the secretion mechanism and the construction of secretion vectors has been significant. The *Schw. occidentalis* signal sequences might be useful entity to construct secretion vectors applied in *Schw. occidentalis* and in other yeasts. Furthermore, an ideal engineered host cell should be able to stably maintain foreign gene under non-selective pressure that is up most important for large-scale production. Although the *Schw. occidentalis ARS* containing vectors showed much higher stability than those of the *S. cerevisiae* replicons, it is necessary to devise

integrating vectors with multiple integration, which could increase gene copies resulting in high yields of gene products (Bergkamp et al., 1992; Rossolini et al., 1992; Le Dall et al., 1994; Kondo et al., 1995; Wery et al., 1997; Kim et al., 1998).

After *Schw. occidentalis* was found to grow on starch (Spencer-Martins and Van Uden, 1977), new amylolytic strains (Laluce et al., 1988) and their amylolytic systems have been studied. As starch hydrolysis is a limiting step to produce ethanol, single cell protein and low calorie beer (Sills et al., 1983; Ryu et al., 1992; 1994), the amylolytic enzymes from *Schw. occidentalis* were considered as good candidates to increase starch hydrolysis. Namely, the amylolytic enzyme genes of *Schw. occidentalis* could be applied to improve the starch hydrolysis by yeasts which have no or low starch hydrolysis capacity. Actually, the *Schw. occidentalis AMY* and *GAM*1 genes have been transferred into *S. cerevisiae* and *Schw. occidentalis* itself to produce more amylolytic enzymes. Both *AMY*1 and *GAM*1 genes of *Schw. occidentalis* under the control of *S. cerevisiae GAL*1 promoter were transformed into *S. cerevisiae*, and the host cells were able to grow efficiently on raw starch (Dohmen et al., 1988). A five-fold increase in α-amylase activity from *Schw. occidentalis* transformants was observed when a *Schw. occidentalis AMY* gene was introduced into the host cells (Dohmen et al., 1989). Invertase is also an important enzyme applied in food industry, and thus the *Schw. occidentalis INV* gene might be used to increase the enzyme production as well. The production of α-amylase and glucoamylase by *Schw. occidentalis* was also repressed by glucose. In order to relieve the glucose repression, *Schw. occidentalis* mutants were developed by mutation with UV irradiation, ethyl methane sulfonate, and nitrosoguanidine (Dhawale and Ingledew, 1983a&b; McCann and Barnett, 1984; Sills et al., 1984; Boze et al., 1989a; Horn et al., 1991; Stobinska et al., 1992). However, no report was found about the cloning of a derepressed amylolytic gene, which could help to understand the expression regulation of the amylolytic genes.

1.2.10 CONCLUSIONS

This review discussed the research achievements that have contributed to our present knowledge of *Schw. occidentalis*, which has been renamed as *Debaryomyces occidentalis* (Klocker). *Schw. occidentalis* is one of the important amylolytic enzyme-producing yeasts that can be applied to produce ethanol as well as single cell protein. Although the study of *Schw. occidentalis* is a new field, an impressive progress has been made in recent years that includes development of transformation systems, gene cloning, gene structures, gene expression, and secretion. To date, no native plasmid was found from *Schw. occidentalis*. Four *Schw. occidentalis* transformation systems based on replicons of the 2-μm plasmid, and *ARS* from *S. cerevisiae* and *Schw. occidentalis* were reported. The yeast contains at least 7 chromosomes with a G+C content of around 35 %. Twenty-one *Schw. occidentalis* genes encoding 14 kinds of enzymes or proteins have been cloned. Although most of the *Schw. occidentalis* genes discussed had high homology with the corresponding genes from *S. cerevisiae*, there was low homology between the operators, promoters, transcription initiation sites, and terminators of *Schw. occidentalis* genes and those of *S. cerevisiae*. However, more data are required to substantiate these hypotheses. *Schw. occidentalis* genes or *ARS* could express in other yeasts and in bacteria. Likewise, the heterologous genes and replicons can function in *Schw. occidentalis*. These achievements are prerequisite to conduct more advanced research of *Schw. occidentalis* at molecular level in the future. *Schw. occidentalis* possess: 1) the ability to use cheap starch as a carbon source to grow with rapid growth rate and high cell mass; 2) the ability to secrete large proteins; 3) low glycosylation; 4) non-measurable extracellular proteases; 5) transformation systems; 6) no modification foreign DNA; and 7) inducible promoters. Consequently, the above advantages justify why *Schw. occidentalis* could be an alternative yeast to *S. cerevisiae* as a promising host system to produce heterologous proteins.

*AMY*1	$_{-281}$**G**TTCT	**T**AC**CG**-165-**G**AAGG	TGC**CG**-11-TATAAA	
*CYC*1$_0$	$_{-516}$**G**TTTTCT	**T**CT**CG**-389-TATATA		
*EG*1	$_{-113}$**G**CATTA	**T**TA**CG**-2-TATAAA		
*GAM*1	$_{-127}$**G**TAAT	**T**TG**CG**-51-TATAAA		
*HAK*1	$_{-766}$**G**CTAG	**T**TA**CG**-521-TATAAT		
*SCR*2	$_{-977}$**G**AAGC	TGA**CG**-178-**G**TTGCA	**T**TT**CG**-348-**G**CACAC	TTA**CG**-259-TATACT

Figure 1.2.1. A putative activator binding site found from *Schw. occidentalis* genes. The consensus sequence is 5'–G-N-N-N-N-T-N-N-C-G-3' (Guarente, 1992). Numbering is upstream from the translation start codon ATG.

ADE2 $_{-97}$TATGAA-19-**CTTT**-14-**CTTT**-2-TAAA-11-TAAA-29-ATG

AMYP $_{-90}$TATAAA-31-**CTCTCT**-4-C<u>AAAGCT</u>-15-TAAATAAA-1-TAAA-2-CAAG-2-ATG

AMYS $_{-89}$TATAAA-29-**TCTCTCT**-4-C<u>AAAGCT</u>-15-TAAATAAA-1-TAAA-2-CAAG-2-ATG

AMYW $_{-90}$TATAAA-31-**CTCTCT**-4-C<u>AAAGCT</u>-15-TAAATAAA-1-TAAA-2-CAAG-2-ATG

CYC1$_0$ $_{-115}$TATATA-38-**TTTTTTTCCTTTTTTTTTTTC**-50-ATG

EG1 $_{-100}$TATAAA-8-**TATTC**-11-<u>TTTAAT</u>-35-TAAA-2-CAAG-10-TAAG-5-ATG

GAM1 $_{-66}$TATAAA-34-**CTCAT**-10-<u>TAAC</u>AAGCAAGATG

GDH $_{-104}$TATAAA-22-**TCACTTTTTTTTC**-1-GAAA-3-GAAA-26-TAAG-8-GAAA-9-ATG

HAK1 $_{-233}$TATAAT-134-**CTATCC TTTT**-4-TAAA-4-GAAG-11-CAAA-21-TAAA-2-GAAG-14-CAAG-5-ATG

HXK $_{-99}$TATATA-19-**CATTTGTCT**-3-GAAA-19-TAAA-25-TAAG-2-CAAAATG

INV $_{-47}$TATAAA-15-**CCAT**-14-<u>AG</u>AAA-23-ATG

LEU2 $_{-124}$TATGAT**TCTTCTCGCTTCC**-72-CAAG-29-ATG

SCR2 $_{-154}$TATACT-15-**CTATTCTCT**-111-TAA<u>A</u>-9-ATG

SWA2 $_{-79}$TATATA-35-**TTTTTTGTTC**-3-<u>A</u>-1-TAAA-3-TAAA-12-ATG

Figure 1.2.2. An alignment of transcription initiation sites of 12 *Schw. occidentalis* genes. The consensus sequences is 5' - TATA box --- (CT)n --- PyAAPu --- ATG - 3'. The bold sequence indicates the CT block. The sequences underlined mean the transcription initiation sites determined by S1-nuclease mapping. The numeral given in the front of each sequence indicates the distance from ATG codon. The numeral found within each sequence indicates the total number of nucleotides between the two segments flanking it.

	-3	1	4 6		-3	1	4 6
ADE2	**ATA**	ATG	**GAT**	*HAK1*	TTG	ATG	TTG
AMY1	**GAC**	ATG	**AGA**	*HXK*	**AAA**	ATG	**GTT**
$CYC1_0$	**ACA**	ATG	CCA	*INV*	**AAC**	ATG	**GTA**
EG1	TGG	ATG	**AAA**	*LEU2*	**GCA**	ATG	**GAG**
GAM1	**AAG**	ATG	**ATT**	*SCR2*	**ATA**	ATG	**GTT**
GDH	**GAG**	ATG	**ATT**	*SWA2*	**ACG**	ATG	**AAA**

Figure 1.2.3. The flanking nucleotides around ATG codon of *Schw. occidentalis* genes. The alignment of these sequences gives a conservation of 5'- (A/G) NN ATG (A/G) N (A/T) – 3'.

A. Nuclear protein

Ribosome protein	*SCR2*	(5) PKTRK					

B. Secreted proteins

			P[a]	Hydrophobic		CS[b]
Amylase	*AMYP*[c]	(33) M	RFSTEGETSK	VVAAILAFSRL	VSA	QPIIF DLR ⇓ DVS
Amylase	*AMYS*[d]	(30) M	RFSTEGFTSK	VVAAILAFSPL	VSA	QPIIF ⇓ DMR DVS
Amylase	*AMYW*[e]	(25) M	RFSTEGFTSK	VVAAILAFSPL	VSA↓	QPIIF DKR DVG
Amylase	*SWA2*	(20) M	KFATILSTT	ALAL	SSL VAS↓	KPI
Glucoamylase	*GAM1*	(23) MIFL	KLIKS	IVIGLGLV	SAI QAA ⇓	PAS
Invertase	INV	(22) MVQVLS		VLVIPLL	TLFFGYVAS ⇓	SSI

Figure 1.2.4. The signal sequences of *Schw. occidentalis* proteins. The signal peptide in A section is called nuclear location sequence, in B section signal sequence. ( ): the number of amino acids in the signal peptide. ↓: the putative cleavage site. ⇓: the putative cleavage site deduced by determining the N-terminus of the purified protein. __: amino acids characteristic in each region. [a]P: positive-charged amino acid. [b]CS: Cleavage site. [c]*AMYP* (Puta et al., 1994). [d]*AMYS* (Strasser et al., 1989). [e]*AMYW* (Wu et al., 1991).

Table 1.2.1. Transformation systems developed for *Schw. occidentalis*.

Host	Vector			Transformation		Stb[b] (%)	Reference
	Name	Marker	Replicon	Method	Eff.[a]		
ade2	pADE	*ADE2* (So[c])	2μ *ORI* (Sc[d])	Spheroplast	10^3	ND[e]	Klein and Favreau, 1988
ade2	pADE-2	*ADE2* (So)	*ARS* (So)	Spheroplast	10^2	ND	Klein and Favreau, 1988
ade2	pADE	*ADE2* (So)	2μ *ORI* (Sc)	Electroporation	10^5	ND	Costaglioli et al., 1994
trp5	YRp7-TRP5	*TRP5* (Sc)	*ARS1* (Sc)	Competent cell	10^0-10^1	1	Dohmen et al., 1989
trp5	pYAS1	*TRP5* (Sc)	2μ *ORI* (Sc)	Competent cell	10^1-10^2	5-10	Dohmen et al., 1989
trp5	YRpJD2	*TRP5* (Sc)	Sw*ARS1* (So)	Competent cell	10^2-10^3	10-20	Dohmen et al., 1989
ura3 or (*ura5*)	pYES2.0	*URA3* (Sc)	2μ *ORI* (Sc)	Competent cell	10^4	ND	Dave and Chattoo 1997

[a]eff: transformation efficiency defined as the number of transformants per μg of DNA used for transformation.

[b]Stb: The vector stability was determined after 15 generations of growing transformants in non-selective condition.

[c]So: *Schw. occidentalis*

[d]Sc: *S. cerevisiae*.

[e]ND: not-detected.

Table 1.2.2. The *Schw. occidentalis* gene cloning.

Enzyme/Protein	Gene	Gene size	(G+C) %	Gene Source	Cloning host	Reference
NP[a]	*EG*1	474	37.75	ATCC 26074	*E. coli*	Prakash and Seligy, 1988
α-Amylase	*AMY*	1,536	37.62	ATCC 44442	Sc[b]	Wang et al., 1989; Wu et al., 1991
α-Amylase	*SWA*2	ND[c]	ND	------[d]	Sc	Puta et al., 1994
α-Amylase	*AMY*	1,536	37.60	ATCC 26077	Sc	Park et al., 1992
α-Amylase	*AMY*1	1,536	37.42	ATCC 26076	Sc	Strasser et al., 1989
α-Amylase	*SWA*1	ND	ND	ATCC 26077	Sc	Abarca et al., 1988; 1989
α-Amylase	*SWA*2	1,521	37.14	ATCC 26077	Sc	Abarca et al., 1991; Claros et al., 1993
ATPase	*ENA*1	3,165	34.91	ATCC 26076	Sc	Banuelos and Rodriguez-Navarro, 1998
ATPase	*ENA*2	3,246	34.57	ATCC 26076	*E. coli*	Banuelos and Rodriguez-Navarro, 1998
Cytochrome c protein	$CYC1_0$	333	40.30	ATCC 26076	Sc	Amegadzie et al., 1990
Glucoamylase	*GAM*1	2,874	36.63	ATCC 26076	*E. coli*	Dohmen et al., 1988; 1990
β-Glucosidase	---	---	---	ATCC 2676	---	Klein et al., 1989a
Hexokinase	*HXK*	1,437	37.71	ATCC 2322	*E. coli*	Rose, 1995
Invertase	*INV*	1,599	35.01	ATCC 2076	*E. coli*	Klein et al., 1989b
β-Isopropylmalate dehydrogenase	*LEU*2	1,140	31.83	ATCC 26077	Sc	Iserentant and Verachtert, 1995
NADP-dependent glutamate dehydrogenase	*GDH*	1,380	40.51	NCYC 953	*E. coli*	De Zoysa et al., 1991
Orotidine 5'-phosphate decarboxylase	*ODC*	ND	ND	ATCC 26076	Sc	Klein and Roof, 1988
Phosphoribosyl aminoimidazole carboxylase	*ADE*2	1,671	35.53	ATCC 26076	Sc	Klein and Favreau, 1988 Gourdon et al., 1995
Potassium transporter	*HAK*1	2,286	32.10	ATCC 26076	Sc	Banuelos et al., 1995
Ribosomal protein	*SCR*1	ND	ND	ATCC 26077	Sc	Del Pozo et al., 1993
Ribosomal protein	*SCR*2[e]	321	34.90	ATCC 26077	Sc	Del Pozo et al., 1993

[a]NP: might be a novel protein.

[b]Sc: *S. cerevisiae*.

[c]ND: not-detected.

[d]---: no report found from cited publication.

[e] The open reading frame of the *SCR*2 is interrupted by a putative intron.

Table 1.2.3. The structures of *Schw. occidentalis* genes.

Gene	Operator		Promoter		Ribosomal binding site		Reference
	G-T-CG[a]	TGACTC	CAAT	TATA	CT block	TIS[b]	
ADE2	---	-131	-389	-97	-72	?[c]	Gourdon et al., 1995
AMY1	-281	---	-115	-89	-54	-42 to -37	Strasser et al., 1989
$CYC1_0$	-516	-466	-164	-115	-71	?	Amegadzie et al., 1990
EG1	-113	---	---	-100	-86	-70 to -65	Prakash and Seligy, 1988
GAM1	-127	---	-132	-66	-26	-11 to -8	Dohmen et al., 1990
GDH	---	---	---	-104	-76	?	De Zoysa et al., 1991
HAK1	-766	-170	---	-235	-95	?	Banuelos et al., 1995
HXK	---	---	---	-99	-74	?	Rose, 1995
INV	---	-339	-302	-67	-42	-28 to -26	Klein et al., 1989b
LEU2	---	---	---	-124	-118	?	Iserentant and Verachtert, 1995
SCR2	-977	---	-176	-154	-133	-10	Del Pozo et al., 1993
SWA2	---	---	-168	-79	-38	-25	Claros et al., 1993

[a]G-T-CG: the sequence of 5`- GNNNNTNCG - 3'.

[b]TIS: transcription initiation start. [c]?: not determined.

Table 1.2.4. The putative TATA box sequences of *Schw. occidentalis* promoters.

Gene	TATA box	Reference
ADE2	TATAAT	Gourdon et al., 1995
AMY1	TATAAA	Strasser et al., 1989
$CYC1_0$	TATATA	Amegadzie et al., 1990
EG1	TATAAA	Prakash and Seligy, 1988
GAM1	TATAAA	Dohmen et al., 1990
GDH	TATAAA	De Zoysa et al., 1991
HAK1	TATAAT	Banuelos et al., 1995
HXK	TATATA	Rose, 1995
INV	TATAAA	Klein et al., 1989b
LEU2	TATGAT	Iserentant and Verachtert, 1995
SCR2	TATACT	Del Pozo et al., 1993
SWA2	TATATA	Claros et al., 1993

Table 1.2.5. The consensus sequences deduced from the 3'-untranslated region of *Schw. occidentalis* genes.

Gene	TAG --- TTT[a]	AAGAAA	AATAAA	AATATA
ADE2	+			
AMY1	+			
$CYC1_0$	+/-	+		
EG1	+	+		+
GAM1	+	+		+
HAK1	+	+		+
HXK	+			+
INV	+			+
LEU2	+/-	+		
SCR2	+		+	
SWA2	+		+	+
GDH	?	?	?	?

[a]TAG --- TTT: 5'- TAG --- TAGT (or TATGT) --- TTT – 3'. The symbol of + means the sequence can be found from the gene. The symbol of +/- means only part of the consensus sequence can be deduced from the gene. The *GDH* gene has a published sequence with only 42 bp in its 3'-untranslated region that might result in no typical terminator structure deduced.

Table 1.2.6. The regulators of *Schw. occidentalis* gene expression.

Gene	Activator	Inactivator	Reference
AMY, *SWA*	β-cyclodextrin, maltose, starch	Glucose	De Mot and Verachtert, 1986; Abarca et al., 1989, 1991; Dohmen et al., 1989; Dowhanick et al., 1987; Wang et al., 1989
ENA1	ATPase	High pH and Na^+	Banuelos and Rodríguez-Navarro, 1998
ENA2	ATPase	High pH	Banuelos and Rodríguez-Navarro, 1998
GAM1	β-cyclodextrin, maltose, starch	Glucose, heat shock	De Mot and Verachtert, 1986; Dohmen et al., 1990; Dowhanick et al., 1990
HAK1	Glucose	Neomycin	Banuelos et al., 1995
INV	Lactose, sucrose	Glucose	Klein et al., 1989b
$CYC1_0$	Oxygen	Glucose*	Amegadzie et al., 1990

*The glucose repression was only in *S. cerevisiae*, but not in *Schw. occidentalis*.

CHAPTER 2.0

A TRANSFORMATION SYSTEM FOR *SCHWANNIOMYCES OCCIDENTALIS*

In order to study the molecular biology of *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*), a transformation system of the yeast is a prerequisite. This chapter describes a developed *Schw. occidentalis* transformation system, which was based on the complementation of *Saccharomyces cerevisiae LEU2* gene in a *leu* auxotrophic mutant of *Schw. occidentalis*.

The major results of this study were summarized and submitted as a manuscript for publication in *Biotechnology Techniques* (1997 11: 307-309). The manuscript entitled "Transformation system for *Schwanniomyces occidentalis*" was co-authored by Tsung-Tsan Wang, and Byong H. Lee. The project was supervised by Dr. Byong H. Lee, while the actual experimental work, and writing of the manuscript were done by Tsung-Tsan Wang. Dr. Byong Lee edited the manuscript prior to its submission for publication.

2.1 ABSTRACT

A new transformation system of *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*) has been developed. After treatment with 1-methyl-3-nitro-1-nitrosoguanidine of *Schw. occidentalis*, a *leu* auxotrophic mutant was obtained. Spheroplast-mediating method was used for the yeast transformation with transformation efficiency of 10^3 transformants/μg DNA. The *leu* mutant was complemented with *Saccharomyces cerevisiae LEU2* gene carried by YEp13. Although low stability, YEp13 could be recovered from yeast transformants and had the same size and restriction enzyme cutting sites like the original one. The 2-μm replicon is proposed to be responsible for YEp13 replication in *Schw. occidentalis*.

2.2 INTRODUCTION

Ingledew (1987) described *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*) as a potential "super yeast". Recently, both academic and industrial interests have focused on *Schw. occidentalis*, because it is able to use cheap starch as a carbon source to grow with rapid growth rate and high cell mass (Moresi et al., 1983; Jamuna and Ramakrishna, 1989; Horn et al., 1992), produce strong amylolytic enzymes extracellularly and secrete large proteins (Ingledew, 1987) without hyper-glycosylation and measurable extracellular proteases (Deibel et al., 1988). These characteristics make *Schw. occidentalis* as a potential host system to replace *Saccharomyces cerevisiae* for producing heterologous proteins. However, the research in molecular biology of *Schw. occidentalis* has been hampered by few transformation systems. Still now, there are only three transformation systems developed for *Schw. occidentalis* (Klein and Favreau, 1988; Dohmen et al., 1989; Dave and Chattoo, 1997). They were based on the complementation of *ade*2, *trp*5, and *ura*3 (or *ura*5) auxotrophic mutants with corresponding genes. *ADE*2, *TRP*5, and *URA*3 genes, respectively. The *ADE*2 gene was cloned from *Schw. occidentalis*, and the *TRP*5 and *URA*3 genes were derived from *S. cerevisiae*. The transformations were carried out by spheroplast-mediating method (Klein and Favreau, 1988), competent cell method (Dohmen et al., 1989; Dave and Chattoo 1997), or electroporation method (Costaglioli et al., 1994) with transformation efficiency of 10^3, 1-10^4, 10^5 transformants/μg DNA, respectively.

As discussed in literature review (1), the *S. cerevisiae LEU*2 gene was the most widely used transformation marker in *S. cerevisiae* and 13 other yeast genera. It is proposed that the *LEU*2 gene might also complement a corresponding leucine auxotrophic mutant. Moreover, a lot of vectors based on the *LEU*2 gene have been constructed. This study aims at establishing a new transformation system based on the complementation of the *LEU*2 gene in a corresponding *leu* auxotrophic mutant of *Schw. occidentalis*. Thus, it is useful that the transformation system can take advantage of the developed *LEU*2-based vectors.

2.3 MATERIALS AND METHODS

2.3.1 Chemicals and enzymes

Unless otherwise specified, all chemical reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Restriction enzymes, agarose and DNA molecular weight markers were obtained from Boehringer Mannheim (Laval, Quebec, Canada). Yeast nitrogen base without amino acids was obtained from Difco Lab. (Detroit, Michigan, USA). Glusulase was obtained from Dupont Co. (Boston, Massachusetts, USA). Other chemicals were obtained from Anachemia (Montreal, Quebec, Canada).

2.3.2 Strains, vectors, and media

The strains and vectors used in this study are listed in Table 2.1. *E. coli* was cultivated in LB medium (0.5 % yeast extract; 1 % Tryptone; 1 % NaCl) at 37°C (Maniatis et al., 1982). The amount of antibiotic added to LB was 100 μg/ml ampicillin, 15 μg/ml tetracycline when needed. Yeasts were cultivated in YPD medium (1 % yeast extract; 2 % Bacto-peptone; 2 % glucose) and MM medium (0.67 % Difco yeast nitrogen base without amino acid; 2 % glucose; 20 μg/ml adenine; 20 μg/ml uracil) at 30 °C (Kaiser et al., 1994). MM medium was supplemented with 30 μg/ml leucine when necessary. For solid media, 2 % Bacto-agar was added into the media. For growing cultures in broth, the cells were grown with agitation by an orbital shaker (Lab-Line Instruments Inc., Melrose Park, Illinois, USA) at 200 rpm.

2.3.3 Yeast mutation

The auxotrophic mutants of *Schw. occidentalis* were obtained by the method of Kaiser et al. (1994), except that 20 μg/ml NTG (1-methyl-3-nitro-1-nitrosoguanidine) was used. Log-phase yeast cells were centrifuged at 4,000 rpm for 15 min with Rotor 215 of IEC Centra-MP4 centrifuge (International Equipment Company, Needham Heights,

Massachusetts, USA), washed with 1 volume of Tris-HCl buffer (0.1 M, pH 7.0) twice, and suspended in 1/10 volume of Tris-HCl buffer. Treated with NTG at 30°C, 30 min, the yeast cells were centrifuged and washed with 1/10 volume of Tris-HCl buffer twice. Suspended and incubated with 1 ml of fresh YDP medium at 30°C for 2 h, the mutated yeast cells were spread on YPD medium plates, and incubated at 30°C for 4 days.

2.3.4 Screen *leu* auxotrophic mutants

Yeast colonies from treatment with NTG were picked up from YPD plates with sterilized toothpicks, replicated on MM and MM plus leucine plates, respectively, and incubated at 30°C for 4 days.

2.3.5 Determination of reversion rate of mutants

Leucine auxotrophic mutants were picked up from MM plus leucine plates, inoculated in 100 ml of YPD broth, and continuously grown for 48 h. Diluted cell cultures (from 10^0 to 10^7) were spread on YPD and MM agar plates, respectively, and incubated at 30°C for 4 days. The reversion rate was defined as the colony number appearing on MM plates divided by the colony number appearing on YPD plates.

2.3.6 Determination of yeast spheroplast generation rate

Yeast spheroplast generation rate was determined by a modified method of Kaiser et al. (1994). Twenty milliliters of yeast cells in log phase and in stationary phase, respectively, were centrifuged at 4,000 rpm for 15 min with Rotor 215 of IEC Centra-MP4 centrifuge, and washed with 50 ml of water. Suspended in 50 ml of SED buffer (1 M sorbitol; 25 mM EDTA (ethylene diamine tetraacetate), pH 8.0; 50 mM DTT (dithiothreitol)) and incubated at 30 °C for 10 min, the cells washed twice with 50 ml of 1.2 M sorbitol. The cell pellet was suspended in 20 ml of SCE buffer (1.2 M sorbitol; 0.1 M sodium citrate, pH 5.8; 10 mM EDTA), added with 0.2 ml of glusulase and incubated at 30 °C for 20 min. From then on, centrifugation was performed under 2,500 rpm for 3

min with the same rotor. The cells were centrifuged, washed twice with 50 ml of 1.2 M sorbitol, suspended in 1 ml of 1.2 M sorbitol, divided into 100 µl aliquots in 1.5 ml of Eppendorf tubes, added with 100 µl of different concentration of sorbitol (1 M, 1.5 M, 2 M, and 2.4 M, respectively) and left at room temperature for 30 min. Diluted (from 10^0 to 10^7) with corresponding concentration (1 M, 1.5 M, 2 M, and 2.4 M, respectively) of sorbitol, an aliquot of diluted cells was transferred into a tube containing 20 ml of pre-warm (48 °C) YPD medium with sorbitol in corresponding concentration (1 M, 1.5 M, 2 M, and 2.4 M, respectively), gently mixed well, poured out into Petri-dish, and incubated at 30 °C for 4 days. A negative control was obtained by using water to replace sorbitol, and positive control was based on the cells without any enzyme treatment. Spheroplast generation rate is defined as colony number from enzymatic treatment divided by colony number from positive control.

2.3.7 DNA isolation.

2.3.7.1 Preparation of vector DNA from *E. coli*

Vector DNA from *E. coli* was prepared with a Midi plasmid purification kit (Qiagen Co., Hilden, Germany) and followed the protocol provided by the company. One hundred milliliters of *E. coli* cells were harvested by centrifugation at 4,000 rpm for 20 min at 4 °C with Rotor 215 of IEC Centra-MP4 centrifuge. The cell pellet was completely suspended in 4 ml of Buffer P1 (10 mM EDTA; 50 mM Tris-HCl, pH 8.0; 100 µg/ml ribonuclease A). Four milliliters of Buffer P2 (1 % SDS (sodium dodecyl sulfate); 200 mM NaOH) were added for cell lysis by gently inverting tube 4 – 6 times and incubated at room temperature for 5 min. To neutralize cell lysate, the tube was added with 4 ml of Buffer P3 (3.0 M potassium acetate) and gently inverted 4 – 6 times and incubated on ice for 15 min. The sample was then centrifuged at 12,000 rpm for 30 min at 4°C (JA-14 rotor of Beckman Model J2-21). During centrifugation, a Qiagen-tip 100 column was equilibrated with 4 ml of Buffer QBT (0.15 % Triton X-100; 15 % isopropanol; 50 mM MOPS (morpholinopropanesulfonic acid), pH 7.0; 750 mM NaCl) and emptied the buffer by gravity flow. After centrifugation, aqueous supernatant was

pipetted to the equilibrated column, and allowed to enter the resin by gravity flow. The column was washed twice with 10 ml of Buffer QC (15 % isopropanol; 50 mM MOPS, pH 7.0; 1.0 M NaCl) and 5 ml of Buffer QF (15 % isopropanol; 50 mM Tris-HCl, pH 8.5; 1.25 M NaCl) was added to elute DNA. The eluted DNA solution was mixed with 3.5 ml of isopropanol and centrifuged immediately at 12,000 rpm for 30 min at 4°C. The DNA pellet was washed with 2 ml of 70 % ethanol, dried at room temperature, and then dissolved in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.3.7.2 Preparation of vector DNA from yeast

Yeast vector DNA was isolated by the method of Kaiser et al. (1994). Fifty milliliters of yeast cells in stationary phase were centrifuged at 4,000 rpm for 15 min with Rotor 215 of IEC Centra-MP4 centrifuge, washed with 50 ml of water, suspended in 50 ml of SED buffer (1 M sorbitol; 25 mM EDTA (ethylene diamine tetraacetate), pH 8.0; 50 mM DTT (dithiothreitol)) and incubated at 30 °C for 10 min. The cells were washed twice with 50 ml of 1.2 M sorbitol, suspended in 50 ml of potassium phosphate buffer (1.2 M sorbitol; 0.1 M potassium phosphate, pH 7.5), and incubated with 0.1 mg of Lysing enzymes (Sigma L2773) at 30 °C for 30 min. The cell pellet after centrifugation was gently suspended in 5 ml of pre-warmed (60°C) lysis buffer (10 mM Tris-HCl, pH 8.0; 10 mM EDTA; 10 % SDS; 100 µg/ml ribonuclease A), and incubated at 60 °C for 30 min. Proteinase K (100 µg/ml) was added into aqueous solution and incubated at 60 °C for 30 min. The aqueous solution was purified with phenol and chloroform. All chloroform used in this protocol was mixed with isoamyl alcohol (24:1, vol/vol). Firstly, the aqueous solution was mixed with 1 volume of pre-warm (60 °C) saturated phenol by gently inverting tube for 5 min, centrifuged at 4,000 rpm for 15 min, and transfer the upper layer solution into a clean tube. The aqueous solution was mixed with 1 volume of saturated phenol/chloroform (1:1, vol/vol) by gently inverting tube for 5 min, centrifuged at 4,000 rpm for 15 min, and the upper layer of solution was transferred into a clean tube. This step was repeated until the white materials on the interface disappeared after centrifugation. The aqueous solution was then mixed with 1 volume of chloroform by gently inverting tube for 5 min, centrifuged at 4,000 rpm for 15 min, and the upper layer

of solution was transferred into a clean tube. The aqueous solution was added 2 volume of ethanol, gently inverted 4 – 6 times, left at room temperature for 5 min. Following centrifugation at 12,000 rpm for 30 min at 4°C, pellet was dried at room temperature, and dissolved in 200 µl of TE buffer. Residues were removed from the DNA solution after centrifugation at 12,000 rpm for 30 min at 4°C. *E. coli* DH10B was used to transfer the total yeast DNA and screened in LB plates containing appropriate antibiotics. The vector DNA was isolated from the *E. coli* transformant.

2.3.7.3 DNA elution, digestion and ligation

The agarose gel containing target DNA was sliced by a sharp knife and put into 3/4 dialysis tubing (GIBCO-BRL, Co.) with a minimum amount of TAE (40 mM Tris-HCl, 20 mM sodium acetate and 20 mM EDTA, pH8.0) buffer as possible and without any air bubble existing inside tube. The dialysis tubing was tied with forceps and put back into electrophoresis tank for further electrophoresis until DNA migrated out of the gel. The solution was pipetted out from dialysis tubing into a new Eppendorf tube, and purified with phenol and chloroform as the procedure shown in isolating yeast DNA. Added with 2 volume of ethanol and 0.1 volume of 3 M sodium acetate, the tube was put in –70 °C for at least 8 h. After centrifugation and drying, the DNA pellet was dissolved in appropriate volume of TE buffer. DNA digestion and ligation followed the instructions for each enzyme provided by Boehringer Mannheim.

2.3.8 DNA Quantitation

A spectrophotometer was used to determine DNA quantity with the reading at wavelength of 260 nm. An OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA (Maniatis et al., 1982).

2.3.9 Agarose gel electrophoresis

Agarose gel (0.8 %) containing 0.5 μg/ml of ethidium bromide was used for DNA electrophoresis, which was carried out in a Mupid 2 electrophoresis tank (Advance, Tokyo, Japan) with TAE buffer. DNA samples were loaded into gel wells with 6× loading buffer (0.25 % bromophenol blue; 40 % glycerol). λ-*Hind*III DNA was applied as standard of molecular weights.

2.3.10 Transformation of microorganisms

2.3.10.1 *E. coli* transformation

E. coli transformation was performed by the competent cell method (Maniatis et al., 1982). A portion (5 ml) of overnight culture was inoculated into 50 ml of fresh LB broth, and grown to OD_{600} of 0.5. After chilling on ice for 10 min, the culture was centrifuged at 4,000 rpm for 20 min at 4°C with Rotor 215 of IEC Centra-MP4 centrifuge. The pellet was suspended in 25 ml of cold (4 °C) 0.1 M $CaCl_2$ and incubated on ice for 30 min. Following centrifugation with 3,000 rpm for 10 min at 4°C, the pellet was suspended in 2 ml of cold 0.1 M $CaCl_2$ and incubated on ice for at least 12 h. The cells were divided into 100 μl aliquots in 1.5 ml of Eppendorf tubes. After addition of 1 – 10 μl DNA, the cells were incubated on ice for 30 min, heated at 42°C water bath for 90 sec exactly, and then chilled on ice for 2 min. Following incubation with 1 ml of LB broth at 37 °C for 60 min without shaking, the cells were plated on LB medium plates containing appropriate antibiotics and incubated at 37 °C for 12 - 20 h.

2.3.10.2 Yeast transformation

Yeast was transformed by a spheroplast-mediating method (Kaiser et al., 1994). Twenty milliliters of yeast cells in stationary phase were centrifuged at 4,000 rpm for 15 min with Rotor 215 of IEC Centra-MP4 centrifuge, and washed with 50 ml of water. The cells were suspended in 50 ml of SED buffer (1 M sorbitol; 25 mM EDTA, pH 8.0; 50 mM DTT), incubated at 30 °C for 10 min, and were washed twice with 50 ml of 1.2 M sorbitol. The cell pellet was suspended in 20 ml of SCE buffer (1.2 M sorbitol; 0.1 M

sodium citrate, pH 5.8; 10 mM EDTA), mixed with 0.2 ml of glusulase and incubated at 30 °C for 20 min. After centrifugation (2,500 rpm, 3 min), the cells were washed twice with 50 ml of 1.2 M sorbitol, with 50 ml of STC buffer (1.2 M sorbitol; 10 mM $CaCl_2$; 10 mM tris-HCl, pH 7.5), and suspended in 2 ml of STC buffer. The cell solution was divided into 100 µl aliquots in Eppendorf tubes, added with 1 – 10 µl of DNA, and left at room temperature for 15 min. Each tube was added with 1 ml of PEG buffer (20 % PEG (polyethylene glycol) 4,000; 10 mM $CaCl_2$; 10 mM tris-HCl, pH 7.5), and left at room temperature for 15 min. After centrifugation, the cells were suspended in 1 ml of 1.2 M sorbitol. Aliquot of 200 µl cells was transferred into a tube containing pre-warmed (48°C) MM medium (1.2 M) sorbitol and poured on Petri dish. The cells were incubated at 30 °C for at least 4 days.

2.3.11 Determination of generation time of yeasts

Yeasts were grown in YPD to stationary phase as seeds. Then, 0.1 ml of seed was inoculated into 100 ml of fresh YPD and incubated. During incubation, 0.1 ml of culture was sampled every 4 h. With appropriately dilution, yeast cells were spread on YPD plates, and incubated at 30°C for 4 days. The generation time was defined by the following equation

$$\text{Generation time} = \ln N_2 - \ln N_1 = k\,(t_2 - t_1)$$

t: growth time

N: cell number at t time.

Thus, generation time = 0.693 / k

2.3.12 Mitotic stability of vector

The mitotic stability of plasmid in yeasts was determined by the method of Stinchcomb et al. (1980). Yeast transformants were grown in MM to stationary phase as

seeds. Then, 0.1 ml of seed was inoculated into 100 ml of fresh YPD and incubated. When the culture grew to stationary phase, another 100 ml of fresh YPD was inoculated with 0.1 ml of stationary-phase culture. During incubation, 0.1 ml of the culture was sampled every 10 h, appropriately diluted, spread on YPD and MM plates, respectively, and incubated at 30°C for 4 days. The mitotic stability was defined as the colony number appeared on MM plates divided by the colony number appeared on YPD plates.

2.4 RESULTS

2.4.1 Characterization of *Schw. occidentalis* mutants

In order to use *S. cerevisiae LEU2* gene as a genetic marker for *Schw. occidentalis* transformation, a corresponding leucine auxotrophic mutant of the yeast is required. Thus, only leucine auxotrophic mutants were screened after treatment of *Schw. occidentalis* ATCC44442 with NTG. Three mutants found were named as DW87, DW88, and DW89, respectively. However, the reversion rates of DW87 and DW89 were almost 100 %, except for DW88 which showed a reversion rate less than 10^{-7}. The morphology observed by microscope (400 ×, Wild Model Photoautomat MPS45, Leitz, Germany) and on YPD plates showed similar cell size and shape between wild type and DW88 (Figure 2.1). These yeasts also had similar generation time of about 2 hours and cell density of 10^8 colonies per milliliter at stationary phase (Figure 2.2). Thus, except for the *leu* allele, *Schw. occidentalis* DW88 was similar to its wild type and chosen as a host for further studies.

2.4.2 Preparation of spheroplast

To make spheroplasts for transformation, an optimal condition for spheroplast generation is required. In this study, sorbitol was used as a spheroplast stabilizer. As shown in Table 2.2, the spheroplast generation rate of *Schw. occidentalis* DW88 in the presence of 1.2 M sorbitol was 98 %, after cells was collected in stationary phase but not

in log phase. No spheroplast was obtained with sorbitol concentration less than 1.2 M. Therefore, the stationary-phase cells of *Schw. occidentalis* DW88 in the presence of 1.2 M sorbitol were chosen to perform transformation.

2.4.3 Transformation of *Schw. occidentalis*

YEp13 carrying *S. cerevisiae LEU2* was transferred into *Schw. occidentalis* DW88 (*leu*) by spheroplast-mediating method. After incubation at 30°C for 4 days on MM plates, Leu^r colonies were obtained with transformation efficiency of 10^3 transformants/μg DNA.

2.4.4 Plasmid construction

YEp13 contains *S. cerevisiae* 2-μm replicon. To make sure whether the 2-μm replicon is responsible for YEp13 replication in *Schw. occidentalis* DW88, a YIp (yeast integrating plasmid) vector, p322L without the 2-μm replicon, was constructed as shown in Figure 2.3. After digestion with *Pst*I, the *LEU2* containing fragment (4,066 bp) of YEp13 was recovered from agarose gel, and ligated with *Pst*I-digested pBR322. *E. coli* DH10B was used to transfer the recombinant DNA and then spread on tetracycline-containing LB plates, because pBR322 kept tetracycline resistant (Tc^r) genotype but lost ampicillin resistance (Ap^r) after insertion of the *LEU2* containing fragment into Ap^r gene. For selecting transformants with phenotype of Tc^r and ampicillin sensitive (Ap^s), transformants were replicated on LB plates including tetracycline and ampicillin, respectively. The vector DNA isolated from one of Ap^s and Tc^r transformants was designated as p322L. After digestion with *Pst*I, p332L offered two DNA fragments (Lane D) with molecular weights of 4.1 and 4.4 kb identical to those from pBR322 (Lane B) and YEp13 (Lane C), respectively (Figure 2.4). Following transforming *Schw. occidentalis* DW88, 2 μg of p322L DNA totally produced only 2 colonies on MM plates.

2.4.5 Fates of vectors in *Schw. occidentalis*

2.4.5.1 Stabilities of YEp13 and p322L in *Schw. occidentalis*

Three *Schw. occidentalis* DW88 Leu$^-$ transformants given by YEp13 and the two transformants by p322L were grown in YPD for 50 h. As shown in Table 2.3, the stability of YEp13 was about 8 % after incubation in non-selective medium for 30 h and decreased with increasing incubation time. However, p322 maintained 96.3 % of stability after 50 h incubation in YPD medium.

2.4.5.2 Structures of recovered vector from *Schw. occidentalis*

When total DNA was isolated from yeast transformants with YEp13 and p322L, respectively, and transformed into *E. coli* DH10B, the former provided transformants on LB plates containing tetracycline, but the latter did not. The recovered YEp13 transformed *Schw. occidentalis* DW88 with the same transformation efficiency (10^3 transformants/μg DNA) as the original YEp13 did. To investigate whether *Schw. occidentalis* modified foreign DNA or not, the original and recovered YEp13 were digested with restriction enzymes *Aat*II, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Kpn*I, *Nru*I, *Pst*I, *Stu*I, and *Xba*I, respectively. Figure 2.5 shows that these two vectors had same molecular weight of 10.7 kb and same profiles on agarose gels.

2.5 DISCUSSION

In order to use the *S. cerevisiae LEU2* gene as a genetic marker for *Schw. occidentalis* transformation, a corresponding leucine auxotrophic mutant was required. In total, three leucine auxotrophic mutants were obtained after mutation, but only *Schw. occidentalis* DW88 possessed a low reversion rate less than 10^{-7}. It means that the mutated *leu* allele of *Schw. occidentalis* DW88 is stable. Both wild type and DW88 showed the same cell size, shape, colony morphologies, generation time, and cell density

in the stationary phase. These results imply that *Schw. occidentalis* DW88 was similar to its wild type except for the mutated *leu* allele.

S. cerevisiae transformation is often performed with log-phase cells (Kaiser et al., 1994). In this study, spheroplasts were obtained from cells harvested in stationary phase but not in log phase. Moreover, the 1.2 M sorbitol (spheroplast stabilizer) using for *Schw. occidentalis* DW88 transformation is higher than that (1.0 M) for *S. cerevisiae* transformation. This hints that *Schw. occidentalis* DW88 has more fragile cell wall than *S. cerevisiae*.

After transformation, YEp13 carrying *S. cerevisiae LEU2* gene allowed *Schw. occidentalis* DW88 to survive on MM plates with a transformation efficiency of 10^3 transformants/μg DNA. This indicates that the *LEU2* gene can complement the *leu* mutation of *Schw. occidentalis* DW88. Compared with other *Schw. occidentalis* transformation systems, this transformation efficiency was similar to that of spheroplast-mediating transformation (Klein and Favreau, 1988), higher than that of a competent cell method ($1\text{-}10^3$) (Dohmen et al. 1989), but less than an electroporation method (10^5) (Costaglioli et al. 1994). This fact also appeared on *S. cerevisiae* transformation (Chapter 1).

Besides pBR322, YEp13 consists of two yeast DNA fragments containing the 2-μm replicon and a *LEU2* gene, respectively. Meanwhile, p322L is composed of the *LEU2* gene inserted into *Pst*I site of pBR322, and p322L did not contain the 2-μm replicon. YEp13 provided *Schw. occidentalis* transformation with the efficiency of 10^3 transformants/μg DNA, but p322 did not. These results imply that 1) the 2-μm replicon is responsible for YEp13 replication extrachromosomally in *Schw. occidentalis*, 2) the replication initiation components of *Schw. occidentalis* recognize foreign replicons, 3) *S. cerevisiae LEU2* gene functions in *Schw. occidentalis*, and 4) *Schw. occidentalis* provides an environment allowing heterologous gene expression.

The 2-μm plasmid contains a replicon and three regions, *REP*1, *REP*2 and *STB* (*REP*3) genes required for plasmid amplification and stable maintenance. The replicon and *STB* gene are cis-acting, but *REP*1 and *REP*2 genes are trans-acting. YEp13 possess the 2-μm replicon and the *STB* gene, but no *REP*1 and *REP*2 genes. *S. cerevisiae* with 2-μm plasmid is thus required to stably maintain 2-μm-based vectors. Otherwise, in a host cell without the 2-μm plasmid, the vectors have only normal amplification, but poor partition resulting in low stability (Soidla and Nevzgliadova, 1987). This is in agreement with Dohmen et al. (1989), who found low stability of YEp-type vectors in *Schw. occidentalis*. This suggests that the yeast lacks the *REP*1- and *REP*2-like functions. To increase vector stability in *Schw. occidentalis*, ribosomal DNA (rDNA)-containing vectors, which perform multiple integration of vectors into host chromosome, are recommended. Recently, this kind of vector has been successfully applied in *Candida utilis* (Kondo et al., 1995), *Kluyveromyces lactis* (Bergkamp et al., 1992; Rossolini et al., 1992), *Phaffia rhodozyma* (Kim et al., 1998; Wery et al., 1997), and *Yarrowia lipolytica* (Le Dall et al., 1994).

Transformants with YEp13 showed that the *S. cerevisiae LEU*2 gene complemented the *leu* allele of *Schw. occidentalis* DW88. Conversely, a putative *LEU*2 gene of *Schw. occidentalis* was cloned in a *leu*2 mutant of *S. cerevisiae* (Iserentant and Verachtert, 1995). These results suggest that *Schw. occidentalis* DW88 is a putative *leu*2 mutant. As mentioned above, the *S. cerevisiae LEU*2 gene is the most widely used genetic marker in yeast genetics. Many vectors based on the 2-μm replicon and the *LEU*2 gene have been constructed (Chapter 1), and thus they can also be applied to *Schw. occidentalis* DW88.

Recovered YEp13 not only transformed *Schw. occidentalis* DW88 to a Leu^+ phenotype, but also showed the same molecular weight and profiles on agarose gel after restriction enzyme digestion as the original YEp13 did. These data suggest that *Schw. occidentalis* does not modify foreign DNA and could be used as a promising host to accommodate heterologous genes for protein productions. This is agreement with an other *Schw. occidentalis* host system (Dave and Chattoo, 1997).

After digestion with *Pst*I, p322L showed two DNA fragments with identical molecular weights to those of pBR322 and YEp13, respectively. This shows that p322-L is the vector expected by design. On MM plates, p322L produced 2 colonies in total, which maintained almost 100 % of Leu$^-$ phenotype even after 50 h growth in YPD medium. Moreover, p322L was not recovered from *Schw. occidentalis* colonies. The results imply that these two colonies might be revertants or transformants due to p322L integration into the host chromosome. *LEU2* genes from *S. cerevisiae* and *Schw. occidentalis* shared a low homology (Iserentant and Verachtert, 1995), suggesting that the two colonies might be revertants, but not transformants.

2.6 CONCLUSIONS

A new transformation system for *Schw. occidentalis* was developed. This system was based on a stable *leu* auxotrophic mutant, *Schw. occidentalis* DW88 and vectors with both *S. cerevisiae* 2-μm replicon and *LEU2* gene. *Schw. occidentalis* DW88 does not modify foreign DNA. Thus, it could be used as a host cell to accept heterologous DNA and produce heterologous proteins.

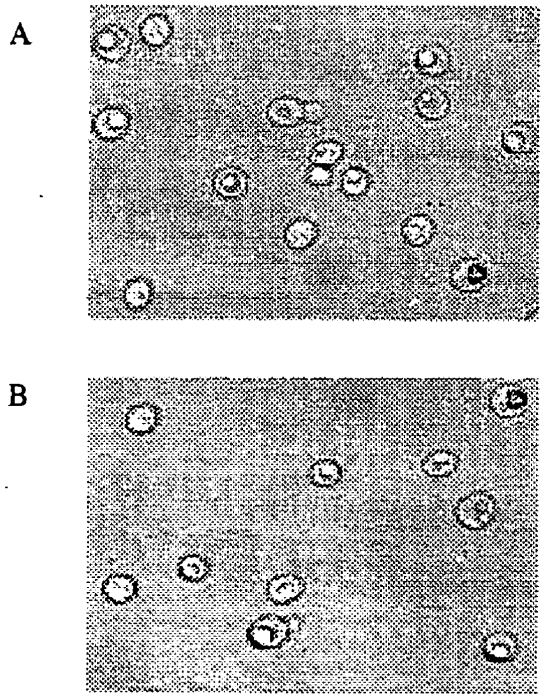

Figure 2.1. *Schw. occidentalis* ATCC44442 (A) and DW88 (B) under microscope (10 × 40).

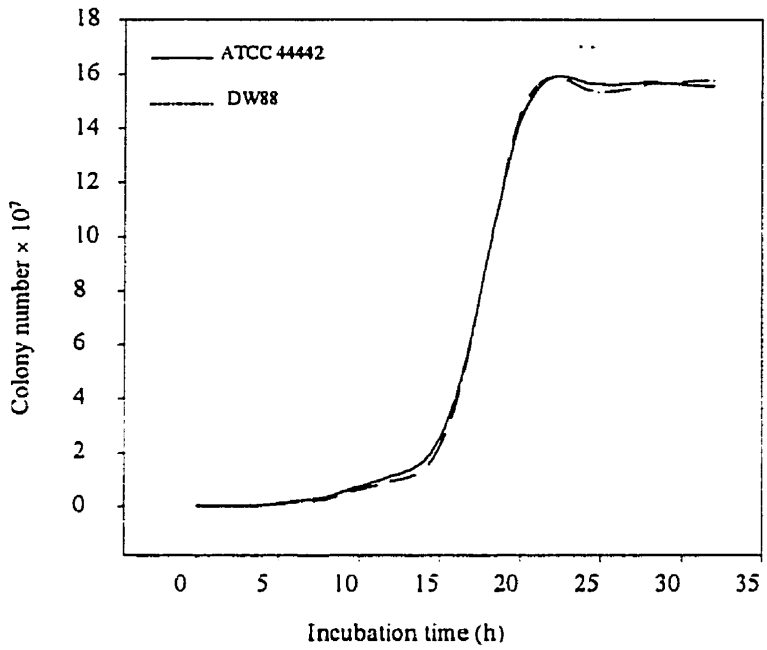

Figure 2.2. The growth curves of *Schw. occidentalis* ATCC44442 and DW88 in YPD.

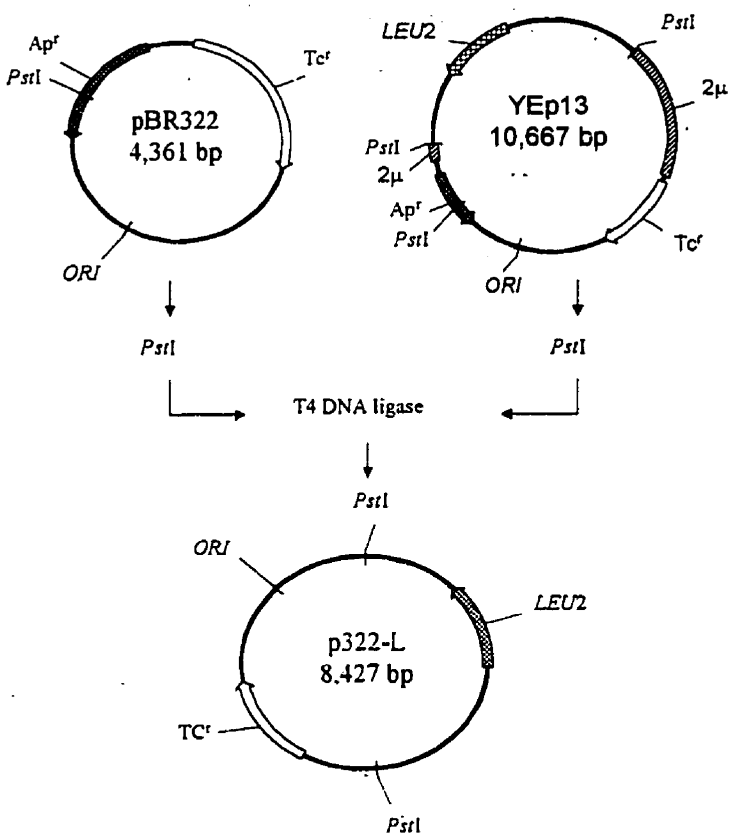

Figure 2.3. Construction of p322-L. The *LEU2* containing fragment (4,066 bp) was cleaved from YEp13 with *Pst*I digestion, and inserted into the *Pst*I site of pBR322. YEp13 contains the replicons of both pBR322 and 2-μm plasmid, but p322L contains only pBR322 replicon.

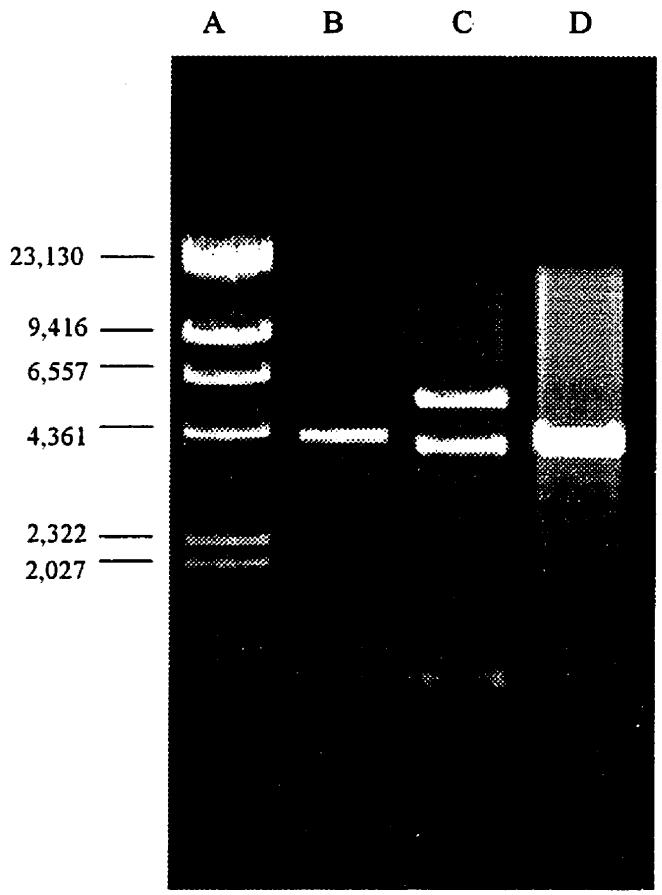

Figure 2.4. Agarose gel electrophoresis of DNA. Lane A, DNA marker λ-*Hin*dIII. Lane B, pBR322 digested with *Pst*I. Lane C, YEp13 digested with *Pst*I. Lane D, p322-L digested with *Pst*I.

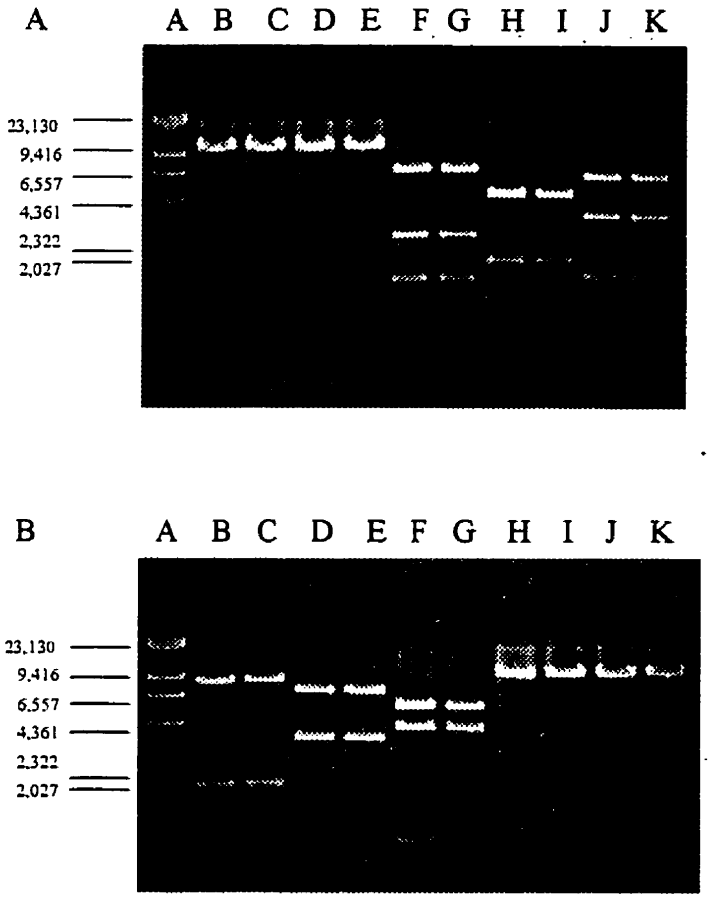

Figure 2.5. DNA profiles of original and recovered YEp13 digested with different restriction enzymes. A. Lane A, DNA marker λ-*Hin*dIII. Lanes B, D, F, H, and J mean original YEp13 digested with *Aat*II, *Bam*HI, *Cla*I, *Eco*RI, and *Eco*RV, respectively. Lanes C, E, G, I, and K mean recovered YEp13 digested with *Aat*II, *Bam*HI, *Cla*I, *Eco*RI, and *Eco*RV, respectively. B. Lane A, DNA marker λ-*Hin*dIII. Lanes B, D, F, H, and J mean recovered YEp13 digested with *Kpn*I, *Nru*I, *Pst*I, *Stu*I, and *Xba*I, respectively. Lanes C, E, G, I, and K mean recovered YEp13 digested with *Kpn*I, *Nru*I, *Pst*I, *Stu*I, and *Xba*I, respectively.

Table 2.1. Microbial strains and vectors used in this study.

Strain or vector	Relevant Characteristics	Source
E. coli DH10B	F⁻ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80*dlac*Z Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*Kλ⁻ *rps*L *end*A1 *nup*G	GIBCO-BRL Co.
Schw. occidentalis ATCC44442	Wild type	ATCC
Schw. occidentalis DW87	*leu*⁻	This study
Schw. occidentalis DW88	*leu*⁻	This study
Schw. occidentalis DW89	*leu*⁻	This study
pBR322	4,361 bp, Ap^r	Bolivar et al., 1977
YEp13	10,667 bp, Ap^r, Tc^r, *LEU2*	Broach et al., 1979
p332L	8,427 bp, Tc^r, *LEU2*	This study

Table 2.2. Spheroplast generation rate* of *Schw. occidentalis* DW88 in different conditions.

	Log phase (10^7 cells/ml)					Stationary phase (10^8 cells/ml)				
Sorbitol Conc. (M)	0	0.5	0.75	1	1.2	0	0.5	0.75	1	1.2
Generation rate (%)	<1	<1	<1	<1	<1	<1	<1	<1	<1	98 ± 2

* Mean of triplicate experiments.

Table 2.3. Vector stabilities in *S. occidentalis* DW88*.

Incubation time (h)	0	10	20	30	40	50
YEp13	96.0	16.1	7.3	6.3	1.9	0.5
p322L	101.2	100.6	97.8	96.1	97.5	96.3

* Mean of triplicate experiments. Stability = (the number of colonies appeared on MM plate) / (the number of colonies appeared on CM plate) × 100%.

CHAPTER 3.0

PUTATIVE PREFERRED CODONS FOR *SCHWANNIOMYCES OCCIDENTALIS* AND A PROGRAM TO CALCULATE CODON BIAS INDEX

Codon usage is an important factor affecting gene expression. Thus, this chapter hypothesizes putative preferred codons for *Schwanniomyces occidentalis* and determines the codon bias indexes for 14 *Schw. occidentalis* cloned genes.

Some results of this study were summarized as part of a publication entitled "The Molecular Biology of *Schwanniomyces occidentalis* Klocker" co-authored by Tsung-Tsan Wang, Ching-Fu Lee, and Byong H. Lee. Some results of this study were submitted as a manuscript for publication in *Molecular Biotechnology* (1998 10: 103-106), and was entitled "A simple program to calculate codon bias index", co-authored by Tsung-Tsan Wang, Wen-Chi Cheng and Byong H. Lee. The project was supervised by Dr. Byong H. Lee, while Professor Ching-Fu Lee as well as Mr. Wen-Chi Cheng who provided some of the data necessary to write this manuscript. The actual experimental work, and writing of the manuscript were done by Tsung-Tsan Wang. Dr. Byong Lee edited the manuscript prior to its submission for publication.

3.1 ABSTRACT

After analysis of 14 cloned *Schwanniomyces occidentalis* genes and comparison of associated genes from both *Schw. occidentalis* and *Saccharomyces cerevisiae*, 25 codons were arbitrarily chosen as putative preferred codons for *Schw. occidentalis*. They are same as those of *S. cerevisiae*, except for TTA for leucine, and AAA for lysine. Based on these preferred codons, the codon bias indexes of 14 *Schw. occidentalis* cloned gene were determined. A computer program (PCBI) was developed to calculate codon bias index (CBI) quickly. PCBI can analyze a gene containing introns. The 22 preferred codons defined from traditional yeast *Saccharomyces cerevisiae* were used in PCBI as a

standard to calculate the CBI values. However, users can modify the preferred codons to suit each organism. The data PCBI provides include DNA sequence of open reading frame without introns, amino acid sequence of gene product, a table of amino acid composition, a table of codon usage and (G+C) content, parameters for calculating CBI, and the value of CBI. PCBI runs on a DOS or Windows environment, and results can be saved in ASCII text format.

3.2 INTRODUCTION

Codons are the nucleotide triplet in messenger RNA, which specifies the amino acid to be inserted in a specific position in the forming polypeptide during translation. The code appears to be universal for all organisms. In total, 64 sets of codons are derived from the random arrangement of 4 nucleotides, A, C, G, and U. Among them, three codons code for chain termination during protein translation, and 61 codons correspond to 20 amino acids. It is clear that the codons are highly degenerate. In other words, all amino acids are designated by more than one codon except methionine and tryptophan, which are coded by only one codon. Although the codons are degenerate, there is often an unequal use of synonymous codons. In both procaryotic and eucaryotic genes, the selection of the synonymous codons is strongly biased (Grantham et al., 1980; 1981; Ikemura 1981a,b; 1982; Bennetzen and Hall 1982). Thus, codon usage is one of important factors affecting gene expression (Kinnaird, et al., 1991; Solomovici et al., 1997). For evaluating the selection of synonymous codons by a specific organism, several measurements such as codon adaptation index (Sharp and Li, 1987), codon bias index (CBI) (Bennetzen and Hall, 1982), intrinsic codon deviation index (Freire-Picos et al., 1994) and effective number of codons (Wright, 1990) have been developed.

In *S. cerevisiae*, 25 codons were arbitrarily chosen as preferred codons (Bennetzen and Hall, 1982), and verified (Ikemura, 1985). Highly expressed genes are more biased using the preferred codons than ones with low expression (Bennetzen and Hall, 1982; Shar, 1986; Sharp and Cowe, 1991). Thus, CBI was introduced to delineate

the extent of using specific codons of *S. cerevisiae* genes (Bennetzen and Hall, 1982). As just one codon encodes methionine and tryptophan, and the codon for aspartic acid exhibits less bias, only 22 preferred codons encoding 17 amino acids were used to calculate CBI based on the following formula

$$CBI = (N_{pfr} - N_{ran}) \div (N_{tot} - N_{ran})$$

where N_{pfr} is the total number of occurrences of preferred codons, N_{ran} the expected number of the preferred codons if all synonymous codons were used equally, and N_{tot} the total number of the 17 amino acids encoded by the preferred codons. A strongly expressed gene has a higher value of CBI and a more biased codon usage than a weakly expressed gene (Bennetzen and Hall 1982). CBI, thus, is a useful tool to evaluate gene expression in a specific species. It easily judges the gene expression level in a specific yeast host as long as the preferred codons of the host can be defined.

Schwanniomyces occidentalis (*Debaryomyces occidentalis*) was described as a potential "super yeast" (Ingledew, 1987). Due to the fact that *Schw. occidentalis* is able to use cheap starch as a carbon source to grow with rapid growth rate and high cell mass (Moresi et al., 1983; Raspor, 1987; Jamuna and Ramakrishna, 1989; Horn et al., 1992), produce extracellularly strong amylolytic enzymes and secrete large proteins (Ingledew, 1987) without hyper-glycosylation and measurable extracellular proteases (Deibel et al., 1988), *Schw. occidentalis* has a high potential to be a useful alternative to *Saccharomyces cerevisiae* in the production of heterologous proteins. However, the study of gene expression in *Schw. occidentalis* is in infancy. It will be very costly, tedious and time-consuming to assay genes one by one to understand their expression in *Schw. occidentalis* without the assistance of CBI. Calculating CBI of a gene requires summarizing a table of codon usage first, and then determining N_{pfr}, N_{ran}, N_{tot}, and N_{ran} based on preferred codons of the specific host. It is tedious to calculate manually, and no computer software was available. Thus, this study was carried out to investigate preferred codons for *Schw. occidentalis* and to develop a computer program (PCBI) to automatically determine CBIs for evaluating gene expression in the yeast.

3.3 MATERIALS AND METHODS

3.3.1 DNA sequences used

The 14 *Schw. occidentalis* genes used in this study are listed in Table 3.1.

3.3.2 Software

The software Hitachi DNASIS for Windows V2.1 (Hitachi Software Engineering Co. Ltd., Japan) was used to analyze DNA sequences and calculate codon usage tables.

3.3.3 Coding PCBI program

The PCBI program was written with Turbo Basic language and compiled as an executable program. It can run on DOS or Window environment. The flow chart of PCBI performance is summarized in Figure 3.1. At first, users have to give PCBI a gene name, gene source and file name to retrieve the DNA sequence, the interval of open reading frame as well as introns if present and the modification of preferred codons if necessary. Users then have to specify a file name where PCBI will save the executed results and choose the executed results to save. After retrieving and performing DNA sequence analysis, PCBI shows the results on screen one by one. During execution, PCBI will inform users if any problem happens.

The entire DNA sequence must be in Text format with no redundant nucleotides, and to be retrieved must start with a translation initiation codon (ATG) and stop with a translation termination codon (TAA, TAG, or TGA). PCBI uses the 22 preferred codons determined by Bennetzen and Hall (1982) from *S. cerevisiae* as standard.

3.4 RESULTS

3.4.1 Codon usage of *Schw. occidentalis* genes

3.4.1.1 For open reading frames

The 14 *Schw. occidentalis* genes encoded 7,653 amino acid residues in total. Their codon usage is listed in Table 3.3 and summarized in Table 3.4. It is clear that ATT (Ile), TTA (Leu), and GTT (Val) are the three most commonly used codons, but CGA, CGC and CGG (all for Arg) are the three most rarely used codons among these 14 genes.

3.4.1.2 For stop codons

These 14 *Schw. occidentalis* genes used all translation stop codons, TAA, TAG and TGA, to terminate protein translation (Table 3.5). Codon TAA was found in the genes *ADE2*, *ENA1*, *ENA2*, *GAM1*, *HAK* and *SWA2*, TAG in the *AMY1*, *CYC1*$_0$, *GDH*, *INV* and *SCR2* genes and TGA in the *EG1*, *HXK*, *LEU2* genes.

3.4.2 Determination of putative preferred codons for *Schw. occidentalis*

Sharp and Cowe (1991) reported that most glycolytic enzymes and ribosomal proteins possess high codon usage bias. Among the 14 *Schw. occidentalis* genes, *HXK* (hexokinase) and *SCR2* (ribosomal protein) genes should be high-expressed gene in the yeast. Nonetheless, based on *S. cerevisiae* CBI measurement, the *HXK* and *SCR2* genes had CBIs of 0.54 and 0.52, respectively. While the CBIs of highly expressed genes are more than 0.6, the moderate ones are around 0.2 (Boucherie et al., 1995). These two genes should thus have low expression in *Schw. occidentalis*. As shown in Table 3.2, the corresponding genes, *HXK1*, *HXK2* (hexokinases), *CYH2*, *RPLs*, *RPSs*, *RPYS*, and *UBIs* (ribosomal proteins) of *S. cerevisiae* have CBIs from 0.61 to 0.93, which are located in the region of high expression. Thus, the low CBIs of the *HXK* and *SCR2* genes might be due to the fact that either they are of weak expression or the CBI measurement of *S. cerevisiae* is not applicable to *Schw. occidentalis*. As shown in Table 3.3, most of *S.*

cerevisiae preferred codons also frequently appeared in the codon usage of the *HXK* and *SCR2* genes, but ones for Leu and Lys did not. If the *HXK* and *SCR2* genes are highly expressed in *Schw. occidentalis*, the preferred codons of *S. cerevisiae* are not those of *Schw. occidentalis*. For Leu and Lys, TTA and AAA were considered as preferred codons for *Schw. occidentalis*, since their frequency was higher than that of TTG and AAG, respectively. Based on this proposition, the CBIs of the *HXK* and *SCR2* genes were 0.62 and 0.78, which are similar to those of corresponding genes of *S. cerevisiae*. Therefore, this proposal of preferred codons for *Schw. occidentalis* (Table 3.6) might be closer to the actual ones than those of *S. cerevisiae*. The preferred codons were used to measure the CBIs of other *Schw. occidentalis* genes such as *ADE2* (0.43), *AMY1* (0.40), $CYC1_0$ (0.74), *EG1* (0.22), *ENA1* (0.44), *ENA2* (0.42), *GAM1* (0.56), *GDH* (0.74), *HAK1* (0.36), *HXK* (0.62), *INV* (0.25), *LEU2* (0.21), *SCR2* (0.78), and *SWA2* (0.50) (Table 3.7).

3.4.3 Performance of PCBI program

The PCBI program provides other useful information in addition to the CBI value. After analyzing of a gene, PCBI can show (1) the DNA sequence of the open reading frame without introns, (2) the amino acid sequence of the gene product, (3) a table showing the amino acid composition of the protein encoded by the gene (Table 3.8), (4) a table displaying codon usage as well as (G+C) content of the gene (Table 3.9), (5) each parameter for calculating the CBI value of the gene, and (6) the CBI value of the gene (Table 3.10). For information, the preferred codons are indicated (*) on Table 3.9. Table 3.10 also summarizes each factor given by users to calculate the CBI value. The results are saved in ASCII text format.

The PCBI program uses the preferred codons of *S. cerevisiae*. These may be different in other organisms (Nagasu and Hall, 1985; Wang et al., 1999). If necessary, users can modify the preferred codons to suit each specific organism. For example, to calculate the CBI value of *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*) genes, GAC has to be substituted with GAT for Asp, TTG substituted with TTA for Leu, and AAG replaced with AAA for Lys (Wang et al., 1999).

The *SCR2* gene was used to demonstrate PCBI performance, because the gene is from non-*Saccharomyces* yeast, *Schw. occidentalis*, and contains an intron. The results, without DNA and amino acid sequences, are shown in Tables 3.8, 3.9, and 3.10. Table 3.11 shows the results of 3 *S. cerevisiae* genes and 3 *Schw. occidentalis* genes analyzed by PCBI. The CBI values obtained by PCBI matched those previously published. PCBI can read a DNA sequence up to 15 kb, which is much bigger than an average gene. Besides, the performance is without a speed limitation. For example, in an 80486 100 MHz personal computer, PCBI took less than one second to analyze a 15 kb DNA sequence with a putative gene of 6 kb.

3.5 DISCUSSION

Codon usage is one of important factors affecting gene expression in a specific host organism. CBI is a concept derived from the bias of using preferred codons that thus could provide a useful criterion to judge gene expression in the host cells. However, the preferred codons defined for *S. cerevisiae* might not be suitable for *Schw. occidenitalis* genes. The putative preferred codons proposed here might be close to the actual ones of *Schw. occidentalis* and could be used to estimate gene expression in this yeast without costly and time-consuming laboratory work. According to these codons, the CBIs of *Schw. occidentalis* cloned genes were determined. Based on the CBIs, the expression of $CYC1_0$(0.74), *GDH* (0.74), *HXK* (0.62), and *SCR2* (0.78) genes should be strong in *Schw. occidentalis* due to CBIs more than 0.6. On the other hand, the *EG1* (0.22) and *LEU2* (0.21) genes with CBIs around 0.2 are expected to have weak expression in the yeast. Within the putative preferred codons, the contents of A and T are 60 %, 56 % and 52 % at the first, second and third position, respectively. Moreover, *Schw. occidentalis* might prefer TAA, TAG codons to terminate protein synthesis. These observations could be useful for specific mutagenesis of genes and other molecular studies. Although the

proposal of putative preferred codons for *Schw. occidentalis* seems to be reasonable, further actual experiments are required to confirm it.

With the help of PCBI, the CBIs of genes can be very quickly determined. Besides, PCBI provides flexibility to integrate user-defined preferred codons if needed and other useful data about target genes. Thus, this program is a good assistance for molecular study of genes.

3.6 CONCLUSIONS

The putative preferred codons of *Schw. occidentalis* are defined. Based on the preferred codons, the CBIs of 14 *Schw. occidentalis* genes were calculated. Moreover, a program (PCBI) to autonomously analyze codon usage frequency and calculate codon bias index has been successfully developed. Both putative preferred codons and PCBI are helpful for molecular study of *Schw. occidentalis*.

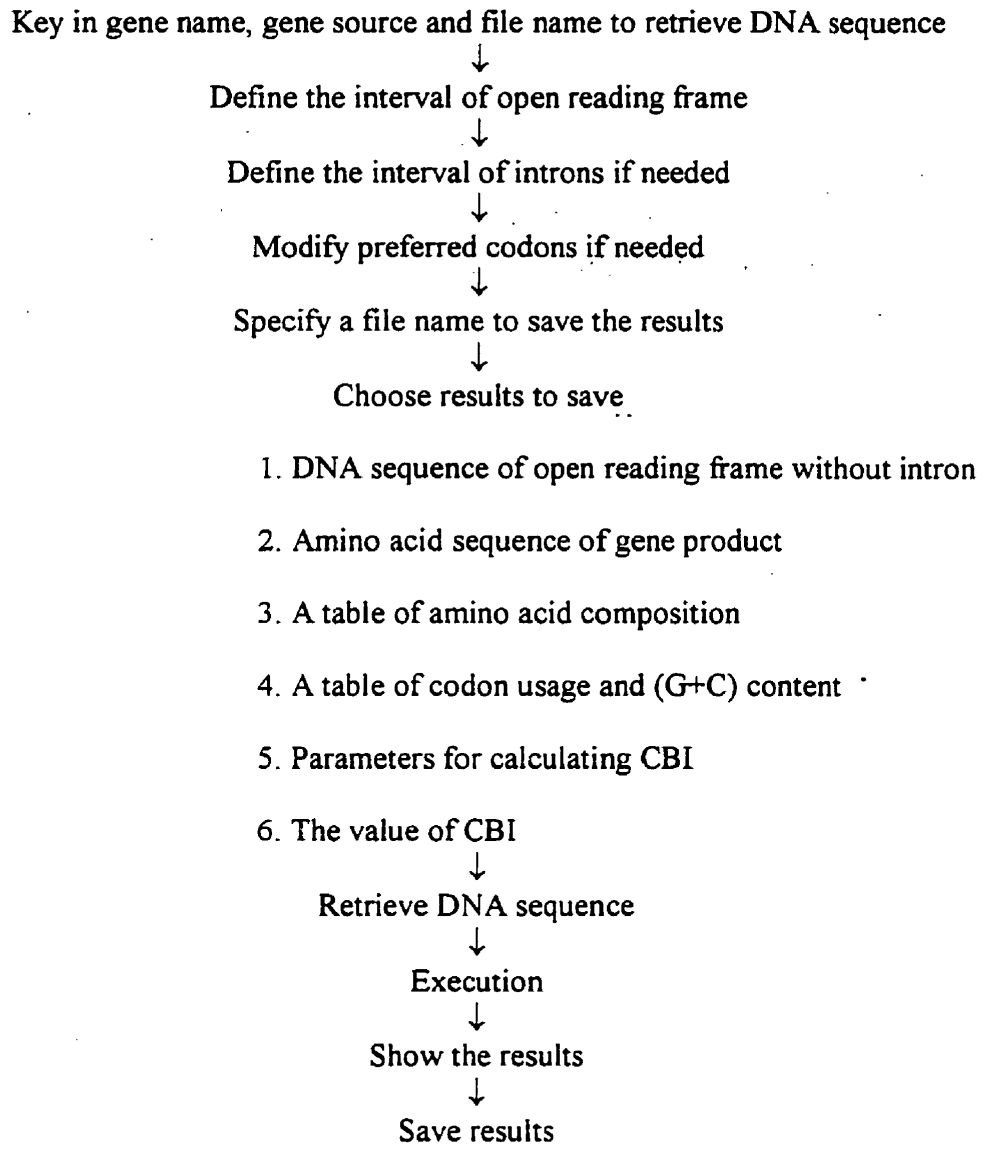

Figure 3.1. The flow chart of PCBI performance.

Table 3.1. The *Schw. occidentalis* genes used in this study.

Gene	Gene product	Gene size (bp)	Gene Source	Accession Number*	Reference
ADE2	Phosphoribosyl aminoimidazole carboxylase	1,671	ATCC 26076	U23210	Gourdon et al., 1995
AMY1	α-Amylase	1,536	ATCC 26076	X16040	Strasser et al., 1989
$CYC1_0$	Cytochrome c protein	333	ATCC 26076	X53770	Amegadzie et al., 1990
EG1	—	474	ATCC 26074	M24078	Prakash and Seligy, 1988
ENA1	ATPase	3,165	ATCC 26076	AF030860	Banuelos and Rodriguez-Navarro, 1998
ENA2	ATPase	3,246	ATCC 26076	AF030861	Banuelos and Rodriguez-Navarro, 1998
GAM1	Glucoamylase	2,874	ATCC 26076	M60207	Dohmen et al., 1988; 1990
GDH	NADP-dependent glutamate dehydrogenase	1,380	NCYC 953	S64476	De Zoysa et al., 1991
HAK1	Potassium transporter	2,286	ATCC 26076	U22945	Banuelos et al., 1995
HXK	Hexokinase	1,437	ATCC 2322	S78714	Rose, 1995
INV	Invertase	1,599	ATCC 2076	X17604	Klein et al., 1989b
LEU2	β-Isopropylmalate dehydrogenase	1,140	ATCC 26077	X79823	Iserentant and Verachtert, 1995
SCR1	Ribosomal protein	---	ATCC 26077	X70807	Del Pozo et al., 1993
SCR2	Ribosomal protein	321	ATCC 26077	X70807	Del Pozo et al., 1993

* Accession number designated by EMBL sequence data bank.

Table 3.2. *S. cerevisiae* genes used in this study.

Gene	Gene product	Gene size (bp)	Accession Number*	CBI	Reference
CYH2	Ribosomal protein L29	450	K01162	0.79	Kaeufer et al., 1983
HXK1	Hexokinases P1	1,578	M14410	0.61	Kopetzki et al., 1985
HXK2	Hexokinases P1	1,461	M14411	0.73	Stachelek et al., 1986
RP51A	Ribosomal protein 51A	411	J01349	0.87	Teem and Rosbash, 1983
RP51B	Ribosomal protein 51A	411	K02480	0.83	Abovich and Rosbash, 1984
RPL2	Ribosomal protein L2	1,089	J03195	0.88	Presutti et al., 1988
RPL4A	Ribosomal protein L4	771	X17204	0.93	Arevalo and Warner, 1990.
RPL16	Ribosomal protein L16	525	X01029	0.87	Leer et al., 1984
RPL17A	Ribosomal protein L17	414	X01694	0.79	Leer et al., 1984
RPL25	Ribosomal protein L25	414	X01014	0.86	Leer et al., 1984
RPL30A	Ribosomal protein 30A	468	K02650	0.83	Mitra et al., 1984
RPL32	Ribosomal protein L32	318	J03457	0.93	Dabeva and Warner, 1987
RPL34	Ribosomal protein L34	342	M23862	0.84	Schaap et al., 1984
RPL45	Ribosomal protein L45	333	J03761	0.88	Remacha et al., 1988
RPL46	Ribosomal protein L46	156	X01963	0.93	Leer et al., 1985
RPS16 Aa	Ribosomal protein 55 Sce-1	561	X02635	0.88	Molenaar et al. 1984
RPS16 Ab	Ribosomal protein 55 Sca-2	561	X01100	0.86	Molenaar et al. 1984
RPS24	Ribosomal protein S24	393	X01962	0.86	Leer et al., 1985
RPS31	Ribosomal protein S31	327	M23819	0.81	Nieuwint et al., 1985
RPS33	Ribosomal protein S33	204	X00128	0.63	Leer et al., 1983
RPYS25	Ribosomal protein YS25	21033	X07811	0.74	Suzuki and Otaka, 1988

* Accession number designated by EMBL sequence data bank.

Table 3.3. Codon usage for 14 *Schw. occidentalis* genes.

AA	Cdn	AD	AM	CY	EG	E1	E2	GA	GD	HA	HX	IN	LE	SC	SW
Ala	GCA	15	4	0	2	25	25	12	5	14	6	8	3	0	8
	GCC	4	4	3	1	9	10	5	14	6	5	1	1	0	6
	GCG	1	2	0	0	6	7	0	0	0	2	1	1	0	2
	GCT	22	16	5	1	40	35	44	22	15	17	10	2	6	21
Arg	AGA	14	10	3	2	23	27	20	13	11	15	8	10	4	11
	AGG	4	2	0	2	4	9	0	0	4	2	2	3	1	2
	CGA	0	0	0	3	2	0	0	0	1	1	0	0	0	0
	CGC	0	1	0	0	0	0	1	0	0	0	0	0	0	0
	CGG	0	0	0	3	1	0	0	0	1	0	1	0	0	0
	CGT	3	0	0	1	5	3	2	2	1	3	3	3	2	2
Asn	AAC	17	10	5	2	12	10	23	22	14	6	9	10	1	13
	AAT	18	20	1	6	51	56	32	4	31	8	33	19	0	21
Asp	GAC	8	13	3	3	10	13	13	5	1	7	12	0	1	8
	GAT	13	30	2	1	49	53	54	12	22	22	27	14	1	30
Cys	TGC	0	2	0	0	5	4	0	0	5	1	0	2	1	3
	TGT	7	6	2	3	11	16	5	6	6	5	1	2	4	5
Gln	CAA	9	20	3	2	15	25	25	15	13	16	15	8	10	19
	CAG	2	3	0	4	5	4	4	0	3	1	6	2	0	0
Glu	GAA	36	13	6	3	54	49	37	36	22	39	13	16	3	13
	GAG	6	5	0	0	13	10	8	1	6	4	10	8	0	2
Gly	GGA	7	11	1	2	13	20	5	6	8	9	13	5	0	6
	GGC	7	4	0	2	4	3	5	4	5	1	0	1	0	5
	GGG	2	2	0	0	3	7	2	0	3	2	2	1	0	2
	GGT	19	21	11	0	48	37	58	40	33	30	17	8	9	24
His	CAC	0	3	3	1	2	6	9	2	2	3	4	3	3	2
	CAT	14	4	0	2	15	15	15	1	12	3	9	3	1	5
Ile	ATA	8	4	0	3	11	12	1	0	14	0	5	14	0	1
	ATC	8	10	1	7	9	15	11	9	16	13	6	7	0	5
	ATT	30	22	2	8	73	63	38	16	63	24	28	18	1	27
Leu	CTA	1	2	0	4	7	4	1	0	10	1	5	7	0	1
	CTC	2	1	0	1	0	0	0	2	1	1	0	1	0	0
	CTG	0	0	0	1	1	0	4	0	0	1	3	2	0	1
	CTT	1	2	0	1	9	7	9	2	11	1	3	5	0	10
	TTA	31	16	5	7	54	63	49	21	42	31	22	17	7	19
	TTG	12	12	2	4	32	23	12	8	19	16	10	13	0	6

Table 3.3. (Continued) Codon usage for 14 *Schw. occidentalis* genes.

AA	Cdn	AD	AM	CY	EG	E1	E2	GA	GD	HA	HX	IN	LE	SC	SW
Lys	AAA	28	10	9	6	40	46	34	23	33	20	18	14	17	9
	AAG	9	8	8	2	21	15	11	14	15	12	12	13	7	7
Met	ATG	18	10	3	4	30	27	10	9	19	10	8	6	1	9
Phe	TTC	4	7	1	3	17	9	31	18	19	11	13	3	3	12
	TTT	10	12	2	5	28	34	23	4	33	9	26	15	1	6
Pro	CCA	20	10	7	4	26	30	40	10	22	20	13	4	1	13
	CCC	2	0	0	0	0	0	1	0	5	0	1	3	0	0
	CCG	1	2	0	3	1	1	0	1	2	2	4	1	0	0
	CCT	9	2	0	3	15	10	13	5	9	5	7	8	0	3
Ser	AGC	0	7	0	1	2	4	4	0	3	1	1	2	0	9
	AGT	5	10	0	1	8	12	10	0	14	0	5	6	0	15
	TCA	18	18	3	4	23	29	24	8	26	9	17	13	0	15
	TCC	3	12	0	2	8	10	8	5	5	4	5	7	2	4
	TCG	3	6	0	3	7	8	6	2	5	0	8	2	0	2
	TCT	8	17	1	4	29	22	37	19	17	12	9	7	0	17
Thr	ACA	10	4	0	3	8	16	9	0	14	5	6	9	0	3
	ACC	3	9	3	0	8	11	14	6	5	7	7	4	5	2
	ACG	0	2	0	1	3	3	1	1	1	0	5	2	0	0
	ACT	12	16	5	2	25	29	36	7	16	11	10	8	3	27
Trp	TGG	2	7	1	3	19	19	17	6	8	4	11	3	0	8
Tyr	TAC	5	9	6	2	6	5	23	13	5	12	6	4	3	23
	TAT	14	25	0	6	19	24	31	3	25	2	17	14	1	15
Val	GTA	8	6	0	1	8	6	9	0	4	1	6	3	0	2
	GTC	8	4	2	1	13	12	12	10	15	4	6	1	1	5
	GTG	6	2	0	5	4	9	1	2	2	2	5	3	0	1
	GTT	30	22	1	7	66	60	49	25	25	19	20	15	6	20

AD: *ADE2*; AM: *AMY1*; CY: *CYC1*$_0$; EG: *EG1*; E1: *ENA1*; E2: *ENA2*; GA: *GAM1*; GD: *GDH*; HA: *HAK1*; HX: *HXK*; IN: *INV*; LE: *LEU2*; SC: *SCR2*; SW: *SWA2*. Codons framed mean the preferred codons of *S. cerevisiae*.

Table 3.4. A summary of codon usage for 14 *Schw. occidentalis* genes.

AA[a]	Codon	%[b]	AA	Codon	%	AMINO ACID	Codon	%
Ala	GCA	1.66	Gly	GGA	1.39	Pro	CCA	2.87
	GCC	0.90		GGC	0.54		CCC	0.16
	GCG	0.29		GGG	0.34		CCG	0.24
	GCT	3.35		GGT	4.64		CCT	1.16
Arg	AGA	2.23	His	CAC	0.56	Ser	AGC	0.44
	AGG	0.46		CAT	1.29		AGT	1.12
	CGA	0.09					TCA	2.70
	CGC	0.03	Ile	ATA	0.95		TCC	0.98
	CGG	0.08		ATC	1.53		TCG	0.68
	CGT	0.39		ATT	5.40		TCT	2.60
Asn	AAC	2.01	Leu	CTA	0.56	Thr	ACA	1.14
	AAT	3.92		CTC	0.12		ACC	1.10
				CTG	0.17		ACG	0.25
Asp	GAC	1.27		CTT	0.80		ACT	2.70
	GAT	4.31		TTA	5.02			
				TTG	2.21	Trp	TGG	1.41
Cys	TGC	0.30	Lys	AAA	4.01	Tyr	TAC	1.55
	TGT	1.03		AAG	2.01		TAT	2.56
Gln	CAA	2.55	Met	ATG	2.14	Val	GTA	0.71
	CAG	0.44					GTC	1.23
							GTG	0.55
Glu	GAA	4.44	Phe	TTC	1.97		GTT	4.77
	GAG	0.95		TTT	2.72			

[a] AA: amino acid.

[b] %: the percentage of the codon occurring in 14 *Schw. occidentalis* genes discussed.

Table 3.5. The translation stop codons used in 14 *Schw. occidentalis* genes discussed.

Stop codon	Gene
TAA	*ADE2*, *ENA1*, *ENA2*, *GAM1*, *HAK1*, *SWA2*
TAG	*AMY1*, *CYC1*$_0$, *GDH*, *INV*, *SCR2*
TGA	*EG1*, *HXK*, *LEU2*

Table 3.6. Putative preferred codons for *Schw. occidentalis.*

Amino acid	Preferred codon	Amino acid	Preferred codon	Amino acid	Preferred codon	Amino acid	Preferred codon
Ala	GCC GCT	Gln	CAA	Leu	TTA*	Ser	TCC TCT
Arg	AGA	Glu	GAA	Lys	AAA*	Thr	ACC ACT
Asn	AAC	Gly	GGT	Met	ATG	Trp	TGG
Asp	GAC	His	CAC	Phe	TTC	Tyr	TAC
Cys	TGT	Ile	ATC ATT	Pro	CCA	Val	GTC GTT

* The preferred codon for the amino acid is different from that of *S. cerevisiae.*

Table 3.7. The values of codon bias index for 14 *Schw. occidentalis* genes.

Gene	N_{pfr}	N_{tot}	N_{ran}	CBI*
ADE2	318	516	168.83	0.43
AMY1	267	452	145.59	0.40
CYC1$_0$	84	101	35.84	0.74
EG1	68	147	46.33	0.22
ENA1	588	947	311.50	0.44
ENA2	590	970	313.58	0.42
GAM1	608	864	282.33	0.56
GDH	352	427	142.50	0.74
HAK1	405	712	232.50	0.36
HXK	324	435	141.63	0.62
INV	241	475	161.50	0.25
LEU2	169	356	118.09	0.21
SCR2	89	103	38.25	0.78
SWA2	297	452	145.00	0.50

* CBI = $(N_{pfr} - N_{ran}) \div (N_{tot} - N_{ran})$ where N_{pfr} is the total number of occurrences of preferred codons, N_{ran} the expected number of the preferred codons if all synonymous codons were used equally, and N_{tot} the total number of the 17 amino acids encoded by the preferred codons.

Table 3.8. An example of the amino acid composition of the product of the *Schw. occidentalis SCR2* gene.

AA	Amount	%	AA	Amount	%	AA	Amount	%
Ala	6	5.66	Gly	9	8.49	Pro	1	0.94
Arg	7	6.60	His	4	3.77	Ser	2	1.89
Asn	1	0.94	Ile	1	0.94	Thr	8	7.54
Asp	2	1.89	Leu	7	6.60	Trp	0	0.00
Cys	5	4.72	Lys	24	22.64	Tyr	4	3.77
Gln	10	9.43	Met	1	0.94	Val	7	6.60
Glu	3	2.83	Phe	4	3.77			

Table 3.9. An example of the codon usage and (G+C) content of the *SCR2* gene of *Schw. occidentalis*.

Codon	No	%	Codon	No	%	Codon	No	%	Codon	No	%
TTT-Phe	1	0.94	*TCT-Ser	0	0.00	TAT-Tyr	1	0.94	*TGT-Cys	4	3.77
*TTC-Phe	3	2.83	*TCC-Ser	2	1.89	*TAC-Tyr	3	2.83	TGC-Cys	1	0.94
*TTA-Leu	7	6.60	TCA-Ser	0	0.00	TAA----	0	---	TGA----	0	---
TTG-Leu	0	0.00	TCG-Ser	0	0.00	TAG----	1	---	*TGG-Trp	0	0.00
CTT-Leu	0	0.00	CCT-Pro	0	0.00	CAT-His	1	0.94	CGT-Arg	2	1.89
CTC-Leu	0	0.00	CCC-Pro	0	0.00	*CAC-His	3	2.83	CGC-Arg	0	0.00
CTA-Leu	0	0.00	*CCA-Pro	1	0.94	*CAA-Gln	10	9.43	CGA-Arg	0	0.00
CTG-Leu	0	0.00	CCG-Pro	0	0.00	CAG-Gln	0	0.00	CGG-Arg	0	0.00
*ATT-Ile	1	0.94	*ACT-Thr	3	2.83	*AAT-Asn	0	0.00	AGT-Ser	0	0.00
*ATC-Ile	0	0.00	*ACC-Thr	5	4.72	AAC-Asn	1	0.94	AGC-Ser	0	0.00
ATA-Ile	0	0.00	ACA-Thr	0	0.00	*AAA-Lys	17	16.03	*AGA-Arg	4	3.77
*ATG-Met	1	0.94	ACG-Thr	0	0.00	AAG-Lys	7	6.60	AGG-Arg	1	0.94
*GTT-Val	6	5.66	*GCT-Ala	6	5.66	*GAT-Asp	1	0.94	*GGT-Gly	9	8.49
*GTC-Val	1	0.94	*GCC-Ala	0	0.00	GAC-Asp	1	0.94	GGC-Gly	0	0.00
GTA-Val	0	0.00	GCA-Ala	0	0.00	*GAA-Glu	3	2.83	GGA-Gly	0	0.00
GTG-Val	0	0.00	GCG-Ala	0	0.00	GAG-Glu	0	0.00	GGG-Gly	0	0.00

(G+C) content = 34.9 %

* The preferred codons used in the DNA analysis.

Table 3.10. An example of a summary to calculate the codon bias index of *Schw. occidentalis SCR2* gene.

Gene Name and Source: *SCR2* gene of *Schwanniomyces occidentalis*

Open Reading Frame: from 1004 to 1777

Intron number: 1

Intron interval: 1007 to 1459

Total codons: 106 not including stop codon

Codon Bias Index:

$CBI = (N_{pfr} - N_{ran}) \div (N_{tot} - N_{ran}) = (89 - 38.25) \div (103 - 38.25) = 0.78$

N_{pfr}: the total number of occurrences of preferred codons.
N_{ran}: the expected number of the preferred codons if all synonymous codons were used equally.
N_{tot}: the total number of the amino acids encoded by the preferred codons.

Table 3.11. The CBI value of some yeast genes.

Gene	Source[a]	Gene product	CBI[b]	Reference
*ADH*1	Sc	Alcohol dehydrogenase I	0.91	Bennetzen and Hall, 1982
*ARG*4	Sc	Argininosuccinate lyase	0.31	Sharp and Cowe, 1991
*THR*4	Sc	Threonine synthase	0.51	Sharp and Cowe, 1991
$CYC1_0$	So	Cytochrome c protein	0.74	Wang et al., 1999
HXK	So	Hexokinase	0.62	Wang et al., 1999
INV	So	Invertase	0.25	Wang et al., 1999

[a]Sc: *S. cerevisiae*. So: *Schw. occidentalis*

[b]To calculate the CBI value of *Schw. occidentalis* genes, the two of standard preferred codons were modified. The codon of TTA substitutes for TTG encoding leucine and AAA replaces AAG for lysine.

CHAPTER 4.0

EXPRESSION OF A *STREPTOCOCCUS THERMOPHILUS* β-GALACTOSIDASE GENE IN *SCHWANNIOMYCES OCCIDENTALIS*

In order to evaluate whether *Schw. occidentalis* DW88 can express a heterologous gene, a heterologous promoter and a heterologous gene were used to carry out this research. A *Streptococcus thermophilus* β-galactosidase gene controlled by a *Saccharomyces cerevisiae ADH*1 promoter was successfully expressed in this yeast.

The major results of this study were summarized and submitted as a manuscript for publication in *Applied Microbiology and Biotechnology*. The manuscript entitled "Expression of a *Streptococcus thermophilus* β-galactosidase gene in *Schwanniomyces occidentalis*" was co-authored by Tsung-Tsan Wang, Young-Jun Choi, Normand Robert, and Byong H. Lee. The project was supervised by Dr. Byong H. Lee. Mr. Young-Jun Choi, and Mr. Normand Robert helped for gel electrophoresis and enzyme assays, while the actual experimental work, and writing of the manuscript were done by Tsung-Tsan Wang. Dr. Byong Lee edited the manuscript prior to its being submitted for publication.

4.1 ABSTRACT

A *Streptococcus thermophilus* β-galactosidase gene controlled by *Saccharomyces cerevisiae ADH*1 promoter was expressed in *Schwanniomyces occidentalis* (*Debaryomyces occidentalis*). The β-galactosidase activity was not detected in the culture supernatant, but from cell lysate. The β-galactosidase produced by *Schw. occidentalis* exhibited the same thermostability as that of *S. thermophilus*. Although *E. coli* produced more β-galactosidase than *Schw. occidentalis*, *Schw. occidentalis* extracts showed 3-fold higher specific activity than those from *E. coli*.

4.2 INTRODUCTION

Saccharomyces cerevisiae, a traditional yeast, is widely used in academic and industrial fields. However, this yeast is not an ideal host producing extracellular heterologous proteins due to certain disadvantages such as:

1) low product yields, even with a strong promoter (Buckholz and Gleeson, 1991),
2) hyper-glycosylated proteins which might affect protein characteristics (Lemontt et al., 1985; Moir and Dumais, 1987; Van Arsdell et al., 1987), and
3) low secretion efficiency when protein is larger than 30 kDa (Smith et al., 1985; Jigami et al., 1986; Moir and Dumais, 1987; De Nobel and Barnett, 1991).

Thus, the molecular studies have been extended to other yeasts than *S. cerevisiae* that makes the genetic engineering of non-*Saccharomyces* yeasts important.

Schwanniomyces occidentalis (*Debaryomyces occidentalis*) was described as a potential "super yeast" (Ingledew, 1987). *Schw. occidentalis* is able to use cheap starch as a carbon source to grow with a rapid growth rate and high cell mass (Moresi et al., 1983; Raspor, 1987; Jamuna and Ramakrishna, 1989; Horn et al., 1992), to produce extracellularly strong amylolytic enzymes and secrete large proteins (Ingledew, 1987) without hyper-glycosylation and measurable extracellular proteases (Deibel et al., 1988). *Schw. occidentalis* has thus a high potential as a useful alternative to *S. cerevisiae* in production of heterologous proteins. However, the molecular study of *Schw. occidentalis* is in its infancy. Heterologous genes expressed in this yeast include only *Saccharomyces cerevisiae LEU2*, *TRP5* and *URA3* genes (Dohmen et al., 1989; Dave and Chattoo, 1997; Wang and Lee, 1997) and a *Clostridium thermocellum* cellulase gene (Piontek et al., 1990). These genes were controlled by their own promoters except the cellulase gene, which was controlled by the *GAM1* promoter of *Schw. occidentalis*.

A new transformation system was recently developed for *Schw. occidentalis* DW88 that does not modify foreign DNA (Wang and Lee, 1997). In order to further verify whether *Schw. occidentalis* DW88 is an ideal host for both academic and industrial

application, it is necessary to study heterologous gene expression in this yeast. Some yeast expression vectors were based on a strong *S. cerevisiae ADH*1 promoter (Bennetzen and Hall, 1982; Vernet et al., 1987). In this study, the *ADH*1 promoter and a *Streptococcus thermophilus* β-galactosidase gene (*GAL*) were used as tools to perform heterologous gene expression in *Schw. occidentalis* DW88.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals and enzymes

Unless otherwise specified, all chemical reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), restriction enzymes, agarose and DNA molecular weight markers were obtained from Boehringer Mannheim (Laval, Quebec, Canada). Yeast nitrogen base without amino acids was obtained from Difco Lab. (Detroit, Michigan, USA). Glusulase was obtained from Dupont Co. (Boston, Massachusetts, USA). Other chemicals were obtained from Anachemia (Montreal, Quebec, Canada).

4.3.2 Strains, vectors, and media

The strains and vectors used in this study are listed in Table 4.1. pVT101-L-H+ (Lee et al., 1990, unpublished) was created in our laboratory. The media and methods to grow *E. coli* and yeasts are same to those in previous research (section 2.3.2). The amount of chemical added to LB was 40 μg/ml of 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) or 40 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) if needed. To prepare β-galactosidase sample, yeast transformants were grown in MM medium supplemented with 13.6 mg/ml KH_2PO_4, pH 7.0; 2 mg/ml $(NH_4)_2SO_4$; 0.5 mg/ml $FeSO_4.7H_2O$; 16 μg/ml thiamine; 800 μg/ml biotin; 16 μg/ml pantothenic acid; 80 μg/ml inositol; 16 μg/ml pyridoxine; 20 μg/ml adenine, 20 μg/ml uracil, 20 μg/ml tryptophan, 20 μg/ml histidine, 20 μg/ml arginine, 20 μg/ml methionine, 30 μg/ml tyrosine, 30 μg/ml

isoleucine, 30 μg/ml lysine, 50 μg/ml phenylalanine, 100 μg/ml glutamic acid, 100 μg/ml aspartic acid, 150 μg/ml valine, 200 μg/ml threonine, and 375 μg/ml serine.

4.3.3 DNA isolation.

4.3.3.1 Preparation of vector DNA from *E. coli*

The method to prepare vector DNA from *E. coli* is same to that of previous chapter (section 2.3.7.1).

4.3.3.2 Preparation of vector DNA from yeast

The method to prepare vector DNA from yeast is same to that of previous chapter (section 2.3.7.2).

4.3.3.3 DNA elution, digestion and ligation

The methods to elute, digest, and ligate vector DNA are same to those of previous chapter (section 2.3.7.2).

4.3.4 Transformation procedures

4.3.4.1 *E. coli* transformation

The method to transform *E. coli* is same to that of previous chapter (section 2.3.10.1).

4.3.4.2 Yeast transformation

The method to transform yeast is same to that of previous chapter (section 2.3.10.2).

4.3.5 Mitotic stability of vector

The method to determine vector mitotic stability is same to that of previous chapter (section 2.3.12).

4.3.6 Preparation of crude enzyme

To isolate intracellular β-galactosidase, a portions (50 ml) of *Schw. occidentalis* DW88 with YEp13-GAL and *E. coli* DH101 with pTV101L-H+ were collected, respectively, by centrifugation at 12,000 rpm for 15 min at 4 °C. The resulting supernatant was collected for the measurement of β-galactosidase activity. The cell pellet was washed twice with 50 ml of 0.05 M sodium phosphate buffer (pH 7.0) and suspended in 2-3 ml of the same buffer. Cell extract was prepared by disrupting cells using an ultrasonic disintegrator (Sonifier 450, Branson, Danbury, Connecticut, USA) for 10 sec with 30 sec intervals on ice bath until cell lysis. The cell lysate was separated from cell debris by centrifugation at 15,000 g for 20 min at 4 °C and the supernatant was used for enzyme assay and protein determination.

4.3.7 Protein Assay

Protein was determined spectrophotometrically by the protocol supplied with the Bio-Rad protein assay kit (Bio-Rad Lab., Mississauga, Ontario, Canada). Aliquots of 800 µl of properly diluted samples were mixed with 200 µl of Bio-Rad dye reagent, put into microplate wells, and read at 595 nm by Bio-Rad Microplate reader (Model 3350-UV). The protein content was estimated by the standard curve established.

4.3.8 β-galactosidase activity assay

β-Galactosidase activity was measured using ortho-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. Samples (50 µl) of cell lysate were mixed with 1 ml of ONPG solution (4 mg/ml ONPG in 0.05 M sodium phosphate, pH 7.0), and

incubated at 37 °C for 20 min. After adding 0.5 ml of 1 M Na_2CO_3 to stop the reaction, the enzyme activity was determined spectrophotometrically by measuring absorbance at 420 nm with Bio-Rad Microplate reader. One unit (U) of enzyme activity was defined as one μM of ONP formed per min at 37 °C from the standard curve and specific activity was expressed as units per μg of protein. The standard curve of ONP was prepared by serial dilution of ONP solution and read at 420 nm. For evaluation of heat stability, aliquots of cell lysate were heated at 55 °C for 30 min before adding substrate.

4.3.9 Electrophoresis

4.3.9.1 Agarose gel electrophoresis

The method to do agarose gel electrophoresis of DNA is same to that of previous chapter (section 2.3.9).

4.3.9.2 Polyacrylamide gel electrophoresis

Electrophoresis in polyacrylamide gel was used to determine molecular weights of proteins isolated. A 4 - 15 % linear gradient native PAGE (Bio-Rad Lab., Richmond, California, USA), 25 mM Tris-glycine buffer (pH 8.3) and 50 volts were used. After electrophoresis, gels were stained with Coomassie blue R250 and destained in methanol-acetate-water (1:1:12, vol/vol/vol). To detect the β-galactosidase band on gel *in situ*, the gel was overlaid with 10 ml of 1 % agarose gel containing 0.5 M potassium phosphate buffer (pH 7.5) and 40 μg/ml X-gal (Lee et al., 1990).

4.4 RESULTS

4.4.1 Cloning of *GAL* in *Schw. occidentalis* DW88

pVT101-L (Vernet et al., 1987), which carries a *S. cerevisiae LEU*2-d gene, was used to clone the *S. thermophilus GAL* gene to form pVT101-L-H+ (Lee et al., 1990, unpublished). The *GAL* gene was located at the *Hin*dIII cutting site between *S. cerevisiae ADH*I promoter and 3' sequence of the *ADH*I gene (*ADH*1-3'). After transformation of *Schw. occidentalis* DW88 with pVT101-L-H+, no transformant was obtained. However, *Schw. occidentalis* DW88 (*leu*) was complemented by YEp13 carrying *S. cerevisiae LEU*2 gene (Wang and Lee, 1997). To take advantage of the *ADH*1 promoter, the cassette of 3.9 kb containing [*ADH*I promoter – *GAL* – *ADH*I-3'] was removed from pVT101-L-H+ by *Sph*I digestion, eluted from agarose gel and cloned into the *Sph*I site of YEp13 (10.7 kb) (Figure 4.1). *E. coli* DH10B was transformed with the recombinant DNA and then spread on LB plates containing ampicillin, IPTG and X-Gal. The vector DNA from one of the blue colonies was isolated and designated as YEp13-GAL (14.5 kb). As shown in Figure 4.2, after digestion with *Sph*I, the YEp13-GAL showed two DNA fragments with molecular weights of 3.9 and 10.7 kb (Lane D), which are identical to those of the corresponding fragments of pVT101-L-H+ (Lane C) and YEp13 (Lane B), respectively.

Following transformation with YEp13 and YEp13-GAL, respectively, *Schw. occidentalis* DW88 survived in MM plates. The vector stability of YEp13 and YEp13-GAL in *Schw. occidentalis* DW88 was about 7 % after 24 h incubation in YPD medium. When these recovered vectors were introduced into *Schw. occidentalis* DW88, transformants were found on MM plates also.

4.4.2 β-galactosidase activity in *Schw. occidentalis*

Since ONPG was used as substrate to measure β-galactosidase activity, a standard curve of ONP was necessary. Figure 4.3 shows a standard curve of ONP with a r^2 of 0.9996683327. To locate the β-galactosidases, culture supernatant and cell lysate from *E. coli* and yeast transformants were assayed (Table 4.2). No detectable β-galactosidase activity was found from all extracellular samples and intracellular sample of *Schw. occidentalis* DW88 with YEp13. The cell lysate samples from *Schw. occidentalis* DW88 with YEp13-GAL and *E. coli* DH10B with pVT101-L-H+ exhibited β-galactosidase

activity with 561.0 and 2,054.3 U, respectively. The crude enzyme solutions of the yeast and *E. coli* contained 0.41 and 4.5 μg/μl, respectively, of total proteins. Accordingly, the β-galactosidase specific activity of extract from *Schw. occidentalis* DW88 with YEp13-GAL and *E. coli* with pVT101-L-H+ were 1,369.0 and 456.9 U/μg, respectively. The specific activity of extract from *Schw. occidentalis* DW88 were 3-fold higher than those from *E. coli* DH10B. Similar β-galactosidase activity was found from *Schw. occidentalis* DW88 with the recovered YEp13-GAL. After heated at 55 °C for 30 min, the β-galactosidases produced by *Schw. occidentalis* and *E. coli* maintained almost 100 % activity (Table 4.3).

Figure 4.4 shows a native PAGE electrophoretogram with Coomassie blue staining and activity staining of cell lysates from yeasts and *E. coli*. Bands with β-galactosidase activity were present in cell lysates from *E. coli* with pVT101-L-H+ and *Schw. occidentalis* DW88 with YEp13-GAL, but not from *Schw. occidentalis* DW88 with YEp13.

4.5 DISCUSSION

Since *Schw. occidentalis* DW88 (*leu*) was complemented by YEp13 (*LEU2*), but not by pVT101-L-H+ (*LEU2*-d), the cassette of [*ADH*I promoter – *GAL* – *ADH*I-3'] was moved from the latter to the former to construct YEp13-GAL. Figure 4.2 shows that YEp13-GAL is composed of the two fragments of 3.9 and 10.7 kb identical with the cassette containing fragment from pVT101-L-H+ and linearized YEp13, respectively. The low stability of YEp13-GAL is similar to those of other YEp vectors in *Schw. occidentalis* (Dohmen et al., 1989; Wang and Lee, 1997). The *Schw. occidentalis* DW88 transformants with the recovered YEp13-GAL also produced β-galactosidase. The results indicate that YEp13-GAL contained the cassette and is extrachromosomally located in *Schw. occidentalis* DW88, and the β-galactosidase activity of *Schw. occidentalis* DW88 transformant was due to the *GAL* gene expression.

As shown in Figure 4.4, no band shows β-galactosidase activity from *Schw. occidentalis* DW88 with YEp13, but *E. coli* DH10B with pVT101-L-H+ and *Schw. occidentalis* DW 88 with YEp13-GAL have bands with activity. Although the cell lysate from *Schw. occidentalis* DW88 with YEp13 contained a certain amount of proteins, no detectable β-galactosidase was found from it (Table 4.2). These results imply that *Schw. occidentalis* DW88 did not produce β-galactosidase, and the β-galactosidase produced by the yeast transformant should be encoded by the *GAL* gene. Moreover, no detectable β-galactosidase activity was found in the culture supernatant from *Schw. occidentalis* DW88 transformants which indicates that the enzyme was not produced extracellularly by the yeast.

As shown in Table 4.2, the total β-galactosidase activity from *E. coli* DH10B with pVT101-L-H+ was 3.7-fold higher than that from *Schw. occidentalis* DW88 with YEp13-GAL. However, the cell lysate from the former had more protein than that from the latter, so that the final specific activity of β-galactosidase from *E. coli* was 3-fold lower than that of *Schw. occidentalis*. Additionally, the *S. thermophilus* β-galactosidase is thermostable (Lee et al., 1990). The β-galactosidase produced by *Schw. occidentalis* kept nearly 100 % activity after heat treatment. This data shows that *Schw. occidentalis* DW88 did not modify the β-galactosidase characteristics. Thus, *Schw. occidentalis* DW88 could be a good candidate to produce heterologous proteins. This *S. thermophilus GAL* is the second bacterial gene expressed in *Schw. occidentalis*.

The *S. cerevisiae ADH*1 promoter is a strong inducible promoter (Ammerer, 1983), but it became non-inducible during the construction of pVT series (Vernet et al, 1987). This constitutive expression characteristic was also found in *Schw. occidentalis* DW88. With the *S. cerevisiae LEU*2 gene, this promoter could be used to construct useful expression vectors for *Schw. occidentalis* DW88.

4.6 CONCLUSIONS

Schw. occidentalis DW88 could serve as a good system for heterologous gene expression without modifying properties of foreign protein. A *S. thermophilus GAL* gene controlled by *S. cerevisiae ADH*1 promoter was successfully expressed in *Schw. occidentalis* DW88. This promoter could be used to construct *Schw. occidentalis* expression vectors for studying gene expression in this yeast.

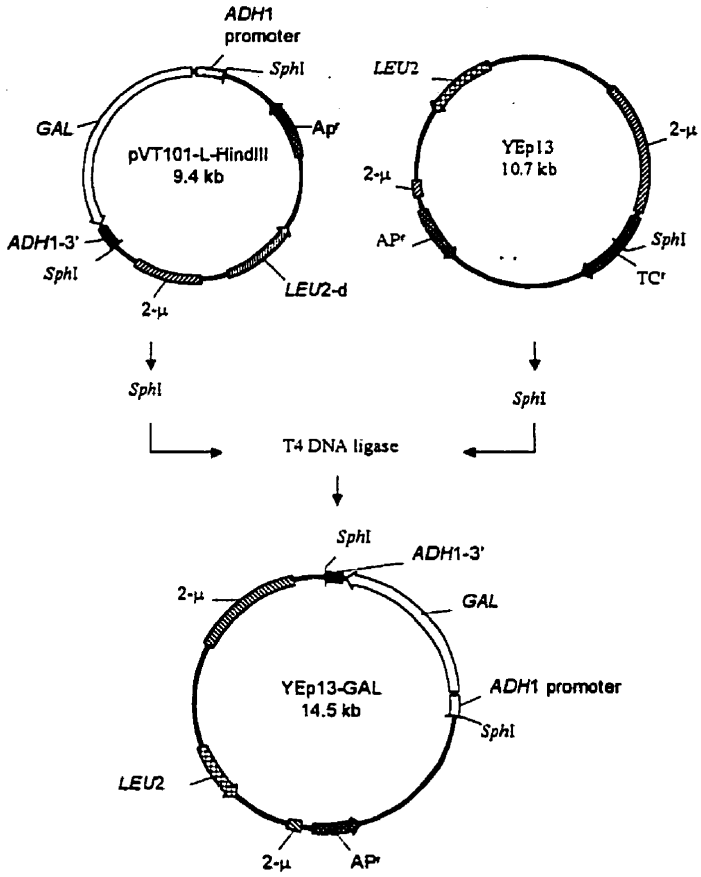

Figure 4.1. Construction of YEp13-GAL. After digestion with *Sph*I, the cassette of [*ADH*I promoter – *GAL* – *ADH*I-3'] was removed from pVT101-L-H+ and ligated with *Sph*I-digested YEp13.

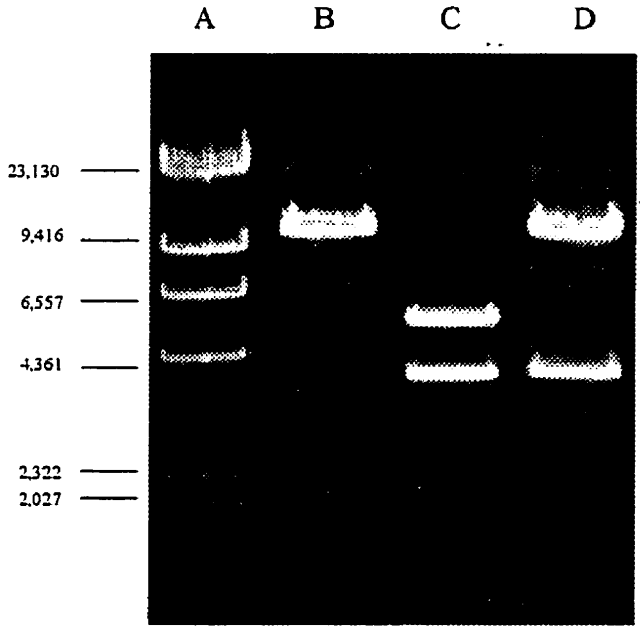

Figure 4.2. Agarose gel electrophoresis of DNA. Lane A, DNA marker λ-*Hin*dIII. Lane B, YEp13 digested with *Sph*I. Lane C, pVT101-L-H+ digested with *Sph*I. Lane D, YEp13-GAL digested with *Sph*I.

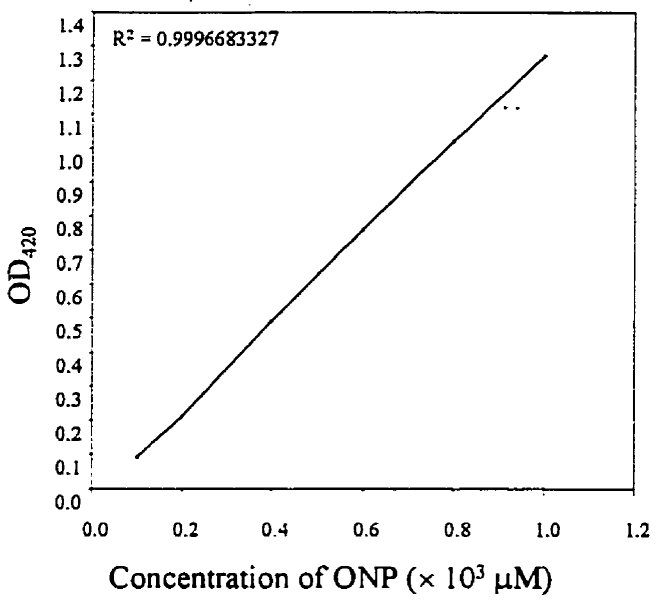

Figure 4.3. A standard curve used for β-galactosidase assay

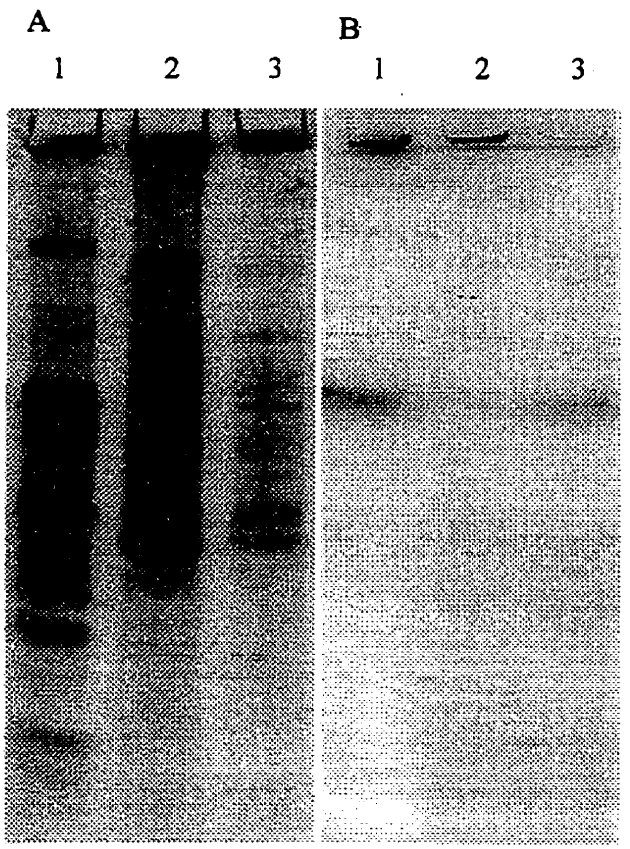

Figure 4.4 Coomassie blue and activity staining on native polyacrylamide gel. Figure A was stained with Coomassie blue and Figure B with X-gal staining. In Figures A and B, Lane 1, cell lysate from *E. coli* DH10B with pVT101-L-H+. Lane 2, cell lysate from *Schw. occidentalis* DW88 with YEp13. Lane 3, cell lysate from *Schw. occidentalis* DW88 with YEp13-GAL.

Table 4.1. Microbial strains and vectors used in this study.

Strain or vector	Relevant Characteristics	Source
E. coli DH10B	F⁻ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80*dlacZ* Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*Kλ⁻ *rps*L *end*A1 *nup*G	GIBCO-BRL
Schw. occidentalis DW88	*leu*⁻	Wang and Lee, 1997
pVT100-L-H+	9.4 kb, Ap^r, *LEU*2-d, *GAL*	Lee et al., 1990, unpublished
YEp13	10.7 kb, Ap^r, Tc^r, *LEU*2	Broach et al., 1979
YEp13-GAL	13.8 kb, Tc^r, *LEU*2, *GAL*	This study

Table 4.2. β-Galactosidase activities[a] from *Schw. occidentalis* and *E. coli.*

Sample		Activity (U)	Protein (μg/μl)	Specific activity (U/μg)
E. coli DH10B - pVT101-L-H+	I[b]	2054.3 ± 41	4.5 ± 0.09	456.9 ± 19
	E[c]	-[d]	-	-
Schw. occidentalis DW88 -YEp13	I	-	0.40 ± 0.01	-
	E	-	-	-
Schw. occidentalis DW88 -YEp13-GAL	I	561.0 ± 10	0.41 ± 0.01	1,369.0 ± 58
	E	-	-	-

[a] Mean of triplicate experiments

[b] Intracellular

[c] Extracellular

[d] non-detected

Table 4.3. Thermostability of β-galactosidase from *Schw. occidentalis* and *E. coli*[a].

Sample	Activity (U)		
	Normal[b]	Heated[c]	Residual activity %[d]
E. coli DH10B - pVT101-L-H+	809.3	784.7	97.0
Schw. occidentalis DW88 -YEp13-GAL	1052.6	1015.8	96.5

[a] Mean of triplicate experiments

[b] Not heated

[c] Heated at 55 °C for 30 min before adding the substrate for the enzyme assay.

[d] Residual activity = (activity units of heated β-galactosidase) ÷ (activity units of normal β-galactosidase) × 100 %

CHAPTER 5.0

CONSTRUCTION OF AN EXPRESSION VECTOR FOR YEAST *SCHWANNIOMYCES OCCIDENTALIS*

In order to express foreign genes in *Schwanniomyces occidentalis*, an expression vector based on *Saccharomyces cerevisiae ADH*1 promoter - multiple cloning sites - *ADH*1-3' sequence and *LEU2* gene was constructed.

The major results of this study were summarized and submitted as a manuscript for publication in *Applied Microbiology and Biotechnology*. The manuscript entitled "An expression vector for *Schwanniomyces occidentalis*" was co-authored by Tsung-Tsan Wang and Byong H. Lee. The project was supervised by Dr. Byong H. Lee, while the actual experimental work and writing of the manuscript were done by Tsung-Tsan Wang. Dr. Byong Lee edited the manuscript prior to the submission for publication.

5.1 ABSTRACT

An expression vector, YEpE81 (6,323 bp), was constructed by substituting *Saccharomyces cerevisiae LEU*2 gene for the *LEU*2-d gene carried by pVT101-L. Similar to pVT101-L, YEpE81 possesses a cassette containing *S. cerevisiae ADH*1 promoter - multiple cloning sites - the 3' sequence of the *ADH*1 gene. This expression vector successfully expressed a β-galactosidase gene of *Streptococcus thermophilus* in *Schwanniomyces occidentalis*.

5.2 INTRODUCTION

Schwanniomyces occidentalis (*Debaryomyces occidentalis*) has been described as a potential "super yeast" (Ingledew, 1987). *Schw. occidentalis* is able to use cheap starch

as a carbon source to grow with a rapid growth rate and high cell mass (Moresi et al., 1983; Raspor, 1987; Jamuna and Ramakrishna, 1989; Horn et al., 1992), produce extracellularly strong amylolytic enzymes and secrete large proteins (Ingledew, 1987) without hyper-glycosylation and measurable extracellular proteases (Deibel et al., 1988). *Schw. occidentalis* thus can be a useful alternative to replace *Saccharomyces cerevisiae* in the production of heterologous proteins, but the study of molecular genetics in *Schw. occidentalis* is far behind than *S. cerevisiae*. Heterologous genes expressed in this yeast include only *S. cerevisiae LEU2*, *TRP5* and *URA3* genes (Dohmen et al., 1989; Dave and Chattoo, 1997; Wang and Lee, 1997), a *Clostridium thermocellum* cellulase gene (Piontek et al., 1990), and a *Streptococcus thermophilus* β-galactosidase gene (*GAL*) (Wang et al., 1999, Chapter 4). These genes were controlled by their own promoters except the cellulase gene and the *GAL* gene, which were controlled by the *GAM1* promoter of *Schw. occidentalis* and the *S. cerevisiae ADH1* promoter, respectively.

A new transformation system was recently developed for *Schw. occidentalis* DW88 (Wang and Lee, 1997). For studying heterologous gene expression in *Schw. occidentalis*, expression vectors are required for this yeast. In previous research, it was demonstrated that the *S. cerevisiae ADH1* promoter drives expression of a *Streptococcus thermophilus* β-galactosidase gene in *Schw. occidentalis* DW88. According to the putative preferred codons of *Schw. occidentalis* (Wang et al., 1999), the codon bias index (CBI) of the *ADH1* gene is 0.71. Since a high expression genes has CBIs more than 0.6 (Boucherie et al., 1995), the *ADH1* promoter should be a strong one for *Schw. occidentalis*. An expression vector for *Schw. occidentalis* with the *ADH1* promoter was thus constructed.

5.3 MATERIALS AND METHODS

5.3.1 Chemicals and enzymes

Unless otherwise specified, all chemical reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), restriction enzymes, agarose and DNA molecular weight markers were obtained from Boehringer Mannheim (Laval, Quebec, Canada). Yeast nitrogen base without amino acids was obtained from Difco Lab. (Detroit, Michigan, USA). Glusulase was obtained from Dupont Co. (Boston, Massachusetts, USA). Other chemicals were obtained from Anachemia (Montreal, Quebec, Canada).

5.3.2 Strains, vectors, and media

The strains and vectors used in this study are listed in Table 5.1. The media and methods to grow *E. coli* and yeasts are same to those in previous research (section 4.3.2).

5.3.3 Isolation of vector DNA from *E. coli*

The method to prepare vector DNA from *E. coli* is same to that of previous chapter (section 2.3.7.1).

5.3.4. DNA elution, digestion and ligation

The methods to elute, digest, and ligate vector DNA are same to those of previous chapter (section 2.3.7.2).

5.3.5 Quantitation of DNA

The method to determine DNA quantity is same to that of previous chapter (section 2.3.8).

5.3.6 Transformation

5.3.6.1 *E. coli* transformation

The method to transform *E. coli* is same to that of previous chapter (section 2.3.10.1).

5.3.6.2 Yeast transformation

The method to transform yeast is same to that of previous chapter (section 2.3.10.2).

5.3.7 Preparation of crude enzyme

The method to prepare crude enzyme solution is same to that of previous chapter (section 4.3.6).

5.3.8 Protein Assay

The method to determine protein quantity yeast is same to that of previous chapter (section 4.3.7).

5.3.9 β-galactosidase activity assay

The method to do β-galactosidase assay is same to that of previous chapter (section 4.3.8).

5.3.10 Agarose gel Electrophoresis

The method to do agarose gel electrophoresis of DNA is same to that of previous chapter (section 2.3.9).

5.4 RESULTS

5.4.1 Vector construction

Following transformation with pVT101-L (*LEU*2-d) and YEp351 (*LEU*2), transformants of *Schw. occidentalis* DW88 were obtained on MM plates from YEp351, but none were obtained with pVT101-L. However, pVT101-L is a yeast expression vector carrying a cassette of [*S. cerevisiae ADH*1 promoter - multiple cloning sites - 3' sequence of *ADH*1 gene]. Thus, to construct an expression vector for *Schw. occidentalis* DW88, it was necessary to substitute the *LEU*2-d gene with the *LEU*2 gene. As shown in Figure 5.1, the DNA fragment of 1,294 bp from *Kpn*I/*Sna*BI-digested YEp351 was eluted from agarose gel, while the DNA fragment of 5,029 bp was prepared from *Kpn*I/*Sna*BI-digested pVT101-L. After ligation of these two DNA fragments, *E. coli* DH10B was transformed with the recombinant DNA and screened on LB plates containing ampicillin. The vector DNA isolated from one of the transformants was designated as YEpE81 (6,323 bp). Figure 5.2 indicates that *Kpn*I/*Sna*BI-digested YEpE81 showed two DNA fragments of 1.3 kb and 5.0 kb (Lane D), which were identical to those from YEp351 (Lane B) and pVT101-L (Lane C) digested with *Kpn*I and *Sna*BI, respectively.

To verify whether the YEpE81 can function as an expression vector in *Schw. occidentalis* DW88 or not, a *Streptococcus thermophilus* β-galactosidase gene (*GAL*) was used as a reporter gene. As shown in Figure 5.3, the *GAL* gene containing DNA fragment of 3.1 kb was cut off from YEp13-GAL by *Hin*dIII and eluted from agarose gel. After ligation of this DNA fragment with *Hin*dIII-digested YEpE81, *E. coli* DH10B was transformed with the recombinant DNA and screened on LB plates containing ampicillin, IPTG and X-Gal. When the *Hin*dIII-digested DNA fragment was inserted into the *Hin*dIII cutting site of YEpE81, two orientations of the inserted fragment were expected. Vectors isolated from three blue-colored transformants were checked for their orientation by *Xba*I digestion. After *Xba*I digestion, each vector would produce either one of two sets of DNA fragments: 1) 0.5 and 8.9 kb; or 2) 2.7 and 6.7 kb. One of the three vectors showed the former, which was desired, on agarose gel (Figure 5.4, Lane E). This vector was designated as YEpE81-GAL (9.4 kb). In Figure 5.4, *Hin*dIII-digested YEpE81-GAL

showed two DNA fragments of 3.1 and 5.3 kb (Lane D), which were similar to those of *Hin*dIII-digested YEp13-GAL (Lane C) and YEpE81 (Lane B), respectively.

5.4.2 Expression of β-Galactosidase gene

Following transformation with YEpE81 and YEpE81-GAL, *Schw. occidentalis* DW88 showed transformants on MM plates with these two vectors. After growth in rich medium, detectable β-galactosidase activity was not found from the cell lysate of YEpE81 transformant, but found from YEpE81-GAL transformant cell lysate with specific activity of 1,210 U/μg.

5.5 DISCUSSION

*S. cerevisiae ADH*1 promoter, a strong promoter (Bennetzen and Hall, 1982), was used to construct series of expression vectors for *S. cerevisiae* (Verent et al., 1987). The cloned genes expressed in *Schw. occidentalis* so far include *S. cerevisiae LEU*2, *TRP*5 and *URA*3 genes (Dohmen et al., 1989; Dave and Chattoo, 1997; Wang and Lee, 1997), a *Schw. occidentalis AMY* gene (Dohmen et al., 1989), a *C. thermocellum* cellulase gene (Piontek et al., 1990) and a *Streptococcus thermophilus GAL* gene (Wang et al., 1999, Chapter 4). These genes functioned in *Schw. occidentalis* with their own promoters except the cellulase gene and the *GAL* gene, which were controlled by *Schw. occidentalis GAM*1 promoter and *S. cerevisiae ADH*1 promoter, respectively. According to the putative preferred codons of *Schw. occidentalis* (Wang et al, 1999), the CBIs of the *ADH*1, *AMY*, *GAM*1, *LEU*2, *TRP*5, and *URA*3 genes are 0.77, 0.40, 0.56, 0.66, 0.49, and 0.25, respectively, in *Schw. occidentalis*. Highly expressed genes have CBIs of more than 0.6, and the moderate ones around 0.2 (Boucherie et al., 1995). Except for *ADH*1 and *LEU*2, these genes are not highly expressed in *Schw. occidentalis*. As the *LEU*2 gene was used as a genetic marker in *Schw. occidentalis* DW88 transformation, the *ADH*1 promoter was a good candidate to construct expression vectors for *Schw. occidentalis* DW88.

The *LEU*2-d resulted from a deletion of the 5'-end sequence prior to –29 bp of *S. cerevisiae LEU*2 gene during vector pMP78 construction (Erhart and Hollenberg, 1983). The *LEU*2-d was used as a genetic marker to construct many high-copy-number vectors for *S. cerevisiae*, such as pJDB219 and pVT series (Erhart and Hollenberg, 1983; Vernet et al., 1987). Unlike YEp351, pVT101-L failed to offer *Schw. occidentalis* DW88 transformants growing on MM plates. This suggests that the *LEU*2-d does not express or can not function well in *Schw. occidentalis* because of different transcription mechanisms present between *S. cerevisiae* and *Schw. occidentalis* for the *LEU*2 gene.

Both pVT101-L and YEp351 possess a DNA fragment containing 2-μm sequence and the *LEU*2 gene. Differences between these two DNA fragments are length and structure. The lengths between the 2-μm sequence and the *LEU*2 gene are 379 bp from pVT101-L and 411 bp from YEp351. To construct an expression vector based on the *ADH*1 promoter for *Schw. occidentalis* DW88, a complete 5' sequence of *LEU*2 gene was used to replace the deleted 5' sequence of *LEU*2-d gene on pVT101-L. The *Kpn*I/*Sna*BI fragment of YEp351 containing the original 5' region of *LEU*2 gene was used to replace the *Kpn*I/*Sna*BI fragment of pVT101-L containing the deleted 5' region of *LEU*2-d gene. After replacement, the resulting vector, YEpE81, possessed both the *ADH*1 promoter and the *LEU*2 gene with the original 5' region and complemented the *leu* mutation of *Schw. occidentalis* DW88. Figure 5.2 shows that *Kpn*I/*Sna*BI-digested YEpE81 had two DNA fragments (Lane D) with same molecular weights as those from *Kpn*I/*Sna*BI-digested pVT101-L (Lane C) and YEp351 (Lane B). It indicates that YEpE81 is the vector desired and has a very similar structure to pVT101-L.

A *S. thermorphilus GAL* gene was used to assess the promoter activity of the expression vector, YEpE81. A *Hin*dIII-digested DNA fragment containing the *GAL* gene from YEp13-GAL was cloned into the *Hin*dIII cutting site of YEpE81 to form YEpE81-GAL. Due to the same cohesive ends at both sides of the DNA fragment, the ligation would produce two kinds of orientations for the inserted DNA fragment. After digestion with *Xba*I, the correct fusion of the *ADH*1 promoter and the *GAL* gene DNA fragment

was confirmed (Figure 5.4, Lane E). In addition, Figure 5.4 showed that *Hin*dIII-digested YEpE81-GAL had two DNA fragments (Lane D) with the same molecular weights as those from *Hin*dIII-digested YEp13-GAL (Lane C) and YEpE81 (Lane B), respectively. These results indicate that the YEpE81-GAL is the desired one. The β-galactosidase activity was detected from *Schw. occidentalis* DW88 carrying YEpE81-GAL with similar specific activity found in previous research (Wang et al., 1999, Chapter 4), but not from transformants with YEpE81. It means that YEpE81 is an expression vector for *Schw. occidentalis*. YEpE81 carries a multiple cloning region containing sites of *Bam*HI, *Hind*III, *Pst*I, *Pvu*II, *Sst*I, *Xba*I, and *Xho*I derived from pVT101-L.

5.6 CONCLUSIONS

The CBI (0.77) of *S. cerevisiae ADH*1 gene based on the putative preferred codons of *Schw. occidentalis* indicates that the *ADH*1 promoter should be strong in *Schw. occidentalis*. The promoter was successfully used to construct an expression vector (YEpE81) for *Schw. occidentalis*. A *S. thermophilus GAL* gene was cloned into YEpE81 and expressed in the yeast. Thus, YEpE81 and *Schw. occidentalis* DW88 can be used to study heterologous gene expressions in *Schw. occidentalis*.

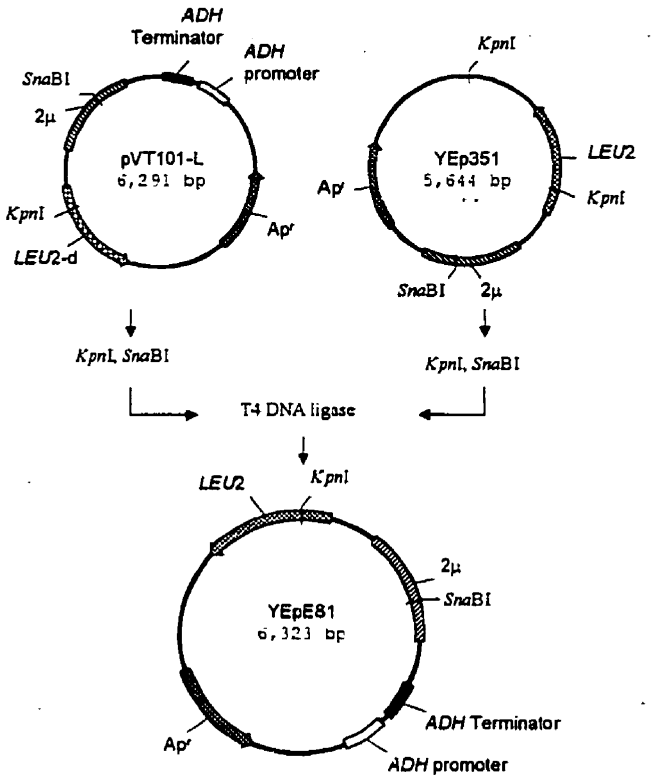

Figure 5.1. Construction of YEpE81. The DNA fragment of 1,294 bp from *Kpn*I/*Sna*BI-digested YEp351 was ligated with the DNA fragment of 5,029 bp from *Kpn*I/*Sna*BI-digested pVT101-L.

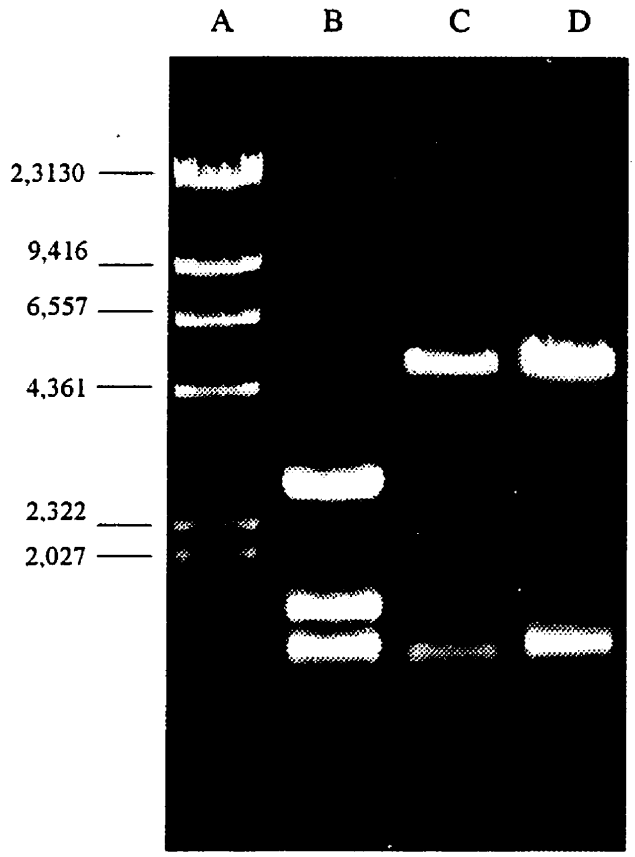

Figure 5.2. Agarose gel electrophoresis of DNA. Lane A, DNA marker λ-*Hin*dIII. Lane B, YEp351 digested with *Kpn*I and *Sna*BI. Lane C, pVT101-L digested with *Kpn*I and *Sna*BI. Lane D, YEpE81 digested with *Kpn*I and *Sna*BI.

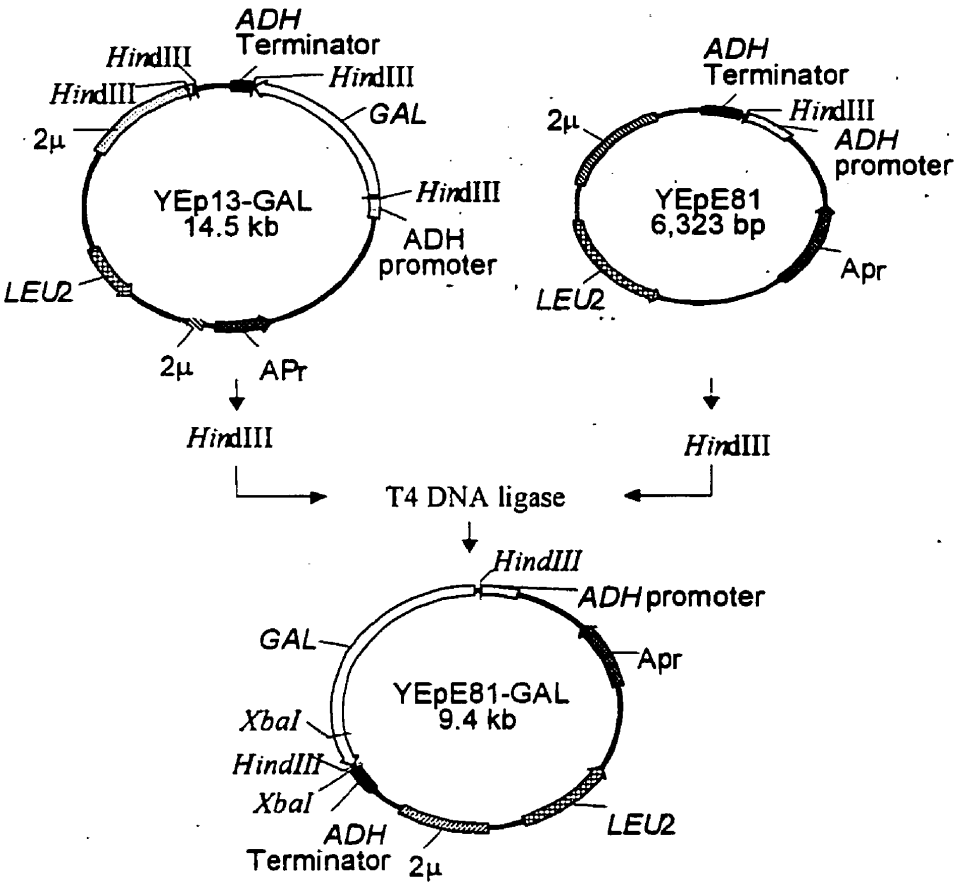

Figure 5.3. Construction of YEpE81-GAL. The *GAL* gene containing DNA fragment of 3.1 kb was cut off from YEp13-GAL by *Hin*dIII and ligated with *Hin*dIII-digested YEpE81.

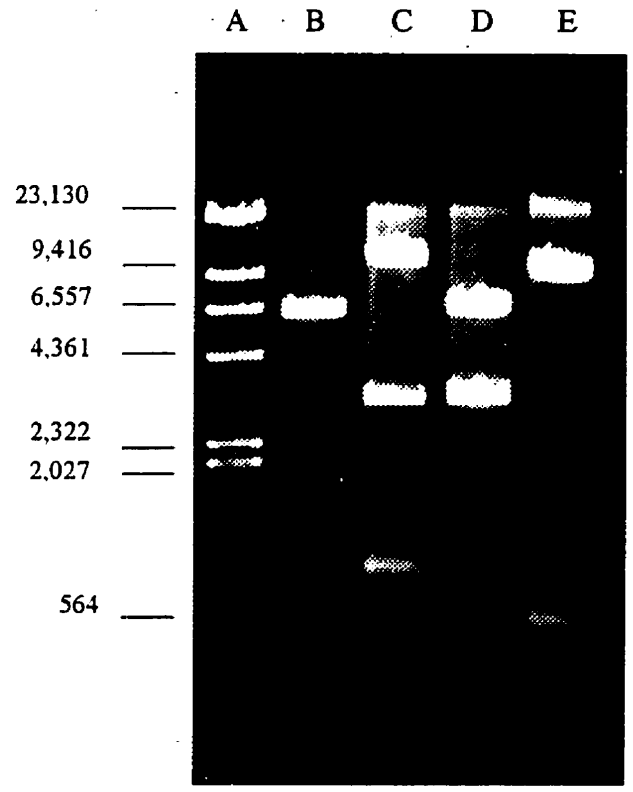

Figure 5.4. Agarose gel electrophoresis of DNA. Lane A, DNA marker λ-*Hin*dIII. Lane B, YEpE81 digested with *Hin*dIII. Lane C, YEp13-GAL digested with *Hin*dIII. Lane D, YEpE81-GAL digested with *Hin*dIII. Lane E, YEp81-GAL digested with *Xba*I.

Table 5.1 Microbial strains and vectors used in this study.

Strain or vector	Relevant Characteristics	Source
E. coli DH10B	F⁻ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80*dlacZ* Δ*lacX*74 *deo*R *rec*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*Kλ⁻ *rps*L *end*A1 *nup*G	GIBCO-BRL
Schw. occidentalis DW88	*leu*	Wang and Lee, 1997
pVT101-L	6,291 bp, Ap^r, *LEU*2-d	Vernet et al., 1987
YEp351	5,644 bp, Ap^r, *LEU*2	Hill et al., 1986
YEp13-GAL	14.5 kb, Ap^r, *LEU*2, *GAL*	Wang et al., 1999, Chapter 4
YEpE81	6,323 bp, Ap^r, *LEU*2	This study
YEpE81-GAL	9.4 kb, Ap^r, *LEU*2, *GAL*	This study

CHAPTER 6.0

SECRETION OF A *SACCHAROMYCES DIASTATICUS* GLUCOAMYLASE BY *SCHWANNIOMYCES OCCIDENTALIS*

To evaluate the possibility of *Schwanniomyces occidentalis* DW88 secretion of heterologous proteins, a *Saccharomyces diastaticus STA3* gene encoding an extracellular glucoamylase was used to carry out this research. The *STA3* gene controlled by its own promoter and the glucoamylase directed by its own signal sequence offered *Schw. occidentalis* DW88 transformant more glucoamylase activity.

The major results of this study were summarized and submitted as a manuscript for publication in *Applied Microbiology and Biotechnology*. The manuscript entitled "Secretion of a *Saccharomyces diastaticus* glucoamylase by *Schwanniomyces occidentalis*" was co-authored by Tsung-Tsan Wang, Young-Jun Choi, and Byong H. Lee. The project was supervised by Dr. Byong H. Lee. Mr. Young-Jun Choi helped for gel electrophoresis and enzyme assay, while the actual experimental work and writing of the manuscript were done by Tsung-Tsan Wang. Dr. Byong Lee edited the manuscript prior to its submission for publication.

6.1 ABSTRACT

After *Saccharomyces diastaticus STA3* gene was cloned into *Schwanniomyces occidentalis* DW88, the transformant yielded more cell mass and glucoamylase activity. The *STA3* gene was controlled by its own promoter and the glucoamylase was directed by its own signal sequence. The *S. diastaticus* glucoamylase secreted by *Schw. occidentalis* DW88 had similar pH and temperature optima as compared with those of wild-type glucoamylase, suggesting that *Schw. occidentalis* DW88 does not change the properties of the heterologous protein. The major glucoamylase activity was detected from culture supernatant, but not from intracellular cell lysate, indicating that *Schw. occidentalis*

DW88 has a strong secretion ability. These results show that *Schw. occidentalis* DW88 should be a good host cell to produce and secrete heterologous proteins.

6.2 INTRODUCTION

Saccharomyces cerevisiae, a traditional yeast, is widely used in academic and industrial fields. However, this yeast is not an ideal host producing extracellular heterologous proteins due to certain disadvantage such as:

1) low product yields, even with a strong promoter (Buckholz and Gleeson, 1991),
2) hyper-glycosylated proteins which might affect protein characteristics (Lemontt et al., 1985; Moir and Dumais, 1987; Van Arsdell et al., 1987), and
3) low secretion efficiency when protein is larger than 30 kDa (Smith et al., 1985; Jigami et al., 1986; Moir and Dumais, 1987; De Nobel and Barnett, 1991).

Thus, the molecular studies have been extended to other yeasts than *S. cerevisiae* that makes the genetic engineering of non-*Saccharomyces* yeasts important.

Schwanniomyces occidentalis (*Debaryomyces occidentalis*) was described as a potential "super yeast" (Ingledew, 1987). *Schw. occidentalis* is able to grow rapidly with high cell mass on cheap starch as carbon source (Moresi et al., 1983; Raspor, 1987; Jamuna and Ramakrishna, 1989; Horn et al., 1992 produce strong amylolytic enzymes extracellularly and secrete large proteins without hyper-glycosylation and measurable extracellular proteases (Ingledew, 1987; Deibel et al., 1988). *Schw. occidentalis* thus has a high potential alternative to *S. cerevisiae* in production of heterologous proteins. However, the molecular study in *Schw. occidentalis* is in infancy. Heterologous protein secreted by the yeast was a *Clostridium thermocellum* cellulase (Piontek et al., 1990). The promoter and signal sequence of a *Schw. occidentalis GAM*1 gene controlled the cellulase-encoding gene expression and protein secretion.

A new transformation system was recently developed on *Schw. occidentalis* DW88 (Wang and Lee, 1997). It was demonstrated that *Schw. occidentalis* DW88 should

be a good host cell for heterologous gene expression, because the strain does not modify heterologous gene and heterologous protein property (Wang.and Lee, 1997; Wang et al., Chapter 4). In industrial scale for enzyme production, secretion has always been emphasized. Secretion not only facilitates protein purification, but also offers secretory pathway for protein folding and post-translational modification environment. Secretion can further prevent host cells from damage if toxic heterologous proteins are present intracellularly.

Schw. occidentalis DW88 is able to simultaneously produce strong extracellular α-amylase and glucoamylase, which can completely hydrolyze starches. The amylolytic ability of *Schw. occidentalis* DW88 could be further improved by introducing cloned amylolytic genes. The purpose of this research was to study the secretion ability and to enhance amylolytic activity of *Schw. occidentalis* DW88 by using a glucoamylase gene of *Saccharomyces diastaticus*.

6.3 MATERIALS AND METHODS

6.3.1 Chemicals and enzymes

Unless otherwise specified, all chemical reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), restriction enzymes, agarose and DNA molecular weight markers were obtained from Boehringer Mannheim (Laval, Quebec, Canada). Yeast nitrogen base without amino acids was obtained from Difco Lab. (Detroit, Michigan, USA). Glusulase was obtained from Dupont Co. (Boston, Massachusetts, USA). Other chemicals were obtained from Anachemia (Montreal, Quebec, Canada).

6.3.2 Strains, vectors, and media

The strains and vectors used in this study are listed in Table 6.1.

The strains and vectors used in this study are listed in Table 6.1. The media and methods to grow *E. coli* and yeasts are same to those in previous research (section 2.3.2). To prepare glucoamylase, yeasts were grown in MS (0.67 % Difco yeast nitrogen base without amino acid; 2 % soluble starch; 20 μg/ml adenine; 20 μg/ml uracil).

6.3.3 DNA isolation.

6.3.3.1 Preparation of vector DNA from *E. coli*

The method to prepare vector DNA from *E. coli* is same to that of previous chapter (section 2.3.7.1).

6.3.3.2 Preparation of vector DNA from yeast

The method to prepare vector DNA from yeast is same to that of previous chapter (section 2.3.7.3).

6.3.4 Transformation procedures

6.3.4.1 *E. coli* transformation

The method to transform *E. coli* is same to that of previous chapter (section 2.3.10.1).

6.3.4.2 Yeast transformation

The method to transform yeast is same to that of previous chapter (section 2.3.10.2).

6.3.5 Preparation of crude enzyme

Portions (50 ml) of *Schw. occidentalis* DW88 with pSTA3-6-4 were collected by centrifugation at 12,000 rpm for 15 min at 4 °C. The resulting supernatant was collected for the measurement of extracellular glucoamylase activity. The method to prepare intracular crude enzyme solution is same to that of previous chapter (section 4.3.6), except for sodium acetate buffer instead.

6.3.6 Protein Assay

The method to determine protein quantity yeast is same to that of previous chapter (section 4.3.7).

6.3.7 Glucoamylase activity assay

Glucoamylase activity was assayed by measuring the amount of glucose released from soluble starch as the substrate (Miller, 1959). The assay mixture contained 100 mM soluble starch in 100 mM sodium acetate buffer (pH 5.0) and 0.2 ml of enzyme solution in total volume of 1 ml. After incubation for 30 min at 50 °C, reducing sugar formed in the reaction mixture was measured by dinitrosalicylic acid (DNS) method. Portions (0.25 ml) of reaction solution were mixed with 0.75 ml of DNS solution (7.5 g dinitrosalicylic acid, 14.0 g NaOH, 216.1 g Rochelle salt, 5.4 ml phenol, and 5.9 g $Na_2S_2O_5$ per liter H_2O), boiled for 5 min, cooled to room temperature and read at 550 nm. One unit (U) of enzyme activity was defined as one μM of glucose formed per min at 50 °C from the standard curve and specific activity was expressed as units per μg of protein. The standard curve of glucose was prepared by using different concentrations of glucose solution to react with DNS solution.

6.3.8 Agarose gel electrophoresis

The method to do agarose gel electrophoresis of DNA is same to that of previous chapter (section 2.3.9).

6.4 RESULTS

6.4.1 Cloning of *STA3* in *Schw. occidentalis* DW88

S. diastaticus STA3 gene was cloned into YEp13 to form pSTA3-6-4 (Yamashita et al., 1985). When the original and recovered YEp13 and pSTA3-6-4 were transferred into *Schw. occidentalis* DW88, respectively, host cells survived on MM plates. Transformants with YEp13 and pSTA3-6-4 were inoculated on MS plates with least amount of cells as possible, and incubated at 30 °C for 5 days. Following the storage of plates for 7 days at 4 °C, clear halo zones appeared around the colonies. The transformants with pSTA3-6-4 (Figure 6.1, B and C) showed bigger colonies and halos than that with YEp13 (Figure 6.1, A).

6.4.2 Glucoamylase activities from different sources

As glucose liberated from soluble starch was used to measure glucoamylase activity, a standard curve of glucose was necessary. Figure 6.2 shows a standard curve of glucose with a r^2 of 0.9997745575. To locate the glucoamylase, culture supernatant and cell lysate from yeast transformants were assayed. The major glucoamylase activities were detected from the extracellular samples of *Schw. occidentalis* DW88 transformants (Table 6.2). The specific activity of glucoamylase from transformant with pSTA3-6-4 was 2.7-fold higher than that of transformant with YEp13. When the same amount of stationary-phase cells of *Schw. occidentalis* DW88 transformants with YEp13 and pSTA3-6-4 were inoculated into fresh MS broth and incubated, the cell mass and glucose content in culture supernatant of transformant with pSTA3-6-4 were higher than those of transformant with YEp13 (Table 3).

The molecular weight of *S. diastaticus* glucoamylase ranged from 53 to 68 kDa determined by SDS-polyacrylamide gel (Yamashita et al., 1984). The molecular weight of *Schw. occidentalis* glucoamylase was around 150 kDa determined by the same method

(Wang et al., 1989, unpublished). To remove the host glucoamylase, a Centriplus concentrators - 100 (Amicon Inc., Beverly, Massachusetts, USA) was used to cut off proteins larger than 100 kDa. After treatment with Centriplus concentrators – 100, the glucoamylase activity was not detected from the sample of *Schw. occidentalis* DW88 with YEp13, but detected from *Schw. occidentalis* DW88 with pSTA3-6-4.

6.4.3. Properties of *S. diastaticus* glucoamylase produced by *Schw. occidentalis*

The enzyme solution of *Schw. occidentalis* DW88 with pSTA3-6-4 treated by Centriplus concentrators - 100 was concentrated to study the properties of *S. diastaticus* glucoamylase produced by *Schw. occidentalis*. The enzyme activities of *S. diastaticus* glucoamylase produced by *Schw. occidentalis* DW88 were determined at different pH and temperatures. The optimal pH for the glucoamylase activity was pH 5 (Figure 6.3) and the optimal temperature was around 55 °C (Figure 6.4).

6.5 DISCUSSION

Since *Schw. occidentalis* DW88 is able to produce both α-amylase and glucoamylase extracellularly, a clear halo zone appeared around its own colony on MS plates and glucoamylase activity was detected from the culture supernatant. *Schw. occidentalis* DW88 with pSTA3-6-4 showed larger colonies and halo zones than the control (YEp13) on MS plates. Higher glucose content, cell mass and the specific activity from the transformants (pSTA3-6-4) were observed as compared with the control. Due to a bigger size (150 kDa) than the membrane cut off size, the *Schw. occidentalis* glucoamylase could be removed from the enzyme sample after treatment with Centriplus concentrators – 100. After treatment with Centriplus concentrators – 100, the enzyme sample of *Schw. occidentalis* DW88 with pSTA3-6-4 still had glucoamylase activity, suggesting that the glucoamylase had a molecular weight less than 100 kDa, which agrees with the published molecular weights (53 – 68 kDa) of *S. diastaticus* glucoamylase (Yamashita et al., 1984). The results strongly indicate that not only the heterologous

STA3 gene was expressed in *Schw. occidentalis* by using its own promoter, but also the heterologous glucoamylase was secreted from *Schw. occidentalis* by using its own signal sequence.

The *S. diastaticus* glucoamylase produced by *Schw. occidentalis* showed pH (5) and temperature (55 °C) optima identical to those of *S. diastaticus* glucoamylase (Yamashita et al., 1985). *Schw. occidentalis* DW88 does not seem to change the properties of *S. diastaticus* glucoamylase. The major glucoamylase activity was detected from the supernatant of *Schw. occidentalis* DW88, but not from intracellular cell lysate, suggesting that *Schw. occidentalis* DW88 is a good host producing and secreting heterologous proteins.

The result of polyacrylamide gel electrophoresis could not be provided in this study as the other evidence of the heterologous protein production in *Schw. occidentalis*. The molecular weights of the *S. diastaticus* glucoamylase ranged from 53 to 68 kDa, which are similar to those of α-amylase produced by *Schw. occidentalis* (Ingledew, 1987; Wang et al., 1989). Moreover, when soluble starch solution was used to perform activity staining of the polyacrylamide gel, the activity staining of *S. diastaticus* glucoamylase produced by *Schw. occidentalis* was interfered by the presence of host α-amylase.

According to putative preferred codons of *Schw. occidentalis* (Wang et al, 1999), the *STA3* gene has a codon bias index of 0.32 in this yeast. While the CBIs of highly expressed genes are more than 0.6, the moderate ones are around 0.2 (Boucherie et al., 1995). The *STA3* gene should thus not have a strong expression in *Schw. occidentalis*, but the signal sequence of the *STA3* gene successfully directed the *S. diastaticus* glucoamylase out of *Schw. occidentalis*. This signal sequence could be helpful to design secretion vectors for the secretion of heterologous proteins by *Schw. occidentalis*.

As many industries are interested in producing strong amylolytic enzymes to hydrolyze starches, the heterologous gene expression of *S. diastaticus STA3* gene can be useful to improve the amylolytic ability of *Schw. occidentalis*.

6.6 CONCLUSIONS

A heterologous gene, *S. diastaticus STA3* controlled by its own promoter was successfully cloned and expressed in *Schw. occidentalis* DW88. The heterologous glucoamylase directed by its own signal sequence was also secreted from the yeast. Due to complete secretion of glucoamylase without modification of the enzyme property, *Schw. occidentalis* DW88 should be a good host cell to produce and secrete heterologous proteins. Furthermore, this gene expression improved the amylolytic activity of *Schw. occidentalis* DW88, though the yeast is able to produce both α-amylase and glucoamylase.

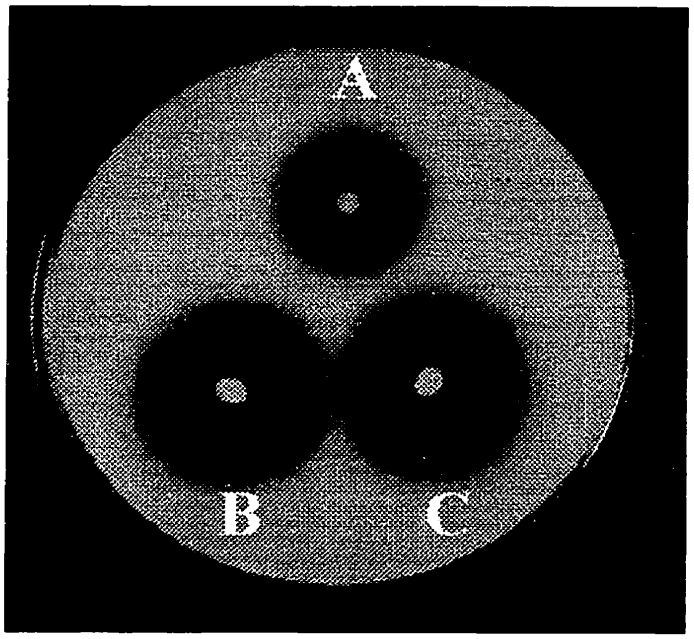

Figure 6.1. *Schw. occidentalis* DW88 transformants grown on MS plate. A, transformant with YEp13. B and C, transformants with pSTA3-6-4. Transformants with YEp13 and pSTA3-6-4 were inoculated on MS plates, first incubated for 5 days at 30 °C, and then for 7 days at 4 °C.

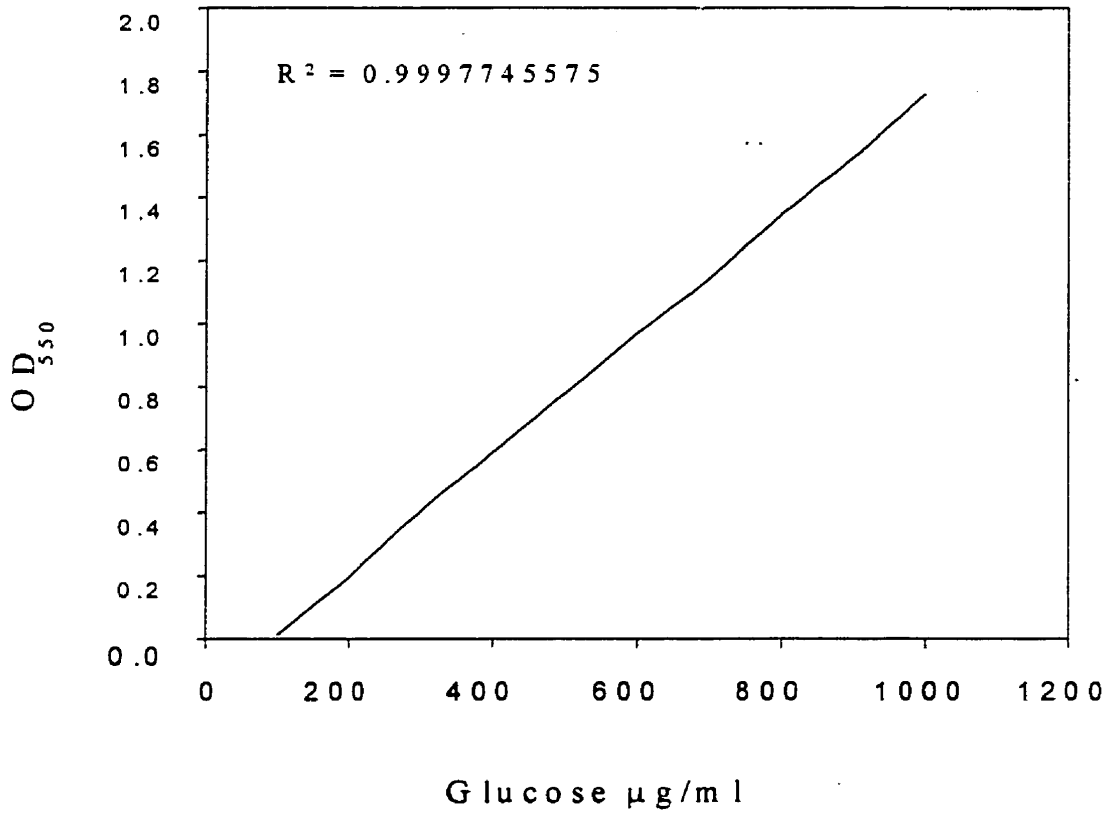

Figure 6.2. A standard curve used for glucoamylase assay.

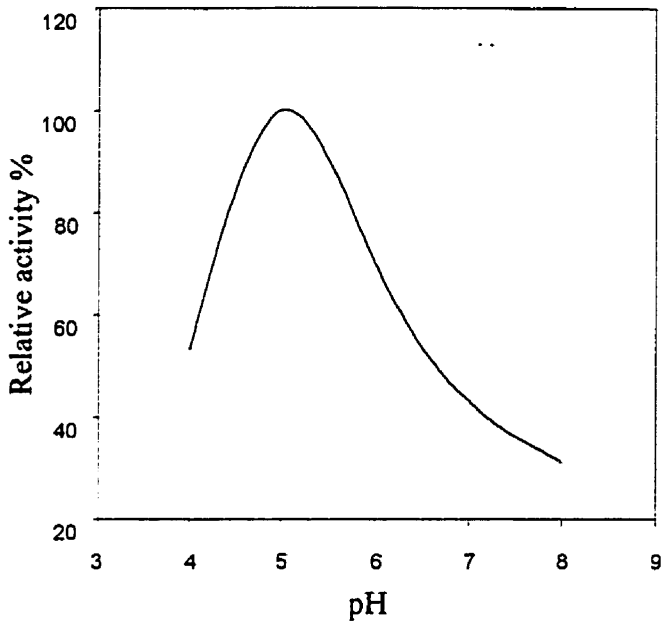

Figure 6.3. pH dependency of *S. diastaticus* glucoamylase produced by *Schw. occidentalis* DW88.

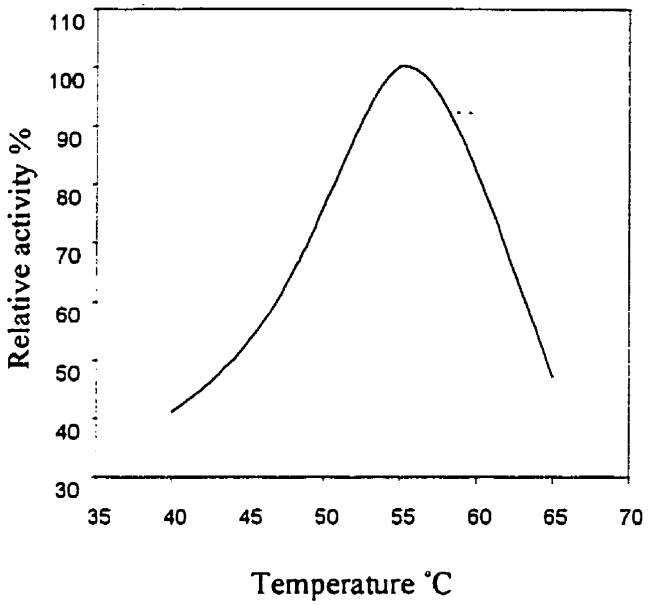

Figure 6.4. Temperature dependency of *S. diastaticus* glucoamylase produced by *Schw. occidentalis* DW88.

Table 6.1. Microbial strains and vectors used in this study.

Strain or vector	Relevant Characteristics	Source
E. coli DH10B	F⁻ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80*dlacZ* Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*Kλ⁻ *rps*L *end*A1 *nup*G	GIBCO-BRL
Schw. occidentalis DW88	*leu*⁻	Wang and Lee, 1997
YEp13	10.7 kb, Ap^r, Tc^r, *LEU2*	Broach et al., 1979
pSTA3-6-4	18 kb, Ap^r, *LEU2*, *STA3*	Yamashita et al., 1985

Table 6.2. Glucoamylase activities* of *Schw. occidentalis* DW88 transformants.

Host	YEp13		pSTA3-6-4	
Location	Intracellular	Extracellular	Intracellular	Extracellular
Activity (U)	0.01	0.28	0.01	0.93
Protein (μg/ml)	0.41	0.13	0.63	0.16
Specific activity (U/μg)	0.02	2.15	0.02	5.81

* Mean of triplicate experiments

Table 6.3. Cell mass and glucose content[a] in yeast culture supernatants.

Incubation time (h)		0	16	20	24	28
YEp13	Cell number	1.6×10^4	0.4×10^6	0.9×10^6	2.1×10^6	4.8×10^6
	Glucose µg/ml	0.2	580	760	850	1,100
STA3	Cell number	1.6×10^4	0.9×10^6	2.2×10^6	5.4×10^6	13.3×10^6
	Glucose µg/ml	0.2	840	1,020	1,390	2,230

[a] Mean of triplicate experiments

GENERAL CONCLUSION

A new transformation system was developed for yeast *Schw. occidentalis* with an efficiency of 10^3 transformants per μg DNA using a spheroplast-mediating method. This system was based on a complementation of *S. cerevisiae LEU2* gene on a stable leucine auxotrophic mutant, *Schw. occidentalis* DW88. The vector YEp13 was replicated extrachromosomally in the yeast host cells and recovered from the yeast transformants without DNA modification.

Putative preferred codons of *Schw. occidentalis* proposed could be useful to evaluate the codon bias index (CBI) for cloned genes, and the CBI could serve to estimate gene expression in the yeast without tedious and time-consuming laboratory work. Moreover, calculating codon bias index manually is time-consuming. A new computer program (PCBI) was developed to overcome the inconvenience. The preferred codons and PCBI program could facilitate gene expression in *Schw. occidentalis.*

A β-galactosidase gene of *Streptococcus thermophilus* controlled by *S. cerevisiae ADH1* promoter was successfully expressed in *Schw. occidentalis* without modification of the intracellular enzyme properties. *S. cerevisiae ADH1* promoter was a strong promoter in *Schw. occidentalis* and was used to construct a *Schw. occidentalis* expression vector.

A *S. diastaticus STA3* controlled by its own promoter was successfully expressed in *Schw. occidentalis* DW88. The heterologous glucoamylase directed by its own signal sequence was also secreted from the yeast. Due of complete secretion of glucoamylase and no modification of its properties, *Schw. occidentalis* DW88 should be a good host to produce and secrete heterologous proteins.

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