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EXPRESSION OF THE GENE ENCODING HEPATITIS B VIRUS SURFACE ANTIGEN IN YEAST BY *PHO*84 PROMOTER

JINTANA WONGWIGKARN

With compliments
of
บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล

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Thesis
entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University

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Jintana Wongwigkarn

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JINTANA WONGWIGKARN; EXPRESSION OF THE GENE ENCODING HEPATITIS B VIRUS SURFACE ANTIGEN IN YEAST BY *PHO*84 PROMOTER. THESIS ADVISORS : CHUENCHIT BOONCHIRD, Ph.D., WATANALAI PANBANGRED, Dr.Eng, SAOVANEE DHARMSTHITI, Ph.D. 165 p ISBN 974-04-1075-8.

*PHO*84 is a low-affinity inorganic phosphate (Pi) transporter. It is regulated under the *PHO* regulatory pathway. When the yeast cells were grown in high phosphate medium (High-Pi), the gene is repressed but derepressed in low phosphate medium (Low-Pi). In this work, the *PHO*84 promoter was employed for the expression of the gene encoding hepatitis B virus surface antigen. Four yeast expression plasmids containing *PHO*84 promoter and *CYC*1 terminator, pPM323-P1 (8.75 kb) and pPM323-P3 (7.33 kb) containing *LEU*2d selectable marker, and pPM323-P2 (9.85 kb) and pPM323-P4 (8.43 kb) containing *LEU*2d and *URA*3 selectable markers were constructed. The PreS2d+S gene, encoded for the middle hepatitis B surface antigen (M HBsAg) in which the protease sensitive site in PreS2 region was deleted, was inserted into the four plasmids to obtain recombinant plasmids pEB-P1, pEB-P3, pEB-P2 and pEB-P4, respectively. The expression of the PreS2d+S gene in the four recombinant plasmids was studied in *Saccharomyces cerevisiae* strain BJ5462 and induction of gene was performed by transferring the recombinant yeasts from High-Pi (11 mM) to Low-Pi (0.22 mM) in early log phase. Western blot analysis of the M HBsAg produced by the four recombinant plasmids demonstrated that the molecular mass of the proteins were 34 kDa and 37 kDa. Comparison of the M HBsAg production, plasmid copy number and plasmid stability among the four plasmids demonstrated that the plasmid pEB-P3 with *LEU*2d selectable marker gave highest value. Optimization of the induction phase for M HBsAg production by the plasmid pEB-P3 with Low-Pi showed that induction at OD_{660} 0.5 was the most suitable. When time-course of the M HBsAg production induced with Low-Pi was investigated, it demonstrated the production of M HBsAg was 100.09 ng/mg protein (146.92 μg/l culture) at stationary phase and the production was growth associated. Furthermore, analysis of the M HBsAg protein bands from the recombinant yeast grown in log to stationary phase indicated that protein bands were still intact throughout the growth phase. In addition, the M HBsAg production was also studied by cultivation of the recombinant yeast in Low-Pi condition. It demonstrated that level of production was slightly lower than in induction condition. The M HBsAg production by cultivation recombinant yeast in Low-Pi condition would be, therefore, convenient for large-scale production.

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จินตนา ว่องวิกย์การ : การแสดงออกของยีนส่วนผิวของไวรัสตับอักเสบชนิดบีในยีสต์ โดย *PHO*84 โปรโมเตอร์ (EXPRESSION OF THE GENE ENCODING HEPATITIS B VIRUS SURFACE ANTIGEN IN YEAST BY *PHO*84 PROMOTER) คณะกรรมการควบคุมวิทยานิพนธ์ : ชื่นจิตต์ บุญเณิด, Ph.D., วัฒนาลัย ปานบ้านเกร็ด, Dr. Eng, เสาวนีย์ ธรรมสถิต, Ph.D., 165 p ISBN 974-04-1075-8.

*PHO*84 เป็นยีนที่สร้างโปรตีนขนส่งสารอนินทรีย์ฟอสเฟต ซึ่งควบคุมการแสดงออกโดยระบบของยีน *PHO* เมื่อเพาะเลี้ยงยีสต์ในอาหารเลี้ยงเชื้อที่มีปริมาณฟอสเฟตสูง (High-Pi) การแสดงออกของยีนจะถูกยับยั้งการแสดงออกแต่สามารถแสดงออกได้เมื่อเพาะเลี้ยงในสภาวะที่มีฟอสเฟตต่ำ (Low-Pi) ในการศึกษานี้ใช้โปรโมเตอร์ *PHO*84 เพื่อควบคุมการแสดงออกของยีนแอนติเจนส่วนผิวของไวรัสตับอักเสบชนิดบี พลาสมิด 4 ชนิดที่มีโปรโมเตอร์ *PHO*84 และเทอมิเนเตอร์ *CYC*1 ได้ถูกสร้างขึ้นซึ่งได้แก่ พลาสมิด pPM323-P1 (8.75 kb) และ pPM323-P3 (7.33 kb) ที่มียีนคัดเลือก *LEU*2d และพลาสมิด pPM323-P2 (9.85 kb) และ pPM323-P4 (8.43 kb) ที่มียีนคัดเลือก *LEU*2d และ*URA*3 เมื่อนำยีนแอนติเจนส่วนผิวขนาดกลาง คือ PreS2d+S (ซึ่งตำแหน่งบน PreS2 ที่สามารถย่อยได้ด้วยโปรตีเอสได้ถูกกำจัดออก) ไปใส่ในพลาสมิดดังกล่าว ทำให้ได้รีคอมบิแนนท์พลาสมิด 4 ชนิดคือ pEB-P1, pEB-P3, pEB-P2 และ pEB-P4 ตามลำดับ การศึกษาการแสดงออกของยีนแอนติเจนส่วนผิวได้ทำในยีสต์ *Sacchromyces cerevisiae* สายพันธุ์ BJ5462 ซึ่งทำให้เกิดการเหนี่ยวนำการแสดงออกยีนโดยการย้ายรีคอมบิแนนท์ยีสต์ที่เพาะเลี้ยงในอาหารเลี้ยงเชื้อ High-Pi (11 mM) ไปใส่ในอาหารเลี้ยงเชื้อที่เป็น Low-Pi (0.2 mM) ในระยะ early log เมื่อนำโปรตีนที่สร้างโดยรีคอมบิแนนท์ยีสต์ที่มีพลาสมิดทั้ง 4 ชนิด มาวิเคราะห์หาขนาดโปรตีนด้วยวิธี Western blot พบว่าโปรตีนมีขนาด 34 kDa และ 37 kDa จากการเปรียบเทียบค่าปริมาณแอนติเจนส่วนผิว จำนวนชุดของพลาสมิด และความเสถียรของพลาสมิดที่ได้จากรีคอมบิแนนท์พลาสมิดทั้ง 4 ชนิดพบว่าพลาสมิด pEB-P3 ให้ค่าสูงสุด เมื่อทำการศึกษาสภาวะที่เหมาะสมในการเหนี่ยวนำการสร้างแอนติเจนโดยพลาสมิด pEB-P3 โดยย้ายเซลล์ที่เพาะเลี้ยงในอาหารเลี้ยงเชื้อ High-Pi ไปใส่ในอาหารเลี้ยงเชื้อ Low-Pi ที่ช่วง OD_{660} ต่างๆ พบว่าที่ OD_{660} เท่ากับ 0.5 เป็นสภาวะที่เหมาะสมที่สุด จากการศึกษาการผลิตแอนติเจนส่วนผิวที่ระยะต่างๆ ของการเจริญเติบโต โดยการเหนี่ยวนำด้วย Low-Pi พบว่าการผลิตสัมพันธ์กับระยะเวลาการเจริญเติบโต และการผลิตสูงสุดอยู่ที่ระยะ stationary คือผลิตได้ 100.09 ng/mg protein (146.92 μg/l culture) นอกจากนั้นโปรตีนที่ผลิตขึ้นในระยะเวลาต่างๆ ของการเจริญเติบโตมีขนาดคงที่ และเมื่อศึกษาการผลิตแอนติเจนส่วนผิวโดยการเพาะเลี้ยงในสภาวะ Low-Pi เพียงอย่างเดียว พบว่ามีการผลิตโปรตีนน้อยกว่าการเพาะเลี้ยงโดยการเหนี่ยวนำใน Low-Pi เพียงเล็กน้อย ดังนั้นเพื่อความสะดวกในการผลิตในระดับอุตสาหกรรม จึงอาจเพาะเลี้ยงรีคอมบิแนนท์ยีสต์เฉพาะในสภาวะ Low-Pi ได้

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LIST OF ABBREVIATIONS

aa	amino acid
Ap^r	ampicillin resistance
bp	base pair
°C	degree Celsius
CIP	calf intestinal alkaline phosphatase
ColE1 ori	origin of replication of bacteria
*CYC*1t	*CYC*1 terminator
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DrfDp	DrfDp protein
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N, N, N, N'-tetra acetic acid
ELISA	enzyme lineked immunodominant specific activity
et al.	Et.alli (Latin), and others
etc.	et cetera (Latin), and other things, and so on
g	gram
Hcs26p	Hcs26 protein
HBV	hepatitis B virus
HBs	hepatitis B surface
HBsAg	hepatitis B surface antigen
h	hour(s)
i.e.	Ed est (Latin), that is

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LIST OF ABBREVIATIONS (Continued)

Kb	kilobase
kDa	kilodalton
l	litre
LB	Lauria-Bertani
LiAc	lithium acetate
M	molar
mg	milligram
min	minute(s)
ml	millilitre
mM	millimolar
MOPS	3-(N-morpholino) propane sulfonic acid
MW	molecular weight
ng	nanogram
nm	nanometre
OD	optical density
pBS	Bluescript plasmid
PEG	polyethylene glycol
Pho2p	Pho2 protein
Pho4p	Pho4 protein
Pho80p	Pho80 protein
Pho81p	Pho81 protein
Pho85p	Pho85 protein
*PHO*84p	*PHO*84 promoter

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LIST OF ABBREVIATION (Continued)

*PHO*84t	*PHO*84 terminator
Pi	inorganic phosphate
*PGK*1p	*PGK*1 promoter
*PGK*1t	*PGK*1 terminator
PMSF	phenylmethylsulfonyl fluoride
rAPase	repressible acid phosphate
rALPase	repressible alkaline phosphatase
rpm	round per minute
SD	synthetic dextrose
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyagarose gel electrophoresis
sec	second(s)
TB	Terrific broth
TBE	Tris-Borate EDTA
TE	Tris-EDTA
TEMED	N-N-N'-N'-tetramethyl ethylenediamine
UV	ultraviolet
v	volume
w	weight
µg	microgarm
µl	microlitre
2µ ori	origin of replication of 2µ plasmid
%	percent

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CHAPTER I
INTRODUCTION

The gene encoding the hepatitis B surface antigen (HBsAg) in recombinant yeast *Saccharomyces cerevisiae* was the first commercial of a recombinant subunit vaccine. The hepatitis B (HB) vaccine produced by recombinant DNA techniques is extremely safe and effective; however, its prohibitively expensive for use in many areas of the world where hepatitis B virus (HBV) is endemic including Thailand. The cost of vaccinating against HBV can exceed the total cost of vaccinating against all other diseases as recommended by the World Health Organization (WHO) (Davis, 1998). Therefore, research and development for low-cost HB vaccine should be carried out for long term supply. Several expression systems have been investigated for regulation of HBsAg expression in *S. cerevisiae*. Although some of them are successfully applied for commercial production, searching for the new expression system for large-scale production is still carried out. The criteria for chosen of expression system for large-scale production are that the induction should be cheap and simple. One convenient and economical inducible method would be changing the inorganic phosphate (Pi) concentration because there is no need to add a high-cost inducer in induction condition. The expression of the PreS2+S gene encoding for the middle (M) HBsAg by this method was previously investigated using *PHO5* promoter (Itoh *et al*, 1986b and US 4945046, 1990). *PHO5* gene encodes repressible acid phosphatase involving in *PHO* regulatory pathway.

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In this study, the expression of the M HBsAg investigated by *PHO*84 promoter. The *PHO*84 encoding high affinity phosphate transporter is also subjected to control by the *PHO* regulatory pathway. When the cells are starved for phosphate, derepression of the *PHO*84 is initiated (Oshima, 1991). Therefore, the induction of gene expression by removal of Pi is attractive system for production of recombinant protein in large-scale. In addition, the expression of the PreS2+S gene by *PHO*84p has never been reported. Since most of host yeasts for construction of recombinant yeast have more than one auxotrophic genes. In order to reduce the addition of amino acids into the culture media of the recombinant yeast, the expression of the PreS2+S gene on plasmid with two selectable markers was also investigated. Furthermore, to obtain high level expression by *PHO*84 promoter, the induction phase should be optimized and the suitable growth phase should be studied. The PreS2+S gene used in this study was the PreS2d+S gene which the protease sensitive site in the PreS2 region was deleted (Foo-trakoon, 2000).

The objectives of this study are :

1. To construct the recombinant yeast strains which could express the M HBsAg by the inducible *PHO*84 promoter.
2. To compare the expression of M HBsAg by yeast expression plasmid containing one and two selectable markers.
3. To optimize the induction phase for high M HBsAg production by recombinant yeast.
4. To study the time-course of cell growth and the production of the M HBsAg.

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CHAPTER II

BACKGROUND

1. Hepatitis B Surface Antigen (HBsAg)

The hepatitis B virus (HBV) is a spherical particle with a diameter of 42 nm which consists of an inner core and outer surface coat. This particle is known as Dane particle. The inner core composes of HBc (core protein) which surrounds the viral DNA and the enzyme polymerase. The outer surface coat composes of several proteins known as HBs (surface protein). HBV infection in humans is associated with the occurrence of Dane particles, filamentous and spherical particles 22 nm in diameter, in the serum. All particles are formed by association of host-derived lipid (Garilanes *et al.*, 1982) with the three HBs proteins called large (L), middle (M), and small (S) (Ueda *et al.*, 1991) (Figure 1). They are encoded by three in-frame ORF, PreS1+PreS2+S, PreS2+S, and S, respectively. As the promoter for the S and PreS2+S specific transcripts are embedded within the PreS1+PreS2+S ORF, transformation of mammalian cells with DNA including the three ORF results in synthesis of all three surface proteins. Among these proteins, the S protein is the major constituent of the envelope (Heermann *et al.*, 1984). The larger proteins, M and L contain the entire S protein with NH_2-terminal extensions of 55 and 174 amino acids, respectively (Bruss and Ganem, 1991). The M protein contains two asparagines in the appropriate sequence [Asn-X-Ser (Thr)] (Lehle and Bause, 1984) for N-glycosylation : Asn4 and Asn201 (Peterson, 1987).

HBsAg is conformational antigen, which is critically dependent on disulfide bonds between cysteines in the envelope proteins. The cysteines are essential for

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assembly and secretion of 20 nm particles. Fourteen cysteines are commonly found in the S protein of HBV (Norder *et al.*, 1992). The M and L proteins carry no additional cysteines in their N-terminal PreS regions (Ganem, 1991). S protein contains three hydrophobic regions, which are separated by two hydropholic domains (Mangold *et al.*, 1997). Assembly of 20 nm particles is initiated by insertion of the hydrophobic segments of the S protein into the endoplasmic reticulum (ER) membrane (Figure 2). Disulfide-linked dimer are formed in the ER, thought to mature by budding into the pre-Golgi compartment and are subsequently secreted from liver cells into the serum (Huovila *et al.*, 1992). The 20 nm particles isolated from carriers have been used as the source of HB vaccine since long time, known as subunit vaccine.

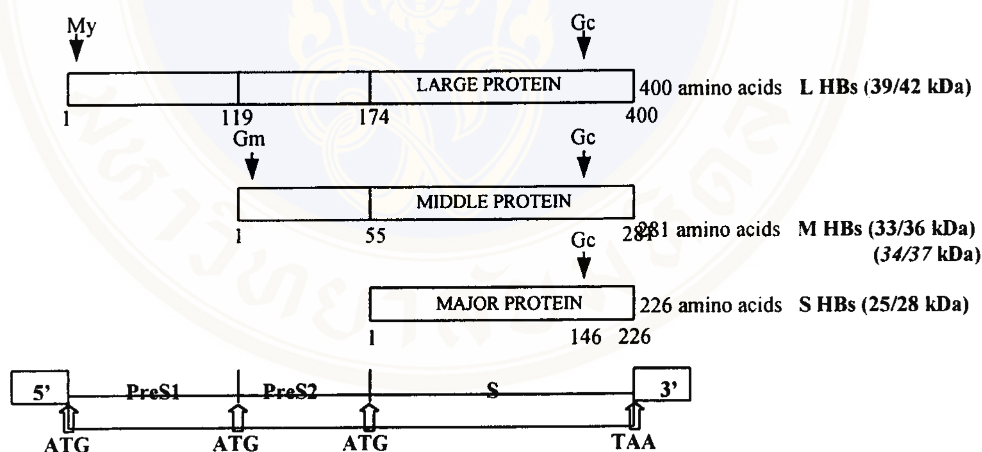

Figure 1. Hepatitis B virus surface antigens : Map representing genomic coding region, and encoded proteins (Tiollais *et al.*, 1985 and McLanchlan, 1991)

The hepatitis B virus surface antigen protein is encoded by a single gene, which is divided into S, PreS2 and PreS1 regions, by three in-frame ATG start codons. Numbers refer to amino acids of the primary translation products. Methionine-1 is missing in L HBs and is replaced by myristic acid (My). Gm, mannose-rich mixed-type glycan linked to asparagine-4; (Gc), facultative glycan of complex type linked to asparagine-146 of S HBs. The molecular mass of M HBs produced in yeast was shown in italic (Kobayashi *et al.*, 1988).

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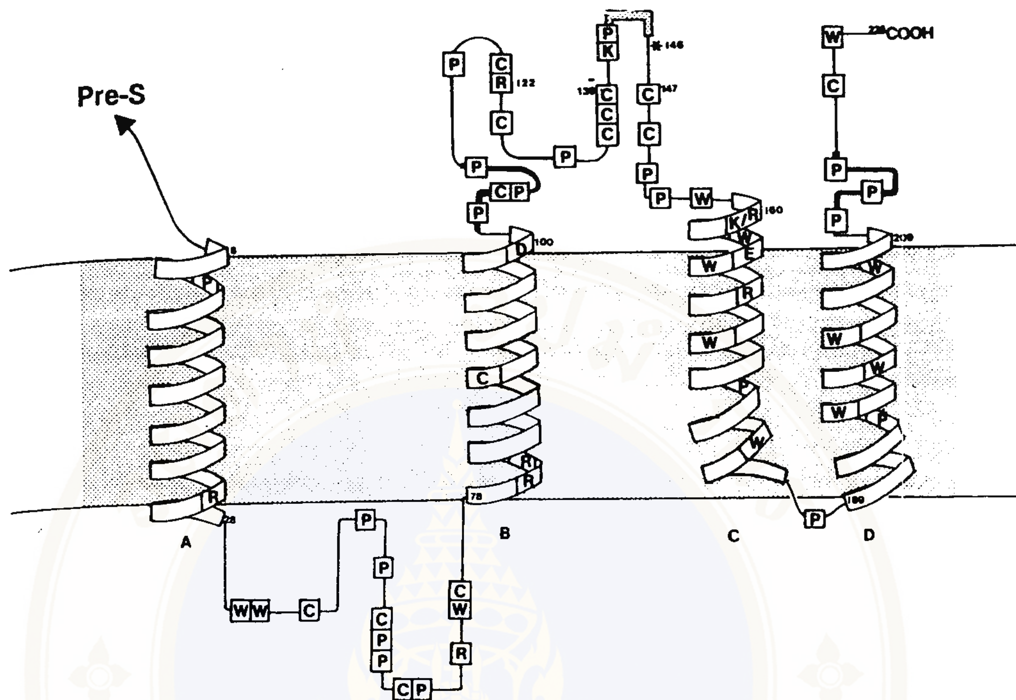

Figure 2. Proposed model for HBsAg in the membrane (Stirk *et al.*, 1992).

*Point of glycosylation. Shaded box represents possible β bend. — = Position of proline helices (residues 105-111 and 217-211). W = Tryptophan; C = cysteine; P = proline and charged residues are marked in single letter code.

2. Expression System for Production of Recombinant Hepatitis B Vaccine

In order to produce the recombinant HB vaccine, it was necessary to insert the gene encodes for HBsAg into an expression plasmid that would direct the synthesis of large quantities of HBsAg in a heterologous host cell. A variety of expression systems have been developed for the HBsAg gene expression, including bacteria, viral vector, mammalian cells, plant cells and yeasts. Either S or PreS2+S or PreS1+PreS2+S genes are used for feasibility to be applied as vaccine.

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2.1 Bacteria

E. coli was the first expression system for expression of the S gene. Its expression was under the control of the *E. coli trp* promoter in the pBR323 plasmid (Edman, 1981; US patent 5196194, 1993). However, it was unsuccessful to be applied as vaccine due to the lack of stability of the S protein and it failured to assemble into 22 nm particles (Korec *et al.*, 1989). Since the immunogenic form of the S protein has to be assembled as 22 nm spherical particles (Peterson *et al.*, 1982). In order to develop the more effective vaccine produce in bacteria, the expression of the PreS1+PreS2 gene in *E. coli* was investigated (Kim *et al.*, 1996) by site-directed mutagenesis of some nucleotide at the 5'end of the PreS1 region gene. The mutant gene was highly expressed in soluble form and the purified polypeptide was demonstrated to exhibit antigenicity and immunogenicity, suggesting that the PreS1+PreS2 was functional.

Many researchers also used attenuated bacterial cells for expressing HBsAg for oral immunization. A non-virulent *Salmonella dublin* was used to express HBsAg epitopes in an immunogenic form. Chimpanzees immunized intramuscularly or orally with the live recombinant bacteria developed antibodies specific to the HBs epitopes (Wu *et al.*, 1989). In addition, the hybrid virus nucleocapsid-PreS (HBcAg-PreS) particle was successfully expressed in the non-virulent *Salmonella thyphimurium* and *Salmonella typhi*. When whole cells of attenuated *Salmonella* expressing recombinant HBsAg were fed to mice, B and T cell immune responses were observed (Schodel *et al.*, 1994). Recently, it was demonstrated that orally administration of the HBsAg presented by non-virulent *S. thyphimurium* showed significantly cell immune response than recombinant HBsAg vaccination, whereas B cell immune response was relatively absent. (Woo *et al.*, 2001). This type of vaccine was suggested to be considering as a

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candidate for therapeutic immunization in chronic HBV infection or immunization of non-responders to recombinant HBsAg vaccine. However, the effectiveness and safety of the recombinant non-virulent bacteria have to be investigated for human used.

2.2 Insect Cells

One system to circumvent the limited amount of viral antigen produced in the other system is the baculovirus. *Autographa californica* nuclear polyhedrosis virus was used as the expression vector for expression of HBsAg in cultured insect cells. Since its replication produced a large inclusion body called polyhedron that accounts for 20% to 50% of the cell protein mass but not essential for viral replication (Millich *et al.*, 1985). Therefore, the large amount of HBsAg was successfully expressed (Lanford *et al.*, 1989). The S protein was not secreted into the medium but was inserted into the membrane, glycosylated, and partially extruded into endoplasmic reticulum as 22 nm lipoprotein particles. The purified S protein was highly antigenic and immunogenic. *Drosophilla melanogaster* Schneider-2 (DS-2) cells were also used to produced the HBsAg. Immunization of BALB/c mice with DS-2 purified HBsAg particles without adjuvant elicited B cell and T cell immune response (Deml *et al.*, 1999). However, the limitation of the baculovirus system is the high cost medium.

2.3 Viral Vectors

Several attenuated virus such as vaccinia, adenovirus have been used as viral vector for expression of the HBsAg (Lubeck *et al.*, 1989). Many studies have provided evidence that L protein could be an ideal candidate for a new HB vaccine with higher efficacy. However, the L protein containing the entire PreS region expressed by viral

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vector in mammalian host cells was not efficiently assembled into viral particles. Recently, an alternative approach to include dominant epitopes of PreS1 and PreS2 into the S protein as fusion proteins was reported (Hui *et al*, 1999). These fusion proteins were efficiently assembled in the viral particles, and displayed S, PreS1 and/or PreS2 epitopes. Nevertheless, envelope of vaccination of BALB/c mice with these viral vector vaccines induced significantly lower antibody titers than did the subunit vaccines. Cho *et al*. (2000) also investigated the expression of HBsAg composing of epitope of S and L region, fused to non-structure protein of poliovirus. It was found that immunization of BALB/c mice with this viral vector elicited significant antigen-specific cytotoxic T cell response to HBsAg. More studies have to be carried out for application of viral vector as HB vaccine.

2.4 Mammalian Cells

The system for producing HBsAg in cultured mammalian cells was developed in mammalian chinese hamster (CHO) cells (Patzer *et al*., 1984). HBsAg was successfully secreted from cells and composed of both unglycosylated and glycosylated polypeptides. Hourvitz *et al*. (1996) found that the HBsAg expression in CHO cells using PreS1+PreS2+S gene. Furthermore, each protein had two forms of glycosylation. The feature of glycosylation of CHO derived HBsAg was different from that of yeast derived HBsAg by which the S, PreS2+S and PreS1+PreS2+S were co-expressed in *H. polymorpha*. The yeast derived HBsAg glycosylate form were formed only in M and L protein but not in S protein. Injection of both HBsAg into mice elicited similar humural immune response. Whereas, the cytotoxic T lymphocyte response to CHO-HBsAg was lower (Diminsky, 1997). The United State Patent Application 6110706

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(2000) disclosed the system for the production of HBsAg particles containing PreS region in monkey cell line (VERO), murine fibroblast and CHO cell. Unfortunately, this system is not preferred because the risk of endogenous proto-oncogenes and retroviruses in mammalian cells.

2.5 Plant Cells

Recently, interest has been focused on the production of HBsAg by plants for edible vaccines. It is particularly advantageous in certain disease prevention for human to produce a vaccine in a foodstuff or juice for ease of administration. Plants which are suitable for the production of vaccine include any dicotyledon and monocotyledon which are edible in part or in whole by human or animal such as apple, banana, carrot, corn, potato, rice, soybean, strawberries, raspberries, and other edible varieties (US patent 5914123, 1999). The expression of HBsAg was studied in tobacco plants using the cauliflower mosaic virus (CaMV) promoter (Mason *et al.*, 1992). The spherical particles with an average diameter of 22 nm were observed. When the tobacco-derived HBsAg was used for parenteral immunization of mice, anti-HBsAg antibody was detected and reacted with an authentic HBsAg from human serum (Thanavala *et al.*, 1995). The United State Patent Application 6136320 (2000) disclosed the introduction of HBsAg gene into cells of tobacco and tomato by using *Agrobacterium tumefaciens*, under the control of CaMV promoter. When the recombinant HBsAg was purified, virus-like particles with an average diameter of 22 nm were observed. The HBsAg level found in tomato leaves was similar to the highest level found in leaves of transgenic potato, whereas the amount of HBsAg in the tomato fruit was lower on a total protein. Unfortunately, production of vaccine antigens in plants often fails to meet

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the minimum level required to produce an immune response when administered orally. To overcome this limitation, Richter *et al.* (2000) studied various ways to increase production of HBsAg in potato. They tested the introduction of a number of signal peptides and 5' and 3' untranslated regions (UTRs) in to HBsAg expression cassette driven by the constitutive CaMV promoter. Sequences tested included 5'UTRs from tobacco virus and tobacco mosaic virus, and 3' UTRs from the soybean and potato genes. The use of different 5' UTRs had little effect on expression levels, but the introduction of the different 3' UTRs increased the amount of HBsAg protein per unit mRNA. The immunogenicity was test in mice, a stronger immune response could be induced triggered when the amount of HBsAg increase to gram per a part of plant. However, it will take several years to established the efficacy of the edible plant vaccine for human.

2.6 Yeast

At present, the most advance recombinant vaccine available in the market is the HB vaccine produced in yeast. Since yeast is safe and more convenient with the scale up production. The S protein produced in yeast was formed in particles and vaccination of the yeast derived HBsAg particles into chimpanzees and mice, induced anti-HBs antibodies that conferred immunity to HBV infection (McAleer *et al.*, 1984). There are several patents disclosed the production of recombinant HBsAg by the baker's yeast *S. cerevisiae*, methylotrophic yeast *P. pastoris* and *H. polymorpha*, and *Y. lipolitica* (Table 1 and 2). The first generation of the recombinant HBsAg by yeast was produced from S gene. Nowadays, many manufacturers as shown in Table 3 produce the HB vaccines supplied all over the world. Either *S. cerevisiae* or *H. polymorpha* are

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employed as the host. Despite the successful of the immunization by the recombinant HB vaccine, improvement the immunogenic potency of conventional vaccine has been attempted. Since, some populations developed the delayed antibody response or were not able to response to the recombinant S HBsAg (US patent application 4769238, 1988). The M protein was, therefore, targeted as another HB recombinant vaccine however the M proteins, contained protease sensitive site in the PreS2 region which was easily cleaved in yeast host. The production of the M protein devoid of the protease sensitive site was, therefore, investigated in order to improve the stability (Itoh and Fujisawa, 1986a; Itoh *et al.*, 1986b, Kuroda *et al.*, 1993, Kuroda *et al.*, 1999). Recently, Yamada *et al.* (2001) confirmed that immunization of mice with the L protein produced as particles in yeast, elicited efficiently and simultaneously the anti-S, anti-PreS2 and anti-PreS1 antibodies. Furthermore, the anti-PreS1 rabbit antibodies were found to recognize various segments of the PreS1 region, including the PreS1 (21-47) segment. These results show the high ability of L particles to induce all antibodies against HBV envelope proteins, therefore promising the future application of L particles for the next generation HB vaccine. Several investigators investigated the expression of M and L protein in *S. cerevisiae* (Table 2). The M protein was found to be glycosylated with a single high-mannose type core oligosaccharide chain and assembled into lipoprotein structures expressing PreS2 encoded antigenic determinants (Imamura *et al.*, 1987, Dehoux *et al.*, 1986 and Kitano *et al.*, 1987). Attempt to produce combine vaccine against HBV and malaria was done by expression the hybrid protein of the gene encoding the circumsporozoite (CS) protein of the parasite *Plasmodium falciparum* and the S gene in yeast *S. cerevisiae* (US patent application 6169171, 2001). Immunization of mice and monkey with the hybrid protein induced

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both anti-HBs and anti-CS antibodies. This vaccine has to be further test for human use.

3. Expression System for HBsAg in Yeast

There are several transcriptional promoters applied for expression the HBsAg in *S cerevisiae*, such as the glycolytic promoter: alcohol dehydrogenase I (*ADH*1), phosphoglycerate kinase (*PGK*) or glyceraldehyde phosphate dehydrogenase (*GAP*/*GLD*). All of them are induced when yeasts are grown in glucose (Valenzuela *et al.*, 1982, US patent application 4803164, 1989, Itoh *et al.*, 1986b and Immamura *et al.*, 1987). The promoter of the acid phosphatase gene (*PHO*5/Apase), the phosphate regulated promoter, was also used for HBsAg expression (Itoh *et al.*, 1986b and Miyanohara *et al.*, 1983). The gene expression is induced after the cells were transferred from high phosphate concentration medium (High-Pi) to low phosphate concentration medium (Low-Pi). The promoter of the alcohol dehydrogenase II (*ADH*2), which is one of glucose repressible promoters, was also applied (US patent application 4816564, 1989). This promoter is tightly regulated in the presence of glucose. When glucose in the medium is depleted, the promoter is derepressed. All the promoters described above are inducible promoters. However, the constitutive promoter of the mating type factor alpha 1 (*MF*α1) was also applied (Baba and Berkower, 1992).

In the yeast *Pichia pastoris*, the alcohol oxidase (*AOX*1) promoter which catalysed the first step in the assimilation of methanol, efficiently expressed the HBsAg (AU patent application 33282/89, 1989). For the *H. polymorpha*, the promoters of methanol oxidase gene (*MOX*) and formaldehyde dehydrogenase gene (*FMD*), which also involve in methanol utilization are used (Janowicz *et al.*, 1991). Recently, the

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expression of HBsAg by *GAP* promoter in *P. pastoris* was also investigated (Vassileva *et al.*, 2001). It was found that the maximal levels of HBsAg expression in case of the single copy *AOX*1 integrant exceeded the levels of antigen produced by the single copy *GAP* integrant. For enhance antigen production levels of the *GAP* clones, the multicopy *Pichia* integrants containing up to four copies of the *GAP* promoter were isolated. The effect of four copy clones resulted in four-fold higher yield of HBsAg.

Table 1. The patent of the expression systems of HBsAg.

Patent number	Host	Promoter gene	Gene
US 4769238 (1988)	*S. cerevisiae*	*ADH*1	S
US 4803164 (1989)	*S. cerevisiae*	*PGK*1	S
AU 31318/89 (1989)	*S. cerevisiae*	*ARG*3	PreS1+PreS2+S
AU 33282/89 (1989)	*P. pastoris*	*AOX*1	PreS2+S
US 4816564 (1989)	*S. cerevisiae*	*GAP* *ADH*2 *MF*α1	PreS2+S
US 4945046 (1990)	*S. cerevisiae*	*PHO*5 *GAPDH*	PreS2+S
US 5196194 (1993)	*E. coli*	*LacZ*	S
US 6110716 (2000)	Monkey kidney cell Murine fibroblast cell Chinese hamster cell	U2 MT H2K	PreS1+PreS2 PreS2
US 6136320 (2000)	Tobacco Tomato	CaMv	S
US 6169171 (2001)	*S. cerevisiae*	*TDH*3	S+CS

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Table 2. The expression systems for HBsAg in yeast.

HBsAg gene	Promoter gene	Host	Yield	References
S	*ADH*1	*S. cerevisiae* XV610-8C	0.001-0.0025 x 10^{-3} g/l	Valenzuela *et al.*, 1982.
	Apase	*S. cerevisiae* AH22	2.5-2.8 g/l	Miyanohara *et al.*, 1983
	MFα*1*	*S. cerevisiae* 2602	1-2 x 10^{-3} g/l	Baba and Berkower, 1992
	MOX	*H. polymorpha*	5-45 unit/100 mg protein x 10^4	Janowicz *et al.*, 1991
	FMD	*H. polymorpha*	10-25 unit/100 mg protein x 10^4	Janowicz *et al.*, 1991
PreS2+S	*AOX*1	*P. pastoris* MP-36	0.038-1.33 g/l	Hardy *et al.*, 2000
	GAP	*P. pastoris* GS115	53.25-66.28 µg/ 100 OD cell	Vassileva *et al.*, 2001
	PHO5	*S. cerevisiae* AH22	0.55 x 10^{-3} g/l	Itoh *et al.*, 1986b
PreS2d+S	*GLD*	*S. cerevisiae* AH22	1.4 x 10^{-3} g/l	Itoh *et al.*, 1986b
	GAP	*S. cerevisiae* AH22	1.1 x 10^{-3} g/l	Immamura *et al.*, 1987
	XPR2	*Y. lipolytica*	0.085 g/l	Hamsa and Chattoo, 1994
	GAP	S. cerevisiae *AH22*	1.19 g/l	Itoh and Fujisawa, 1986a Kuroda *et al.*, 1993
PreS1+PreS2+S	*GAP*	*S. cerevisiae* AH22	5-109 µg/5x10^7 cells	Kuroda *et al.*, 1999
	*TRP*1	*S. cerevisiae* AH22	<0.1-8.4 g/l	Itoh *et al.*, 1992
	GAP	S. cerevisiae YI	1.25 g/l	Shiosaki *et al.*, 1991

Table 3. Hepatitis B vaccine manufacturer.

Tradename	Manufacturer	Country
Biken-HB	Research Foundation for microbial disease	Japan
Bimmugen	Kaketsuken	Japan
Comvax	Merck	US
Engerix-B	SmithKline Beecham	US
GenHenvac B	Pasteur	France
HBY*	GreenCross (Member of Rhein-Biotech Group)	Korea
Heberbiovac-HB**	Cuban Vaccine Manufacturer	Cuba
R-HB	Mitsubishi Chemical Corp.	Japan
Recombivax	Merck	US

Note : All vaccines are produced by *S. cerevisiae* except * by *H. polymorpha* and ** by *P. pastoris*.

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4. Comparison of the Yeast-Derived HBsAg Particle with Human HBsAg

As described above the yeast HBsAg is assembled into 20 nm particles and immunologically similar to its human counterpart. However, glycosylation of the surface protein produce in *S. cerevisiae* differs from that observed in mammalian cells. The S protein is not glycosylated form whereas the M and the L protein are produced in both N-linked glycosylated and non-glycosylated forms. N-linked chains of the high-mannose type were identified, as well as O-linked oligosaccharide chains (Itoh *et al.*, 1986b and Langley *et al.*, 1988). The high-mannose oligosaccharides account for all the N-glycans released from PreS2+S : structures include Man7GlcNAc2, Man8GlcNAc2, and Man9GlcNAc2 isomers in the ratios of 3:6:1. Approximately 80% of the oligosaccharides contain the C2, C6-branched trimannosyl structural element typical of yeast high-mannose oligosaccharide but not usually found in high-mannose oligosaccharides in animal glycoproteins (Chrlotter *et al.*, 1992). When extracted from yeast, both the S and M proteins were recovered as lipoprotein particle closely resembling the 22 nm particles present in serum of human HBV patients (Valenzuela *et al.*, 1982). The L protein was also recovered in lipoprotein structure, but their exact nature was not been elucidated (Dehoux *et al*, 1986). Later, the internal structure of the recombinant yeast HBsAg particle was investigated by small-angle neutron scattering (SANS) using the contrast variation method. SANS analysis showed that the HBsAg is a spherical particle with a diameter of 290 Å, in which two different regions in terms of scattering density were distributed radially. The lipids and carbohydrates form a spherical cluster with a diameter of 240 Å in the core region of the particle, and the surface antigen protein was present in the peripheral region (Sato *et al.*, 1995). Recently, Yamaguchi *et al.*, (1998), elucidated that the ultrastructure of HBsAg

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particles produced by recombinant yeast were larger than human HBsAg particles by both negative staining and ice embedding, electronmicroscopy. Because of molecular differences in polypeptide structure whereas the central pores found on both yeast and human HBsAg particles appeared similar (Figure 3).

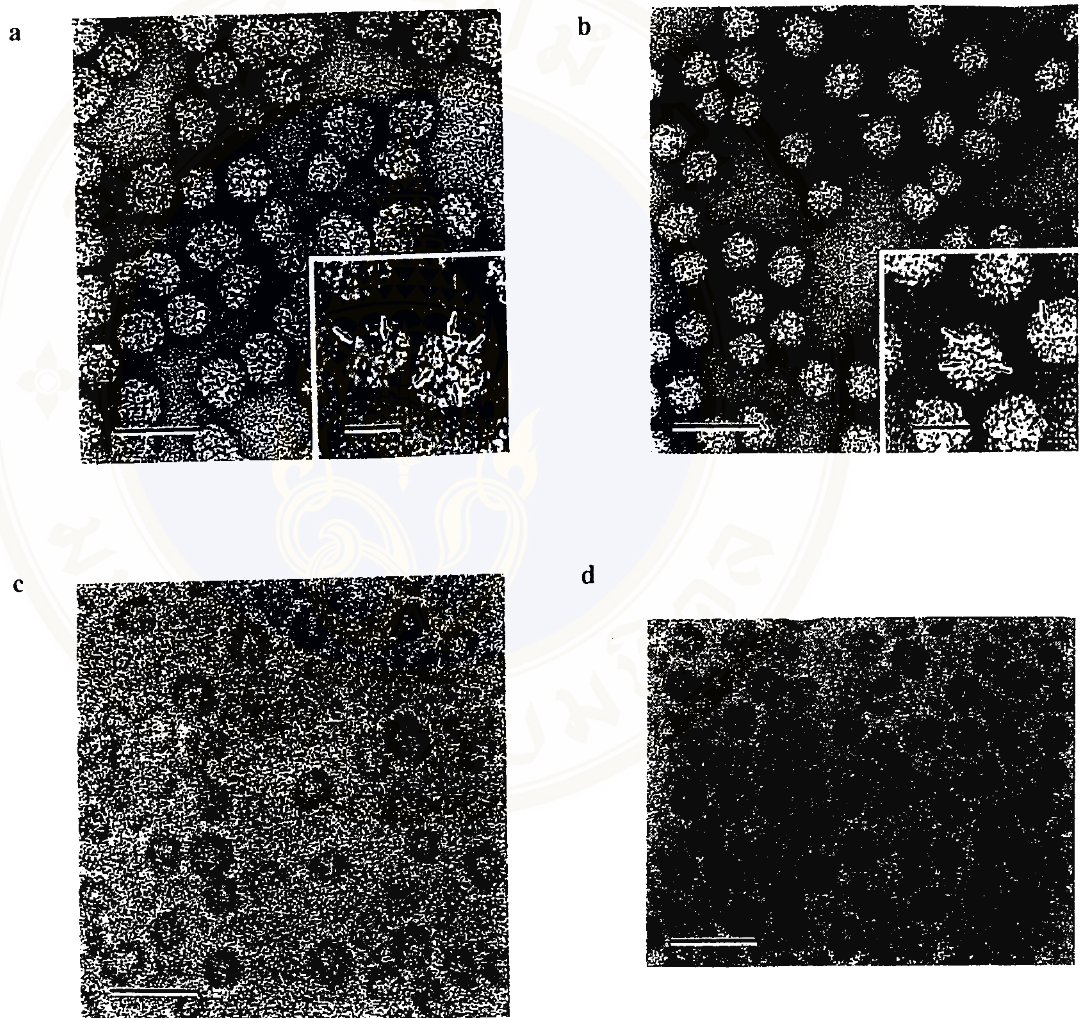

Figure 3. Negative stained and ice embedded HBsAg particles isolated from recombinant yeast and human plasma (Yamaguchi *et al*, 1998). Arrows indicate subunit structures with pores.

a) Negative stained HBsAg particles isolated from recombinant yeast (Bar = 50 nm, Inset : Bar = 20 nm).
b) Negative stained HBsAg particles isolated from human plasma (Bar = 50 nm, Inset : Bar = 20 nm).
c) Ice embedded HBsAg particles isolated from recombinant yeast (Bar = 50 nm).
d) Ice embedded HBsAg particles isolated from human plasma (Bar = 50 nm).

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5. Phosphate Regulation System in Yeast

5.1 Phosphatase Enzymes and Genes

The preferred phosphate source of yeast cells is inorganic phosphate (Pi). However, in the absence of Pi, yeast can obtain phosphate from a variety of organic compounds through the cleavage of phosphoester bonds catalysed by phosphatases (Oshima, 1982, Yoshida *et al*., 1987, Vogel and Hinnen, 1990). Phosphate is needed in large amount for nucleic and phospholipid biosynthesis. However, when it is excess, it is stored in cells as polyphosphate. Many enzymes responsible for phosphate acquisition are encoded by multiple genes which are regulated by several proteins. S. *cerevisiae* posses two different kinds of enzymes for phosphate acquisition acid (optimum pH 3-4) phosphatases and alkaline (optimum pH 8) phosphatases. There are several phosphatases of both types (Table 4). Four different genes encode acid phosphatases. The major acid phosphatase is encoded by *PHO*5; two less abundant acid phosphatases which are similar to Pho5p, are encoded by *PHO*10 and *PHO*11. These three enzymes have broad substrate specificities. The three acid phosphatases are so called repressible acid phosphatase (rAPase). A fourth acid phosphatase, encoded by *PHO*3, may function to acquire thiamine. *PHO*5 and *PHO*3 are tightly linked on the right arm of chromosome II and encode similar proteins. There are two different alkaline phosphatases in yeast, nonspecific alkaline phosphatase encoded by *PHO*8 and specific alkaline phosphatase encoded by *PHO*13. They are so called repressible alkaline phosphatase (rALPase). Expression of genes for acquisition of phosphates is repressed by Pi. Yeast cells contain two transporters, one with high affinity for Pi (*K*m 8 μM) encoded by *PHO*84, and another with low affinity (*K*m 770 μ M) (Tamai *et al*., 1985). Activity of the *PHO*84 encoding high-affinity transporter is

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repressed about 60-fold by Pi (Ueda and Oshima, 1975; Tamai *et al.*, 1985; Bun-ya *et al.*, 1991).

The Pi signals are conveyed to these genes by regulatory system consisting of products of at least five genes, *PHO*2, *PHO*4, *PHO*80, *PHO*81 and *PHO*85 (Oshima, 1991). Each component of the *PHO* gene regulatory mechanism is summarized in Table 4.

5.2 *PHO* Gene Regulation

Transcription of *PHO*5, *PHO*10, and *PHO*11 is repressed by Pi (Bostain *et al.*, 1980; Kramer and Andersen, 1980; Lemire *et al.*, 1985; Bun-ya *et al.*, 1991). *PHO*5, which is responsible for the majority of the acid phosphatase, appears to be more highly expressed and more severely repressed by Pi than are *PHO*10 and *PHO*11 (Bajwa *et al.*, 1984). *PHO*3 is modestly repressed by thiamine and appears to be regulated reciprocally to *PHO*5. *PHO*3 is expressed only when *PHO*5 is not, which makes *PHO*3 appear to be induced by Pi (Rogers *et al.*, 1982; Tait-Kamradt *et al.*, 1986).

The regulation of *PHO* gene expression by Pi is accomplished by several regulatory proteins acting in a cascade of positive and negative events superficially similar to those of the *GAL* gene regulatory mechanism. In a current model for *PHO* regulation (Figure 4) (Oshima *et al.*, 1996), a positive regulator Pho4p, coded by *PHO*4, is indispensable for transcriptional activation of the *PHO* structural genes. An additional factor, Pho2p (Bas2p or Grf10p), is also involved in the transcription of all the *PHO* structural genes except *PHO*8. In high-Pi medium, a complex of negative regulators, Pho80p and Pho85p, inhibits the Pho4p function by hyperphosphorylation of it, and so

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Table 4. *PHO* genes and regulators (Johnston and Carlson, 1992)

Gene	Product	Regulation	References
Phosphatase and permease genes			
*PHO*3	acid phosphatase 57 kD (thiamine phosphatase)	constitutive	Nosaka, 1990
*PHO*5 (p60)	acid phosphatase, 60 kD (major)	Pi repressible	Bajwa *et al.*, 1984
*PHO*10 (p58)	acid phosphatase, 58 kDa (minor)	Pi repressible	Venter and Horz, 1989
*PHO*11 (p56)	acid phosphatase, 56 kDa (minor)	Pi repressible	Venter and Horz, 1989
*PHO*8	fructose-2,6-bisphosphate, 66 kDa	Pi repressible	Plankert *et al.*, 1991
*PHO*13	alkaline phosphatase, 60 kDa (specific)	constitutive	Kaneko *et al.*, 1989
*PHO*84	permease, low *K*m	Pi repressible	Bun-ya *et al.*, 1991
*PHO*89	permease, low *K*m Pi repressible	Pi repressible	Martinez *et al.*, 1998a
Positive regulators			
*PHO*2 (*BAS*2)	DNA-binding transcriptional activator		Sengstag and Hinnen, 1987; Berben *et al.*, 1988
*PHO*4	DNA-binding transcriptional activator		Ogawa and Oshima, 1990
*PHO*82 (*PHO*4^c)	constitutive mutations in *PHO*4		Toh-e *et al.*, 1981
*PHO*81	inhibitor of Pho80p/ Pho85p function	Pi repressible	Yoshida *et al.*, 1989b
*PHO*6 (*THI*2)	required for *PHO*3 expression, regulator of thiamine biosynthesis		Nishimura *et al.*, 1992
*PHO*7	required for *PHO*3 expression		Toh-e *et al.*, 1975
*PEP*4 (*PHO*9)	protease for Pho8p maturation		Kaneko *et al.*, 1982
Negative regulators			
*PHO*80	inhibitor of Pho4p		Toh-e and Shimauchi, 1986; Madden *et al.*, 1988
*PHO*85	inhibitor of Pho4p; protein kinase		Uesono *et al.*, 1987; Toh-e *et al.*, 1988

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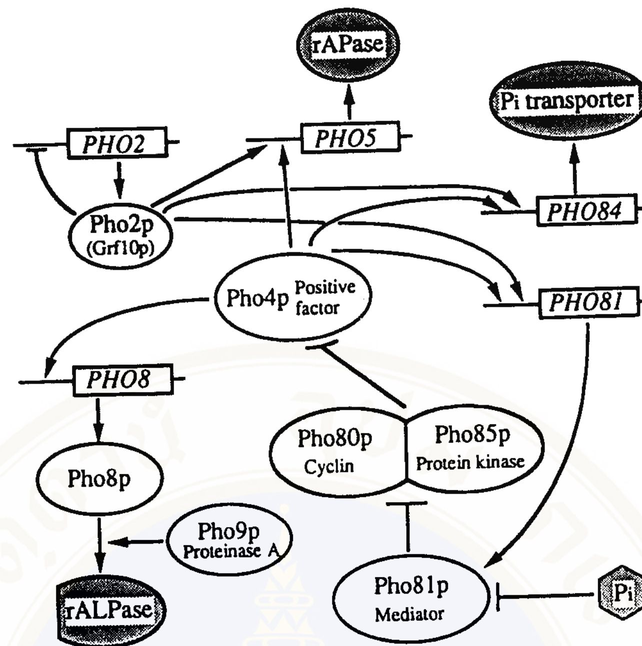

Figure 4. The regulation circuit for phosphate genes (Oshima *et al.*. 1996).

The protein factors, enzymes, and enzyme precursors in the regulatory circuit are shown in open ovals, and the genes regulated by these proteins are shown in boxes. The factors in the shaded hexagon and ovals, Pi, rAPase (repressible acid phosphatase). rALPase (repressible alkaline phosphatase), and Pi transporter are the input signal to and outputs of the regulatory circuit, respectively. The arrowheads in the regulatory circuit indicate a positive or stimulating function of the factors, bars, indicate repressive or inhibitory functions. Pho9p (Pep4p), proteinase A, is required for processing of the Pho8p polypeptide.

Pho4p is unable to activate the transcription of the *PHO* genes (Kaffman *et al.*, 1994; Ogawa *et al.*, 1995). When the Pi concentration in the medium is sufficiently low, Pho81p inhibits the function of the Pho80p-Pho85p complex (Schneider *et al.*, 1994; Ogawa *et al.*, 1995), thus allowing Pho4p, in collaboration with Pho2p, activates the transcription of the *PHO* genes. The *PHO*4, *PHO*80 (Yoshida *et al.*, 1989a,b; Madden *et al.*, 1990), and *PHO*85 (Madden *et al.*, 1990; Uesono *et al.*, 1992) genes are transcribed constitutively at low levels. *PHO*2 transcription is also low level but is self regulated (Yoshida *et al.*, 1989a). Transcription of *PHO*81, which encodes Pho81p, is

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regulated by Pi via the same *PHO* regulatory system, indicating that the regulatory circuit forms a positive feedback loop (Yoshida *et al.*, 1989b; Ogawa, 1993).

5.2.1 Positive Regulators Pho4p and Pho2p

The nucleotide sequence of *PHO4* suggests that Pho4p consists of 312 amino acid residues (Yoshida *et al.*, 1989a). It has a basic region beside and amphipathic helix-loop-helix structure in the 85 amino acid C-terminal region (Ogawa and Oshima, 1990). These structures are known to function as a DNA-binding domain and homodimer formation (Murre and Baltimore, 1992). Pho4p binds at a specific *cis*-regulatory site, UAS of the *PHO* genes. The N-terminal region (amino acid 1-109), which is rich in acidic amino acid, is suggested to act as the activation domain for gene transcription and a region from amino acid 203 to 227 might be involved in oligomer formation (Ogawa and Oshima, 1990).

The *PHO2* nucleotide sequence predicts that Pho2p is composed of 519 (Yoshida *et al.*, 1989a) or 559 (Sengstag and Hinnen, 1987) amino acid residues, and contains a homeo box from amino acid 76 to 135 or from amino acid 77 to 163 (Burglin, 1988). A portion of the homeo box of Pho2p has a similar amino sequence to that of a short stretch in Pho4p, Pho2p also has a stretch of Gln residues (14 of 18 amino acid) on the N-terminal side of the homeo box.

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5.2.2 Specific Nucleotide Sequences for Pho4p Binding

*PHO*8 promoter

Hayashi and Oshima, 1991 found a 6 bp sequence CACGTG, at position –535 of *PHO*8 promoter is proposed to be the Pho4p-binding site in the promoter sequence of *PHO*8 because only Pho4p but not Pho2p is required for its transcription. The result was confirmed by Barbaric *et al*., (1992), Ogawa *et al*., (1994).

*PHO*5 promoter

Three putative UASs in the *PHO*5 promoter, UASp1 located in the region from nucleotide -373 to -347, UASp2 located from nucleotide -262 to -239 and UASp3 from -202 to -182 (Figure 5), have been detected (Vogel *et al*., 1989). These regions contain one copy of, respectively, the CACGTT, CACGTG and CATGTG motifs.

*PHO*81 promoter

For *PHO*81 promoter, one copy of CACGTG motif in the -349 is found (Ogawa *et al*., 1993).

*PHO*84 promoter

The promoter region of *PHO*84 bears five copies of 6 bp motif at nucleotide positions -880 (site A), -587 (site B), -436 (site C), -414 (site D), and -262 (site E) relative to the ATG start codon (Figure 5). Deletion analysis of the promoter and analysis with base substitutions in the 6 bp motif revealed that the 6 bp motifs at site C or D and E are essential and sufficient for full regulation of *PHO*84 transcription, whereas sites A and B are dispensable.

The nucleotide sequences in the promoter regions of *PHO*5, *PHO*8, *PHO*81, and *PHO*84 are defined by Pho4p protection from DNase I digestion *in vitro* (Ogawa *et al*., 1994). The sequences are listed in Figure 6 along with the 6 bp sequence formed in

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the distal site in the *PHO*8 promoter (Barbaric *et al.*, 1994). So for eleven UASs have been estimated; six have the CACGTG motif (group 1) and three have the CACGTT motif (group 2) in the center of the protected regions, and two remaining ones were not to be involved in *PHO* regulation.

The transcriptions of these *PHO* structural genes except *PHO*8, depend on the presence of Pho2p. Several attempts to detect a Pho2p-binding site in the *PHO*5 promoter were unsuccessful. However, a region of the *PHO*5 promoter from nucleotide –381 to –351 bearing UAS_p1 (GCACGTTTT) which is homology to the E site of the *PHO*84 promoter, whereas *PHO*8 has no such sequence, although it does have a GCACGCTTA sequence at distal position (Figure 5). These finding suggest the possibility that the Pho4p-Pho2p complex actually bind to the GCACGTTTT sequence.

5.2.3 Structures and Functions of Pho80p and Pho85p

*PHO*80 and *PHO*85 encode negative regulators of the *PHO* genes. Pho80p and Pho85 are thought to inhibit Pho4p and/or Pho2p function but not the expression of *PHO*4 and *PHO*2 (Oshima, 1982; Yoshida *et al.*, 1989a). Pho80p is a 34-kDa protein (Uesono *et al.*, 1992) composed of 293 amino acid residues as deduced from the nucleotide sequence of *PHO*80 (Toh-e and Shimauchi, 1986). A coordinate increase of *PHO*80 dosage canceled the dosage effect of *PHO*4 on expression of *PHO*5 (Yoshida *et al.*, 1989b) and a mutation occurring in *PHO*80 suppressed a $PHO4^c$ mutation (Okada and Toh-e, 1992). Based on these findings, it was thought that Pho80p directly interacts with Pho4p for transduction of the Pi signals. On the other hand, Pho85p, is a 36-kDa compose of 305 amino acids protein and known as a homolog of Cdc28p, a cyclin-dependent protein kinase (cdk) of *S. cerevisiae* (Uesono *et al.*, 1987, 1992; Toh-

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e *et al.*, 1988). It was thought that Pho85p kinase phosphorylates Pho80p, resulting in Pho80p activation (Useono *et al.*, 1992). Recent reexamination of the amino acids sequence of Pho80p; however, has revealed that it has significant similarities to these of two yeast cyclins, Hcs26p (Ogas *et al.*, 1991) and OrfDp (Fröhlich *et al.*, 1991) in a conserved region of the cyclins (Kaffman *et al.*, 1994). It was demonstrated that Pho80p and Pho85p form a complex and that the complex, but not either protein alone, efficiently phosphorylated Pho4p *in vitro* and *in vivo* (Kaffman *et al.*, 1994).

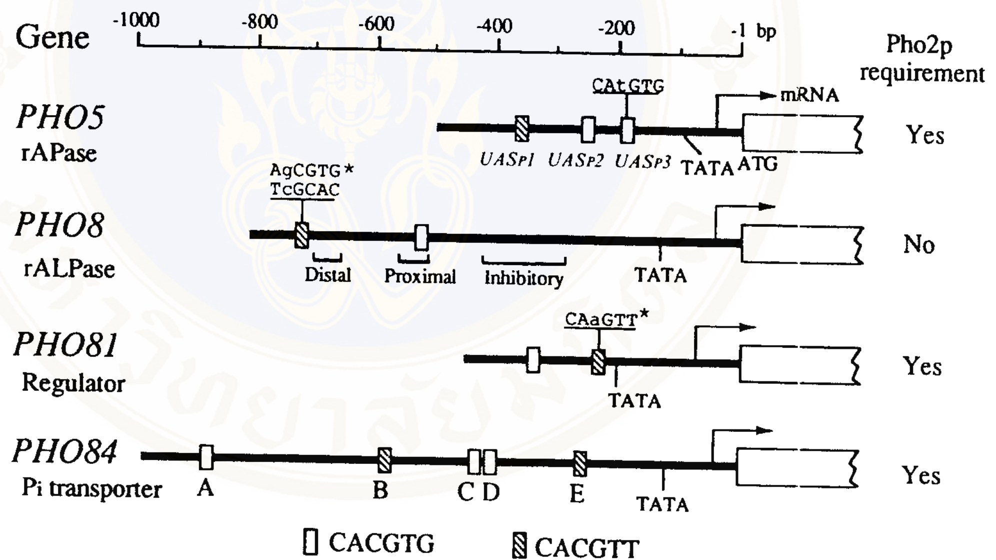

Figure 5. Distribution of the 6-bp motif, CACGTK (K=G or T), in the promoter regions of an ORF (Oshima *et al.*, 1996).

The open reading box at the right end of individual genes represents an ORF. Short open and hatched boxes indicate CACGTG and CACGTT motifs, respectively. Three 6 bp sequences, analogous as the 6 bp motif, shown in the *PHO5*, *PHO8*, and *PHO81* promoters are indicated with their sequences (lower case letters indicate deviation from the 6 bp motifs). The function and Pho4p binding ability of the sequence analogous to the 6 bp motif in the *PHO8* promoter (marked with and asterisk) were described by Barbaric *et al.* (1992), but that of the *PHO81* promoter has not yet been examined.

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Site	*UAS* activity	Pho4p-binding sequence	Affinity to Pho4p
Group 1			
PHO5 UASp2	+	-258 a C t C a C A C A C G T G G G A C T A G C -240	High
PHO84 Site D	+	-421 T T T C C A G C A C G T G G G G C G G A -402	High
PHO81 UAS	+	-350 T T A T G G C A C G T G C G A A T A A -332	High
PHO8 Proximal	+	-520 G T G A T C G C T G C A C G T G G C C C G A -541	High
PHO5 UASp3	±	-182 T A A T T T G G C A T G T G C G A T C T C -202	Low
PHO84 Site C	±	-426 A C G T C C A C G T G G A A C T A T T -444	Low
PHO84 Site A	−	-870 T T T A T C A C G T G A C A C T T T T T -889	Low
Consensus 1		t t - - - G C A C G T G G G - c - a	
Group 2			
PHO5 UASp1	+	-370 T A A A T T A G C A C G T T T T C G C -352	Medium
PHO84 Site E	+	-251 A A T A C G C A C G T T T T T A A T C T A -271	Medium
PHO84 Site B	−	-592 T T A C G C A C G T T G G T G C T G -573	Low
PHO8 Distal	±	-719 T T A C C C G C A C G C T T A A T A T -737	Low
Consensus 2		a a t - - G C A C G T T T T	

Figure 6. Regions in the *PHO* promoters protected from DNase I digestion *in vitro* by Pho4p (Oshima *et al.*, 1996).

The protected sequences observed in previous studies (Ogawa *et al.*, 1993, 1994) are listed for strands having sequences conforming most closely to GCCGTGGG or GCACGTTTT along with the data of Barbaric *et al.* (1992) for the similar sequences at the distal site in the *PHO*8 promoter. These sequences were classified into two group depending on whether they contained the CACGTG (group 1) or CAGTT (group 2) sequence in the core region. The relative affinity of each sequence to Pho4p was estimated from the amount of Pho4p protein required for protection from DNAse I digestion.

5.2.4 Structure and Function of Pho81p

*PHO*81 encodes a 134 kDa Pho81p composed of 1179 amino acids. Transcription of *PHO*81 is regulated by Pi signal via the *PHO* regulatory system including both Pho4p and Pho2p. The Pho81 is thought to be an inhibitor of Pho80p-Pho85p kinase complex, because the inability of *pho*81 mutants to express the *PHO* genes is suppressed by *pho*80 or *pho*85 mutations (Ueda *et al*, 1975). Pho81p presumably inhibits Pho80p-Pho85p function postranslationally, because it does not regulate *PHO*80 transcription, and transcription of *PHO*80 and *PHO*85 is not regulated by Pi levels (Toh-e and Shimauchi, 1986, Uesono *et al.*, 1987, Madden *et al.*, 1988). Although there are some suggestive data that might support and enzymatic role for

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Pho81p (Yoshida *et al.*, 1989a), the mechanism by which Pho81p inhibits Pho80p/ Pho85p is not clear.

It was demonstrated that Pho81p interacted with the Pho80p-Pho85p kinase complex and inactivated in low-Pi conditions (Schneider *et al.*, 1994; Ogawa *et al.*, 1995). Pho81p is inactive as an inhibitor in cells grown in high-Pi medium and that thus the Pho80p-Pho85p complex inactivates Pho4p by its hyperphosphorylation, and preventing the transcription of the *PHO* structural genes. In low-Pi medium, Pho81p binds with and inhibits the kinase activity of the Pho80-Pho85 complex, and allows underphosphorylated Pho4p to activate transcription of *PHO* structural genes. Hirst *et al.* (1994), however, claimed that Pho81p interacts directly with Pho4p, along with Pho80p, under conditions of *PHO* repression and that Pho80p and Pho81 dissociate from Pho4p under derepression conditions.

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CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and Reagents

Chemicals and reagents used in this study were analytical and biotechnological grade obtained from Amersham (Buckinghamshire, England), Bio-Rad (California, USA), BRL (Gaithersburg, USA), Promega (Madison, USA), and Sigma (St. Louis, USA).

All of them were absolute ethanol, acetic acid, acrylamide, agarose, ammonium acetate, ammonium heptamolybdate, ammonium persulfate, ammonium sulfate, ampicillin, bisacrylamide, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), bromophenol blue, calcium chloride, chloroform, Coomassie Brilliant blue, copper (II) sulfate pentahydrate, ethidium bromide, ethylene diamine tetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether) N, N, N, N'-tetraacetic acid (EGTA), glass beads (diameter 0.45 mm, acid washed), D-glucose, glycerol, glycine, human serum albumin, hydrochloric acid, iron (II) sulfate heptahydrate, isopropanol, N-lauroyl-sarcosine, lithium acetate, leupeptin, magnesium sulfate heptahydrate, maleic anhydride, manganese chloride, manganese chloride tetrahydrate, 2-mercaptoethanol, methanol, 3-(N-morpholino) propane sulfonic acid (MOPS), N-N-N'-N'-tetramethyl ethylene-diamine (TEMED), pepstatin A, phenol, phenylmethylsufonylfluoride (PMSF), poly-ethylene glycol (PEG), potassium acetate, potassium chloride, potassium dihydrogen phosphate, potassium hydroxide, potassium iodide, dipotassium hydrogen phosphate, potassium iodide, rubidium chloride, sodium acetate, sodium bisulfate,

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sodium chloride, sodium dodecyl sulfate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, disodium tetraborate decahydrate, Tris-hydromethyl amino methane (Tris), Triton X-100 and Tween 20.

2. Enzymes, Miscellaneous Materials and Culture Media

Enzymes for molecular work and kits or reagents for analysis were obtained from different sources. Lysozyme, proteinase K and RNase A were provided by Sigma (St. Louise, USA). Zymolyase 20T was purchased from Seikagaku Corporation (Tokyo, Japan). Calf Intestinal Alkaline phosphatase (CIP), restriction endonuclease, T_4 DNA ligase, DNA molecular weight marker III and DIG labeling and detection kits were supplied by Boehringer Mannheims (Mannheim, Germany). Bradford® reagent, nitrocellulose membrane and Quantum Prep® Gel Slice kit were purchased from Bio-Rad® (California, USA). Nylon membrane was purchased from BRL Life Technologies, Inc. (Gaithersburg, USA), ProtoBlot® II AP System was obtained from Promega (Madison, USA). Prestained protein molecular weight markers was supplied by BioLab® (USA). The ELISA kit for detection of HBsAg was obtained from Sorin Biomedica (Saluggia, Italy). Standard human HBsAg was obatianed from Auszyme® monoclonal diagnostic kit (Abbot Laboratories, Abbot Park, USA). The purified HBsAg from human carriers was provided by Mrs. Jittaporn Wattanaseree, Government Pharmaceutical Organization. Mouse anti-S-monoclonal antibody was kindly provided by Dr. Kruavan Bholachandra, Department of Medical of Medical Science, National Institute of Health. Mouse anti-PreS2 monoclonal antibody 2-12F2 clone was a gift from Prof. W.H. Gerlich, Institute of Medical Virology, Justus-Liebig University, Germany.

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Culture media and constituents were obtained from Difco (Detroit Michigan, USA), Merck (Darmstadt, Germany) and Sigma (St. Louis, USA).

3. Microorganism Strains and Plasmids

The *Escherichia coli*, *Saccharomyces cerevisiae* strains, recombinant yeasts and plasmids used in this study were summarized in Table 5 and Table 6, respectively.

Table 5. List of microorganisms.

Microorganism	Strain	Genotype/Plasmid	Source
Escherichia coli	DH5α	*F*ϕ80d*lac*ZΔM15,Δ(*lac*ZYA-*arg*F)u169 deoR, *rec*A1, *end*A1, *hsd*R17(r_{K-},m_{K+})*Sup*E44, λ-*thi*-1, *gyr*A96, *rec*1A1	BRL
Saccharomyces cerevisaie	BJ5462	*MAT*α, *ura*3-5, *trp*1*leu*2Δ1, *his*3Δ 200,*pep*4::*HIS*3, *prb*1Δ1.6R *can*1 *GAL*	Yeast Genetic Stock Center, University of California Berkley.
	B1P1 to P1P10 B2P1 to B2P10 B3P1 to B3P10 B4P1 to B4P10 M5B1	BJ5462 recombinant strain harboring plasmid pEB-P1 pEB-P2 pEB-P3 pEB-p4 pEB-M5-2	This study.

Table 6. List of Plasmids.

Plasmid	Characteristic	Sources
pBSII+SK (2.96 Kb)	Ap^R, *Col*E1 ori, *LacZ*, *f1*(+) ori	Strategene®
pYC301 (6.5 Kb)	Ap^R, *Col*E1 ori, 2μ ori, *PHO*84p, *PHO*84t, *URA*3	Gift from Dr. Chulee Yompakdee, King Mongkut's University of Technology Thonburi.
pTB326 (6.18 Kb)	Ap^R, ColE1 ori, 2μ ori, *ADH*1p, CYC1t, *TRP*1	Gift from Dr. Qui Hongfang, Department of Health & Human Services, National Institue of Health, Bethesda.

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Table 6. List of Plasmids (continued).

Plasmid	Characteristic	Sources
pJW1 (3.86 kb)	pBSII+SK + *CYC*1t from pTB326	This study.
pJW2 (4.72 kb)	pJW1 + *PHO*84p from pYC301	This study.
pSP72 (2.462 kb)	Ap^R, *Col*E1 ori, SP6p and T7p	Promega®
pJW3 (4.22 kb)	pSP72 + *PHO*84p and *CYC*1t from pJW2	This study
pPM323 (10.6 kb)	Ap^R, ColE1 ori, 2μ ori, *PGK*1p, *PGK*1t, *URA*3, *LEU*2d	Gift from Dr. Qui Hongfang, Department of Health & Human Services, National Institue of Health, Bethesda.
pSF5-1 (3.89 kb)	pBSII+SK + 0.9 Kb PreS2d+S	Constructed by Ms. Sunsanee Foo-trakoon (2000).
pPM323-P1Δ (7.49 kb)	pPM323 ΔU*RA*3, Δ part of *LEU*2d (1.26 kb) + *PHO*84p and *CYC*1t from pJW3	This study
pPM323-P1 (8.75 kb)	pPM323 ΔU*RA*3 + *PHO*84p and *CYC*1t from pJW3	This study.
pPM323-P2 (9.85 kb)	pPM323 + *PHO*84p and *CYC*1t from pJW3	This study.
pPM323-P3Δ (6.07 kb)	pPM323 ΔU*RA*3, Δpart of vector (1.42 kb), Δ part of *LEU*2d (1.26 kb) + *PHO*84p and *CYC*1t from pJW3	This study
pPM323-P3 (7.33 kb)	pPM323 Δ*URA*3, Δpart of vector (1.42 kb) and *CYC*1t from pJW3	This study.
pPM323-P4 (8.43 kb)	pPM323 Δpart of vector (1.42 kb) + *PHO*84p and *CYC*1t from pJW3	This study.
pEB-P1 (9.65 kb)	pPM323-P1 + PreS2d+S from pSF5-1	This study.
pEB-P2 (10.75 kb)	pPM323-P2 + PreS2d+S from pSF5-1	This study.
pEB-P3 (8.23 kb)	pPM323-P3 + PreS2d+S from pSF5-1	This study.
pEB-P4 (9.33 kb)	pPM323-P4 + PreS2d+S from pSF5-1	This study.
pEB-M5-2 (10.26 kb)	Ap^R, *Col*E1 ori, 2μ ori, *MF*α1p, *CYC*1t, *URA*3, PreS2+S	Constructed by Mr. Thainchai Lakornrach (unpublish data)

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Methods

1. Microorganisms and Maintenance Conditions

1.1 *Escherichia coli*

E. coli host strain was maintained on Luria Bertani agar (LB agar : 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl pH 7.0, and 1.5% agar), while the recombinant strains were maintained on LB agar supplemented with 50 mg/ml ampicillin. They were incubated at 37 °C for overnight and kept at 4 °C for routine use. For long term preservation, the bacteria were cultivated in LB at 37 °C until mid to late log phase of growth, then 500 μl of the culture was transferred to a vial containing 500 μl of 30% glycerol, mixed and kept at -80 °C.

For selection of recombinant *E. coli*, LB agar was supplemented with 50 μg/ml ampicillin. When blue-white selection required, the plate was overlayed with 100 μl of X-gal (20mg/ml in dimethylformalmide) was used.

1.2 *Saccharomyces cerevisiae*

S. cerevisiae host strain BJ5462 was maintained on Yeast Peptone Dextrose agar (YPD : 1.0% yeast extract, 2.0% peptone, 2.0% glucose, and 2.0% agar). The recombinant *S. cerevisiae* clones were usually selected and grown on Synthetic Dextrose Minimal agar (SD agar: 0.67% yeast nitrogen base w/o amino acid, 2.0% glucose, and 2.0% agar) supplemented with 30 mg/ml leucine, 30 mg/ml tryptophan and 20 mg/ml uracil (SD+leucine+tryptophan+uracil) or 30 mg/ml tryptophan (SD+ tryptophan) or 30 mg/ml tryptophan and 20 mg/ml uracil (SD+tryptophan+uracil). They were incubated at 30 °C for 3-4 days kept at 4 °C for routine use. For long term

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preservation, they were grown in SD broth supplemented with required amino acids at 30 °C until mid to late log phase of growth (24 h), then 500 μl of the culture was transferred to vial containing 500 μl of 30% glycerol, mixed and kept at -80 °C.

2. Preparation of DNA

2.1 Small Scale Preparation of *E. coli* plasmid

The rapid boiling method of Holmes *et al.*, 1981 was employed for small scale preparation of plasmid. A single colony of *E. coli* was inoculated into 3 ml of LB broth containing 50 mg/ml ampicillin and incubated overnight at 37 °C. with vigorous shaking. The cells were packed by centrifugation at 5,000 rpm for 1 min in a microcentrifuge tube. The supernatant was discarded and the cell pellet was resuspended in 350 μl of STET solution (8% sucrose, 5% triton X-100 (v/v), 50 mM EDTA and 50 mM Tris-HCl pH 8.0), using vortex mixer. Cells were lysed by adding 25 μl of STET solution containing 10 mg/ml lysozyme and immediately mixed by inverting the tube 3 to 4 times. The tube was boiled for 1 min and immediately centrifuged at 10,000 rpm for 10 min. The slimy gelatinous pellet was then removed by picking with sterile toothpick. An equal volume of phenol/chloroform (1:1) saturated with Tris-HCl pH 8.0 was added to the supernatant and the tube was vigorous shaken with vortex mixer followed by centrifugation at 10,000 rpm for 5 min. The protein and other impuritied were removed by this step. Subsequently, an aqueous phase was transferred to a new tube and equal volume of chloroform was added, mixed and recentrifuged. The 1:10 volume of 3 M sodium acetate pH 7.5 and 0.5 volume of isopropanol were added to the supernate to precipitate DNA. The solution were mixed by inversion and kept at -80 °C

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for at least 15 min. After the tube was spun at 10,000 rpm for 10 min, the DNA pellet was washed once with cold 70% ethanol, air dried and resuspended in 20 μl of TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA). To remove RNA in the sample, the DNA was incubated in 1 μl of 10 mg/ml RNase A (boiled at 65 °C for 5 min before use). This DNA was then ready for restriction endonuclease analysis.

2.2 Large Scale Preparation of Plasmid *E. coli* plasmid

The modified alkaline lysis method described by Birnhoim and Doly, 1979 was used to prepare large scale preparation of plasmid. Fresh *E. coli* cells were grown in 200 ml of Terrific Broth (TB: 12 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4) containing 50 mg/ml ampicillin at 37 °C with shaking for overnight. The cells were harvested by centrifugation at 5,000 rpm at 4 °C for 5 min. Then the pellet was completely resuspended in 8 ml of Solution I (25 mM Tris-HCl pH 7.5, 10 mM EDTA, 15% sucrose, and 2 mg/ml lysozyme) and incubate on ice for 10 min. To lyse the cells, 16 ml of freshly prepared solution II consisting of 0.2 N NaOH and 1% SDS was added to the cell suspension, the mixture was mixed by gentle rocking and kept on ice for 10 min. The mixture was subsequently neutralized by 12 ml of ice-cold solution III (3M potassium acetate pH 5.2), mixed and incubated on ice for another 10 min. The supernatant was collected by centrifugation at 10,000 rpm at 4 °C for 20-25 min and subsequently filtered through layers of gauze cloth. To precipitate DNA, 0.6 volume of isopropanol was added to the filtrate. The mixture was spun at 10,000 rpm at 4 °C for 10 min. The DNA pellet was washed once with cold 70% ethanol and dissolved in 6 ml TE buffer. The DNA solution was incubated with 50-100 μg/ml boiled RNase A and 100-200 μg/ml of Proteinase K at 37 °C for 1 h each,

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respectively. The protein and other impurities were removed by extraction with equal volume of phenol/chloroform (1:1), vigorously shaken and centrifuged at 5,000 rpm at 4 °C for 5 min. The DNA in the aqueous solution was removed to a new tube. The process was repeated until the white interface disappeared. The final extraction was washed with an equal volume of chloroform, shaken with vortex mixer and recentrifuged. The supernatant containing DNA was precipitated with 1:10 volume of 3 M sodium acetate pH 5.2 and 2 volume of chilled absolute ethanol, mixed and kept at –80 °C for at least 15 min. The nucleic acid was collected by centrifugation at 10,000 rpm at 4 °C for 10 min then rinsed twice with cold 70% ethanol. The pellet was dried and dissolved in 0.2 ml TE buffer and the DNA solution was stored at 4 °C or –20 °C.

2.3 Minipreparation of Yeast DNA

Yeast DNA minipreparation was done using the method described in Method in Yeast Genetics (Rose *et al.*, 1990). Yeast cells were grown in 10 ml of SD broth containing appropriate amino acids at 30 °C overnight with vigorous shaking. Then the cells were collected by centrifugation at 3,000 rpm for 5 min, washed once with 1 ml of 50 mM EDTA pH 7.5 and resuspended in 0.5 ml of 1 M sorbitol, 0.1 M EDTA pH 7.5. After the cells were transferred to microcentrifuge tube, 20 μl of 3 mg/ml Zymolyase 20 T in 1 M sorbitol, 0.1 M EDTA pH 7.5 was added and mixed. The mixture was incubated at 37 °C for 60 min. The cells were converted to spheroplast in this step. After the incubation was completed, the spheroplast was spun down at 5,000 rpm for 1 min and resuspened gently in 0.5 ml of 50 mM Tris-HCl, 20 mM EDTA. To lyse the spheroplast, 50 μl of 10 % SDS was added to the suspension, the mixture was incubated at 65 °C for 30 min.

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A 0.2 ml of 5 M potassium acetate was added and the tube was placed on ice for 60 min. After centrifugation at 10,000 rpm for 5 min, the DNA in supernatant was transferred to a fresh microcentrifuge tube. One volume of isopropanol was added and the tube was allowed to stand at room temperature for 5 min. Subsequently, the DNA pellet was collected by spinning the tube briefly, washed with cold 70% ethanol, air-dried and dissolved in 20-30 µl of TE buffer. The yeast DNA was kept at 4 °C.

3. DNA Manipulation and Analysis

3.1 Restriction Enzyme Digestion

Restriction enzyme digestion of plasmids was performed according to the manufacturer's recommendations (Boehringer Mannheim). In principle, the reaction mixture was consisted of appropriate buffer of particular pH and salt, the restriction enzyme (not more than 10 % of total volume) and the DNA. The reaction was terminated either by heating at 65 °C for 10 min or extracting with phenol-chloroform depended on the nature of restriction enzyme. When a double digestion of DNA with 2 different buffer conditions was required, one each of phenol extraction and ethanol precipitation (as described in method 2.2) were required after the first enzyme digestion.

The restriction enzymes used in this study including their recognition sequences and their optimal conditions are shown in Table 7 and 8.

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Table 7. Restriction enzymes with their recognition sequences and optimal conditions.

Enzyme	Buffer	Optimal temperature (°C)	Recognition sequence
*Kpn*I*	L, A	37	5'-GGTAC↓C-3'
*Pvu*II	M	37	5'-CAG↓CTG-3'
*Bam*HI	A, B, L, M	37	5'-G↓GATCC-3'
*Hind*III	B, M	37	5'-A↓AGCTT-3'
*Cla*I	A, B, L, M, H	37	5'-AT↓CGAT-3'
*Eco*RI	A, B, H	37	5'-G↓AATTC-3'
*Sal*I	H	37	5'-G↓TCGAC-3'
*Xba*I	A, B, L, M ,H	37	5'-T↓CTAGA-3'

Note : * Requires addition of bovine serum albumin, 100 μg/ml.
↓ represents the cleavage site of restriction endonuclease.

Table 8. Restriction enzymes buffer composition.

Buffer Component	Final Concentration in mM				
	Buffer A	**Buffer B**	**Buffer L**	**Buffer M**	**Buffer H**
Tris acetate	33	-	-	-	-
Tris HCl	-	10	-	10	50
Mg-acetate	10	-	10	-	-
$MgCl_2$	-	5	-	10	10
K-acetate	66	-	10	-	-
NaCl	-	100	-	50	100
Dithioerythritol (DTE)	-	-	1	1	1
Dithiothreitol (DTT)	0.5	-	-	-	-
2-Mercaptonethanol	-	1	-	-	-
pH at 37 °C	7.9	8.0	7.5	7.5	7.5

Note: All buffers were stored at –20 °C.

3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed by standard method (Maniatis *et al.*, 1982). It was used for restriction analysis and isolation of DNA fragments. The condition usually used in this study was 0.8 % agarose gel in TBE buffer (0.089 M Tris, 0.089 M Borate, and 0.002 M EDTA). Agarose concentration was occasionally varied depend on the size of DNA fragments. DNA samples were mixed in a ratio of

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3:1 with gel loading buffer type III (0.25 % bromophenol blue, 30 % glycerol in water), before loading. The minicell electrophoresis sets (Mupids, Tokyo, Japan) were commonly used for routine analysis. The gel was run at a constant voltage of 50 or 100 volts for 30-40 min or until the loading dye reached 1 cm from the bottom of the gel. The gel was then removed from the tray and stained with 0.5 μg/ml ethidium bromide solution for 10-15 min then destained with distilled water for 10-30 min. The DNA was visualized with a long wavelength transluminator WL300-310 nm (Fotodyne, New Berlin, USA) and photographed using polariod camera (Polariod MP4, Fotodyne, New Berlin, USA) with polariod film type 667 (Hertfordshire, England). The size of DNA fragments were estimated by standard marker, λ DNA cut with *Hind*III and *Eco*RI, which contained 12 fragments of 21.2, 5.1, 4.9, 4.2, 3.5, 2.0, 1.9, 1.5, 1.3, 0.94, 0.83, and 0.56 kb.

3.3 Recovery of DNA Fragment

Plasmid DNA was digested with one or two restriction endonuclease(s) to yield desired fragments. Isolation of the DNA fragments from the agarose gel was done by the Quantum Prep® Gel Slice kit (Bio-Rad®) according to manufacturer's recommendation. Briefly, the desired DNA band was excised with a razor blade in the smallest possible size and put into a 1.5 ml microcentrifuge tube. A 900 μl of matrix were added onto the sliced gel and the tube was, then, incubated at 55-70 °C to dissolved the gel. The tube was centrifuged at 3,500 rpm for 1 min and the supernatant was discarded. Afterward, the DNA bound matrix was washed twice with 1 ml of isopropanol and centrifugation at 3,500 rpm for 1 min. The matrix pellet was subsequently mixed with 1 ml of cold Quantum Prep wash buffer and applied onto the

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Quantum Prep Midi spin column, which placed into a 2 ml microcentrifuge tube. The column was spun at 3,000 rpm for 30 sec and the flow-through was discarded. The remaining ethanol in the wash buffer was completely removed by centrifugation at 12,000 for 1 min. Finally, the bound DNA in the column was eluted by incubation with the 30 μl elution buffer (distilled water or TE buffer) at room temperature for 2 min. Then the column was placed into a new 1.5 microcentrifuge tube and centrifuged at 13,000 rpm for 4 min. The aqueous DNA in the flow-through was finally collected for ligation or labelling.

3.4 Dephosphorylation of DNA

To remove the 5'-phosphate from both ends of the linear DNA, dephosphorylation of DNA was performed. This procedure was required in order to decrease self-recirculalization of DNA vector when it was digested with one restriction enzyme. The plasmid DNA in the amount of approximately 1-20 pmol was completely digested with restriction enzymes, phenol/chloroform extraction and precipitated with ethanol at –80 °C for 15 min. The precipitated DNA was dissolved in 50 μl TE buffer, then dephosphorylation was performed in 10x dephosphory late buffer [25 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 50 % glycerol (w/v) pH 7.6] and 0.1 unit of CIP. The mixture was incubated at 37 °C for 1 h, then enzyme was inactivated by heating at 65 °C for 15 min. The DNA solution was extracted twice with phenol/chloroform and once with chloroform. Finally, the DNA was precipitated with ethanol and dried. It was ready to use for ligation.

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3.5 Ligation of DNA

Typically, the digested vector or digested-dephosphorelated vector DNA was mixed with DNA fragment in a molar ratio of 1:3. The mixed DNA was precipitated with 3 M sodium acetate pH 5.2 and cold absolute ethanol at –20 °C for at least 5 min. After centrifugation at 10,000 rpm for 10 min, the DNA pellet was washed with cold 70 % ethanol and dried. Routinely, the ligation reaction was performed in 10 μl with 10x ligation buffer (0.5 M Tris-HCl pH 7.6, 100mM $MgCl_2$, 100mM dithiothreitol, 500 μ g/ml bovine serum albumin). The DNA solution in 8 μl TE was, then, warmed at 68 °C for 10 min, immediately chilled on ice for 1 min and finally mixed with 1 unit of T_4 DNA ligase (10 unit). The ligation reaction was allowed to occur at 16 °C for at least 3 h to overnight.

4. Transformation of *Escherichia coli*

4.1 Preparation of Competent *E. coli* Cells

The competent *E. coli* cells were prepared by using the modified standard method (Hanahan, 1983). A single colony of fresh *E. coli* was grown in 3 ml LB at 37 °C for overnight with vigorous shaking. A 1% of this culture was inoculated into fresh LB and further grown with the same condition. The culture was chilled on ice bath for 5 min then spun at 3,000 rpm at 4 °C for 10 min. The supernate was discarded and then cell pellet was resuspended with 2:5 volume of filtered sterile Tfb1 solution (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, adjust pH to 5.8 with 0.2 M acetic acid) and kept on ice bath for 5 min. The cells were pelleted by centrifugation at 3,000 rpm at 4 °C for 5 min and subsequently resuspended with 1:50 volume of filtered sterile Tfb2 solution (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$

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and 15% glycerol, adjust pH to 6.5 with KOH). The suspended cells were chilled on ice bath for 10 min. An aliquot of 200 µl was into a sterile ice-cold microcentrifuge tube and stored at –80 °C until used.

4.2 Transformation Technique

The transformantion of *E. coli* was done by using the standard method (Maniatis *et al.*, 1982). The frozen competent cells in microcentrifuge tube were thawed on ice bath for 15-20 min and transferred into a chilled glass tube (13x100 mm). Then, 20-100 ng of cold ligated DNA or control plasmid was added and mixed. After incubation on ice bath for 30 min, the tube was heated at 42 °C for 90 sec (or 37 ° C for 4 min) and immediately chilled on ice bath for 1 min. A 800 µl of LB was, then, added to each tube and the transformed cells were further incubated at 37 °C for another 1 h. After incubation has been completed, the cells were spun at 10,000 rpm for few sec and the cell pellet was resuspended in 100-200 µl of LB. Finally, the cell suspension was spread on the LB agar supplemented with 50 mg/ml ampicillin. When blue-white selection was required, the plate was overlayed with 100 µl of X-gal (20mg/ml in dimethyl-formalmide) prior to spreading the cell suspension. The transformants were allowed to grow at 37 °C for overnight.

5. Transformation of *Saccharomyces cerevisiae*

The whole cell method of yeast transformation described by Ito *et al.*, 1983 was used with some modification (Daniel *et al.*, 1991). A single colony of fresh *S. cerevisiae* on YPD plate was grown in 5 ml of YPD broth at 30 °C with vigorous shaking for overnight. Then, the culture was diluted with 20 ml fresh YPD broth to an

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OD_{660} of 0.10-0.15. The culture was further shaken at 30 °C until OD_{660} value reach the level of 0.4-0.5 which was corresponding to $2x10^6$ cells/ml. Subsequently, the cells were harvested by centrifugation at 3,000 rpm for 5 min. The harvested cells were washed with 1.0 ml of distilled water and 1.0 ml of TE/LiAc solution (100mM lithium acetate, 10 mM Tris-HCL pH 8.0 and 1 mM EDTA), respectively. The cells were, then, resuspened in 200 µl of TE/LiAc solution. This cell suspension was sufficient to transform 4 plasmids. An aliquot of 50 µl was distributed into a microcentrifuge tube. A 50 µl of yeast cell suspension was mixed with 1 µg of DNA and 50 µg of 10 mg/ml single stranded Salmon sperm carrier DNA which was prepared prior to use by boiling for 5 min and chilled immediately on ice bath. Afterward, 300 µl of sterile 40% PEG 4000 solution (40% PEG 4000, 100mM lithium acetate, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA) was added to the cells and mixed throughly. The suspended cells were incubated at 30 °C with agitation for 30 min and transformed cells were consequently spun down at 5,000 rpm for few sec. The cell pellet was resuspended in 100 µl of distilled water and an appropriate volume was spread on the SD agar supplemented with required amino acids. The transformants were allowed to grow at 30 °C for 3-4 days.

6. Confirmation of Yeast Transformants

6.1 Replica Plating

The replica plating technique was used to confirm the auxotrophic marker of yeast transformants. The yeast tranformants showing big colonies on selective medium plates were picked and patched onto a fresh selective medium. The auxotrophic markers of the transformants were checked by replicating the patches on SD agar

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supplemented with one or two amino acid(s) using a sheet of velvet cloth. The desired yeast transformants must grow only on the plate supplemented with required amino acids such as the transformants BJ5462 harboring pPM323 based plasmid must grow only on SD supplemented with tryptophan and uracil, but not with tryptophan, or with uracil alone. After checking the auxotrophic markers, the yeast transformants were subsequently subcultured on another SD supplemented with required amino acids to obtain single colonies for further study. The selected yeast transformants must be preserved immediately at –80 °C as described in method 1.1. In addition, each selected yeast transformant was stocked in several aliquots.

6.2 Back Transformation of *Escherichia coli*

To confirm the presence of yeast expression of plasmid harboring the PreS2+S gene fragment in yeast cells, the back transformantion of *E. coli* was done using yeast DNA minipreparation as the transformed DNA. The preparation of competent *E.coli* cells and transformation technique were performed as described in methods 2.3, 4.1 and 4.2.

7. Cultivation of Transformants for Expression of Recombinant Gene

The cultivation of yeast transformant under the control of *PHO*84p was performed according to the method described by Bun-ya *et* al., 1991 and Hiraoka *et* al., 1994. A 500 µl of frozen stock culture was precultured in 10 ml High-Pi medium [25% +P stock solution (appendix A), 2% glucose, 0.5 % $(NH_4)_2SO_4$) and vitamin mix (appendix A, added before use)] supplemented with required amino acids at 30 °C with vigorous shaking for 18-20 h. Then, the preculture was diluted with 100 ml of the

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same medium to an OD_{660} of 0.1. The culture was allowed to shake at 30°C until an OD_{660} value reached the level of 0.5-1.0 (early logarithmic phase). The cells were harvested by centrifugation at 3,000 rpm at room temperature for 5 min, washed once with 1:10 volume of Low-Pi medium [0.5% +P stock solution, 24.5% -P stock solution (appendix A), 2% glucose, 0.5% $(NH_4)_2SO_4$ and vitamin mix (appendix A, added before use)] centrifuged at 3,000 rpm for 5 min. Then, cell pellet was resuspended in the same volume of Low-Pi medium supplemented with required amino acids. The culture was continuously shaked at 30 °C until an OD_{660} value reached the level of 5.0-6.0 (late logarithmic phase). Subsequently, the cells were harvested by centrifugation at 3,000 rpm at 4 °C for 5 min, wash once with cold phosphate buffer pH 7.2 (0.5 M KH_2PO_4 and 0.5 M K_2HPO_4) and stored at -80 °C. To analyse the expressed protein, the cells should not kept more than 6 months before use.

8. Southern Blot Analysis

Southern blot analysis was performed to determine the copy number of recombinant plasmids in yeast cells. The DNA labeling and detection was performed with the DIG labeling and detection kit from Boehringer mannheim.

8.1 Southern Blot Transfer

Southern blot transfer was performed by the standard method (Maniatis *et al.*, 1982). Total DNA from yeast transformants harboring plasmid pEB-P1, pEB-P2, pEB-P3 and pEB-P4 extracted by the method 2.3 was digested with restriction endonuclease for overnight to obtain complete digestion. DNA fragments were separated on 0.8% agarose gel electrophoresis. After taking photograph, the gel was denatured by soaking twice in denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 15 min with gently

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shaking. Subsequently, the gel was neutralized by soaking twice in neutralizing solution (0.5 M Tris-HCl, pH7.5 and 3 M NaCl) for 15 min with shaking gently. The DNA from agarose gel was transferred into a nylon membrane by using Blot Transfer System (Gibco, BRL Life Technologies, Inc., USA). A wick of two layers Whatman 3 MM paper was cut to form salt bridge standing on a tray containing 20x SSC (3M NaCl and 0.3 M sodium citrate). The gel was carefully placed on the damp wick to avoid air bubbles, then wet nylon membrane cut exactly to the size of the gel was placed on top of the gel. All air bubbles trapped between the gel and the filter were carefully removed with glass rod.

Consequently, wet two layers of Whatman 3 MM paper and a stack of paper towel cut to the same size as the gel were placed on top of the membrane. A 500 g weight was placed on top of the stack of paper towel. The objective of this arrangement was to set up a flow of liquid from the reservoir through the gel and the membrane by capillary force. The transfers of DNA fragments were allowed to occur for 12-24 h. Next day, the positions of wells were marked on the membrane with pencil. The DNA was fixed to nylon membrane by exposing the side that faced the gel to short-wave UV length for 30 sec in a UV-crosslink (UVItec, England). The membrane can be used immediately for hybridization or air dried prior to storage in a sealed plastic bag at 4 °C until used.

8.2 Labeling DNA with Digoxigenin

The DIG-DNA labeling kit was used to prepare the DNA probes. A 10 ng to 1 μ g of 0.9 Kb *Bam*HI purified PreS2d+S gene fragment from pSF5-1 or 0.42 Kb *Hind*III-*Xba*I purified *TRP*1 gene fragment from pTB326 were labeled in a total volume of 20 μ

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l of reaction mixture (10x Hexanucleotide mixture, 10x dNTP labeling mixture and 2 units/μl Klenow enzyme) and incubated at 37 °C for overnight. After labeling, the reaction was stopped with 2 μl of 0.2 M EDTA pH8.0 and the DIG-labeled DNA probes were precipitated with 0.6 volume of isopropanol and 0.3 M sodium acetate pH 5.2 at –80 °C for at least 30 min. The DIG-labeled DNA was pelletted by centrifugation at 12,000 rpm at 4 °C for 15 min and washed once with 100 μl 70% cold ethanol. Then, the precipitated probes were resuspended in 40 μl of TE buffer. The labeled DNA probes can be used immediately or stored at –20 °C until used. The PreS2d+S probe was used to hybridize to the multicopy plasmid borne gene and the *TRP*1 probe to hybridize to the single copy chromosomal gene.

8.3 Hybridization

Prehybridization:

The membrane was placed in a hybridization roller tube containing prehybridization solution [5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% Blocking reagent (Boehringer Mannheim)] and incubated in a Hybaid Micro-4 (Hybaid, England) at 68 °C for 2 h.

Hybridization step:

Prehybridization solution was removed and replaced with the same volume of prehybridization. The DNA probes denatured by boiling for 10 min and chilled on ice immediately, was immediately, added to final concentration of 5-25 ng/ml. Hybridization procedure was continued at 68 °C for overnight.

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Washing step:

The hybridized membrane was washed twice with a washing solution I (2x SSC and 0.1% SDS) at room temperature for 5 min and followed by twice with a washing solution II (0.1x SSC and 0.1% SDS) at 68 °C. The membrane was subsequently subjected to detection step or air dried prior to storage in a sealed plastic bag at 4 °C until used.

Detection step:

The detection step was performed in a petri dish with small volume of solution. The membrane was soaked in washing buffer [Maleic buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) and 0.3% tween 20] prior to incubation in blocking solution [1% (w/v) blocking reagent in maleic acid buffer] at room temperature for 1 h. Subsequently, it was incubated in anti-Digoxigenin-AP solution (diluted to 1:5000 in blocking solution) at room temperature for 30 min. After incubation has been completed, the unbound antibody was removed from the membrane by washing twice with washing buffer for 15 min. The membrane was equilibrated in detection solution (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl) for 2 min. Finally, colorimetric detection step was performed in 30 ml detection solution containing 45 µl of 4-Nitroblue Tetrazoluim chloride (NBT) and 35 µl of 5-bromo-4chloro-3-indolyl-phosphate (X-phosphate) in detection solution. The membrane was incubated in the dark until the desired bands were detected. The detection reaction was terminated, when the color was developed to the desired intensity by washing the membrane with deionized water.

After detection, the intensity of the two differently sized bands on the membrane corresponding to chromosomal and plasmid bands were scan with Bio imaging system (Syngene, England). The area of the two bands were equally located

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before scanning. The ratio of the intensity of the plasmid and chromosomal bands was finally calculated, which would represent the average plasmid copy number in the yeast cell population.

9. Preparation of Protein Extracts

The yeast cell pellet from 2 ml culture (method 7) was resuspended in 200 μl of ice-cold extraction buffer containing protease inhibitor cocktail (50 mM phosphate pH 7.2, 140 mM NaCl, 0.1% Triton X-100, 0.5 mM PMSF, 2 μM pepstatin A, 2 μM leupeptin, 2 mM EDTA, 0.5 mM EGTA, and 10 mM sodium bisulfide) (Imamura *et al.*, 1987). To break the cells, chilled glass beads (diameter 0.45 mm) were added to two thirds of the height of the menicus, and the tube was vortexed intervally at full speed for 10 min (vortexed 30 sec, and placed on ice 30 sec). After centrifugation at 10,000 rpm at 4 °C for 10 min, total protein concentration in the protein extracted was then determined. The protein extract was immediately proceeded for quantitative determination or detection of the PreS2+S gene product.

10. Determination of Protein Concentration

Protein concentration was determined by a procedure described by Bradford, 1976. A 1 ml of Coomassie Brilliant blue Reagent (Bradford®), containing 10% Coomassie Brilliant blue R, 95% ethanol and 85% phosphoric acid was added to 20 μl of sample or standard bovine serum albumin. After mixing, the mixture was allowed to stand at room temperature for 5 min for color development. The absorbance of blue color was measured at wavelength 595 nm against a blank preparation with the same extraction buffer. The concentration of protein was determined by comparing with

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standard curve of bovine serum albumin (BSA). The concentration of BSA was 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml

11. Quantitative Determination of the Hepatitis B surface Antigen (HBsAg)

Quantitative determination of the hepatitis B surface antigen was based on ELISA technique using Sorin Biomedica ETI-MAK-3 kit which detected ***a*** epitope in the S domain of HBsAg. Therefore, the quantitative of the PreS2+S gene product was expressed as HBsAg concentration in ng/mg protein. The method was a direct, non-competitive (sandwich) assay using anti-S mouse monoclinal antibody and sheep-anti-S conjugated to horse raddish peroxidase. A 100 µl of negative control (human plasma non-reactive for all HBV markers), positive control (heat-inactivated recalcified human serum), standard human HBsAg and protein extracts with appropriate dilution in extraction buffer were dispensed into their respective wells of microtiter plate which coated with anti-S. A cardboard sealer was applied in order to prevent evaporation. The wells were incubated at 37 °C for 1 h. After incubation had been completed, the cardboard sealer was discard and the wells were rinsed five times with 250 µl wash buffer. The wash buffer (provided by kit) was prepared by diluting 1:25 with distilled water. Then, 100 µl of diluted enzyme tracer containing sheep anti-S conjugated to horse raddish peroxidase (provided by kit) was dispensed into all wells except for the blank well. The enzyme tracer (provided by kit) was prepared by diluting 1:50 with tracer diluent. The wells were rinsed five times with 250 µl wash buffer. The color was developed by adding 100 µl of chromogen/substrate into all wells followed with incubating at room temperature for 30 min in dark. The chromogen/substrate was

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prepared prior to use by mixing chromogen and substrate in a ratio 1:1. At the end of incubation, the reaction was stopped by adding 100 μl of blocking reagent.

The absorbance of samples were measured with a microplate reader at wavelength 450 and 630 nm within one hour of adding the blocking reagent. The value obtained from subtracting the absorbance value at 630 nm from that at 450 nm absorbance value was used to interpret the result. The presence of HBsAg was determined by relating the subtraction value of samples to that of cut-off value, which was, obtained by adding 0.050 to the mean absorbance of the negative control values (NCx). In order to validate the criteria, the mean absorbance of negative and positive control were considered, i.e., the mean absorbance of negative control should be less than 0.050 and more than -0.010, the mean absorbance of positive control should be more than 0.550, and the positive-negative difference should be greater than or equal to 0.500. The quantity of HBsAg was determined by comparing with standard curve of human HBsAg. To prepare standard curve for quantification of HBsAg, human HBsAg concentration of 9 mg/ml was diluted with the same buffer as used for preparation of protein extracts from yeast cells. The concentration after dilution was 0.5, 1, 1.5, 2, 2.5 and 3 ng/ml. The linearity of the standard curve was between $OD_{450-630}$ 0.1-0.7.

12. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Proteins

SDS-PAGE used in this study was based on the procedure described by Laemmli (1970).

12.1 Slab Gel Preparation

The SDS-PAGE was carried with 15% resolving gel pH 8.8 using ATTO dual mini slab kit (Japan). Glass plates were washed with detergent, rinsed through with

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water, then with ethanol, and wiped dry with tissue. Plastic spacers were placed on two edges of the plate. Two glass plates were clamped together in a mini slab gel set. Preparations of resolving gel and stacking gel were shown in Table 9. The resolving gel solution was poured into the assembled glass plate to 30 mm from a top of edges. Distilled water was layed carefully across the top of the resolving gel solution while polymerization was occurring. The stacking gel was spread over the top of resolving gel until the level reached the top of the edges. Then the sample-well-comb was inserted into the gel at the top of the plates. After polymerization, the comb was carefully removed and the wells were rinsed several rimes with distilled water to remove unpolymerized gel and gel debris.

12.2 Sample Preparation and Electrophoresis

The samples were prepared by mixing an appropriate volume of protein sample with 5x SDS sample buffer [0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (w/v) SDS and 0.025% bromophenol blue] and the total volume was adjusted with distilled water. The molecular weight marker (Prestained Protein Marker, BioLabs®) of protein and the purified HBsAg, (positive control), were prepared as same as the sample protein. The molecular weight marker contained 7 sizes of 172, 87, 42.5, 32.5, 25, 16, 6.5 kDa. Usually, one well of mini slab gel could be loaded with sample upto 30 μl. Prior to loading, the samples were heated in boiling water for 5 min and the wells were washed with electrophoresis buffer (0.25 M Tris-HCl, 0.192 M glycine pH 8.3 and 0.1% SDS). The electrophoresis was performed at a constant voltage (110 volt) and allowed to proceed until the dye reached the bottom of the gel. After, the power was turned off, the gel plated was removed from the tank and the top glass plate was carefully

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separated from the gel with thin spatula. The gel slab was carefully peeled off from the remaining plate and placed into a shallow tray containing protein staining solution or proceed to Western blotting.

Table 9. Preparation of resolving gel and stacking gel for ATTO dual mini slab kit (Laemmli, 1970).

Stock Solution	15% resolving gel	4% stacking gel
Distiled water	2.5 ml	3 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	-
0.5 M Tris-HCL, pH 6.8	-	1.25 ml
10% w/v SDS	100 µl	50 µl
Acrylamide/Bis (29.2 g/100 ml acrylamide, 0.8 g/100963.*-ml N'N'-bis-methylene-acrylamide, make to 100 ml with distilled water)	5 ml	0.67 ml
10% ammonium persulfate	50 µl	50 µl
TEMED	5 µl	5 µl
Total volume	10 ml	5 ml

12.3 Protein Staining

For analysis of protein bands, the gel was stained with Coomassie Brilliant blue R-250 staining solution [0.1% Coomassie blue R-250 in fixative solution (40% methanol 10% acetic acid)] for 1-2 h with gentle shaking. Then the gel was destained by soaking in several changes of destaining solution (40% methanol, 10% acetic acid) for 1-3 h each with shaking. The stained gel was air dried in double layers of cellophane.

13. Western Blot Analysis of the M HBsAg

After SDS-PAGE, the protein bands from gel were transferred to nitrocellulose membrane by Western Blot using a Transblot apparatus (Bio-Rad®, Califormia USA) at a constant volt (35 volt) at 4 °C for 16 h in a buffer (192 mM glycinem 25 mM Tris-

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HCl pH 8.3 and 20% methanol). For the membrane blot from miniprotein gel, the following step were performed in small volume of reagent of about 10 ml in a glass petri disk. After the trasfer has been completed, the membrane was removed and rinsed with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween 20). Then the membrane was soaked in blocking solution containing 5% skim milk in TBST at room temperature for 1 h with agitation. Afterward, the membrane was incubated with TBST containing anti-S (1:500) or anti-PreS2 mouse monoclonal antibody for 6 h with gentle agitation. After removal of unbound primary antibody by washing three times in TSBT for 5 min each, the membrane was transferred into TBST containing 1:4000 dilution of anti-mouse IgG AP conjugate and incubated for 1 h with gentle agitation. To remove unbound secondary antibody, the membrane was washed in TBST three times for 5 min each. Afterwards, the blot was rinsed briefly in two changes of TBS (20 mM Tris-HCl pH 7.5 and 150 mM NaCl) to remove Tween 20 from the membrane surface. The ProtoBlot® II AP system (Promega) was employed to detect HBsAg. The reaction was started by incubation the blot in Western Blue Stabilized Substrate (nitroblue tetrasolium and 5-bromo-4-chloro-3-indolylphosphate) with agitation until the bands of interest had reached the desired intensity. Typically, the reactive areas would turn purple color within 1-15 min. When the color had developed to the desired intensity, the reaction was stopped by washing the membrane several times with deionized water.

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14. Dot Blot ELISA

The dot blot ELISA was performed by applying protein directly onto nitrocellulose membrane. Several protein extracts could be analysed on the same sheet of membrane. The total protein from yeast cells was diluted to 0.4 mg/ml with the extraction buffer prepared by method 9. About 4 µg of total yeast protein, negative control and positive control were blotted onto the same sheet of nitrocellulose membrane with Hybridot® Manifold (BRL Life Technologies, Inc., Gaithersburg, USA). The dots were then air dried. Afterwards, the membrane was subjected to subsequent steps as described in method 13.

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CHAPTER IV

RESULTS

1. Construction of Yeast Expression Plasmid with *PHO*84 promoter

In this work, the expression of the PreS2d+S gene encoding middle Hepatitis B surface antigen (M HBsAg) in *S. cerevisiae* was investigated using the *PHO*84 promoter (*PHO*84p) and *CYC*1 terminator (*CYC*1t). Two types of yeast expression plasmid harboring one or two selectable markers were used. They were either *LEU*2d alone or *LEU*2d and *URA*3. Since the series of desired expression plasmid with *PHO*84p and one or two selectable markers were not available, therefore they were constructed using pPM323 harboring *PGK*p, *PGK*t and *LEU*2d and *URA*3 selectable markers as the starting plasmid. Furthermore, pPM323 contained some part of *E. coli* vector about 1.42 Kb which was not necessary for plasmid function both in *E. coli* and yeast. Therefore, smaller pPM323 based expression plasmid with one and two selectable markers were also constructed by deletion of non-necessary part. Altogether, four types of yeast expression plasmid were constructed. They were plasmids pPM323-P1 (8.75 kb) and pPM323-P3 (7.33 kb) harboring *LEU*2d, and pPM323-P2 (9.85 kb) and pPM323-P4 (8.43 kb) harboring *LEU*2d and *URA*3. All of them contained an expression cassette with *PHO*84p and *CYC*1t.

1.1 Construction of an Expression Cassette with *PHO*84p and *CYC*1t

1.1.1 Construction of Plasmid pJW1

To construct an expression cassette, firstly the DNA fragment 0.90 kb containing *PHO*84p was isolated from plasmid pYC301 by *Hind*III and *Eco*RI

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digestion. This fragment was ligated to the linearized plasmid pBSII+SK at the corresponding sites to obtain pJW1. The construction diagram was shown in Figure 7.

The ligated DNA was transformed into *E. coli* strain DH5α and the transformants were selected on LB agar plate with ampicillin and X-gal. The plasmids from selected white colony of transformants were extracted by rapid boiling method and subjected to restriction endonuclease analysis on 0.8% agarose gel electrophoresis. When the plasmid pJW1 was cleaved with *Hind*III and *Eco*RI, two bands were obtained. As shown in Figure 8 (lane 6) the upper band about 2.96 kb was corresponded to the pBSII+SK (lane 4) and the lower band about 0.90 kb was corresponded to the lower band of the plasmid pYC301 (lane 2) cleaved with the same enzymes. The result of restriction analysis was summarized in Table 10.

1.1.2 Construction of Plasmid pJW2

The second step of an expression cassette construction was to ligate the *CYC*1t fragment into the plasmid pJW1. The *CYC*1t fragment was obtained from plasmid pTB326 by *Eco*RI and *Xba*I double digestion. This fragment was inserted into the plasmid pJW2 at the corresponding sites. The newly constructed plasmid was name pJW2 and the construction diagram was illustrated in Figure 9.

The ligated DNA was transformed into *E. coli* strain DH5α and the transformants were selected on LB agar plate with ampicillin. The plasmids from selected transformants were subjected to restriction enzyme digestion with *Eco*RI and X*ba*I which generated two bands as shown in Figure 8 (lane 10). The upper band about 3.86 kb was equivalent to the plasmid pJW1 (lane 8) and the lower band about 0.86 kb was equivalent to the lower band of the plasmid pTB326 (lane 7) cleaved with the same enzymes. To confirm that the plasmid pJW2 contained the *PHO*84p and *CYC*1t gene

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fragment, the plasmid were analysed with *Hind*III digestion which generated two bands about 3.56 kb and 1.16 kb (lane 11). The result of restriction analysis was summarized in Table 10.

1.1.3 Construction of Plasmid pJW3

The third step of an expression cassette construction was to modified the 3' end of the *CYC*1t fragment to be compatible to the cloning sites, which were *Sal*I and *Pvu*II, in the plasmid pPM323. The plasmid pJW2 was cleaved with *Cla*I and *Xba*I to isolate a 1.76 kb fragment containing *PHO*84p and *CYC*1t gene. This fragment was inserted into plasmid pSP72 at the corresponding sites to obtain plasmid pJW3. The construction diagram was shown in Figure 10.

The ligated DNA was transformed into *E. coli* DH5α and the plasmid from transformants were subjected to double digest with *Cla*I and *Xba*I which generated two bands about 2.46 kb and 1.76 kb, as shown in Figure 11 (lane 7). The upper band was corresponded to the plasmid pSP72 (lane 3) and the lower band was corresponded to the lower band of the plasmid pJW2 double digested with *Cla*I and *Xba*I (lane 5). The result of restriction analysis was summarized in Table 11.

1.2 Construciton of Yeast Expression Plasmid with *PHO*84p and *CYC*1t

1.2.1 Construction of Plasmid pPM323-P1 and pPM323-P3

To clone the 1.76 kb expression cassette containing the *PHO*84p and *CYC*1t into plasmid pPM323. The 1.76 kb fragment was prepared from plasmid pJW3 by *Cla*I and *Sal*I double digestion. This fragment was inserted into plasmid pPM323 at the corresponding sites to obtain vector pPM323-P1Δ. This plasmid lacked part of *LEU*2d gene 1.26 kb. The construction diagram was illustrated in Figure 12.

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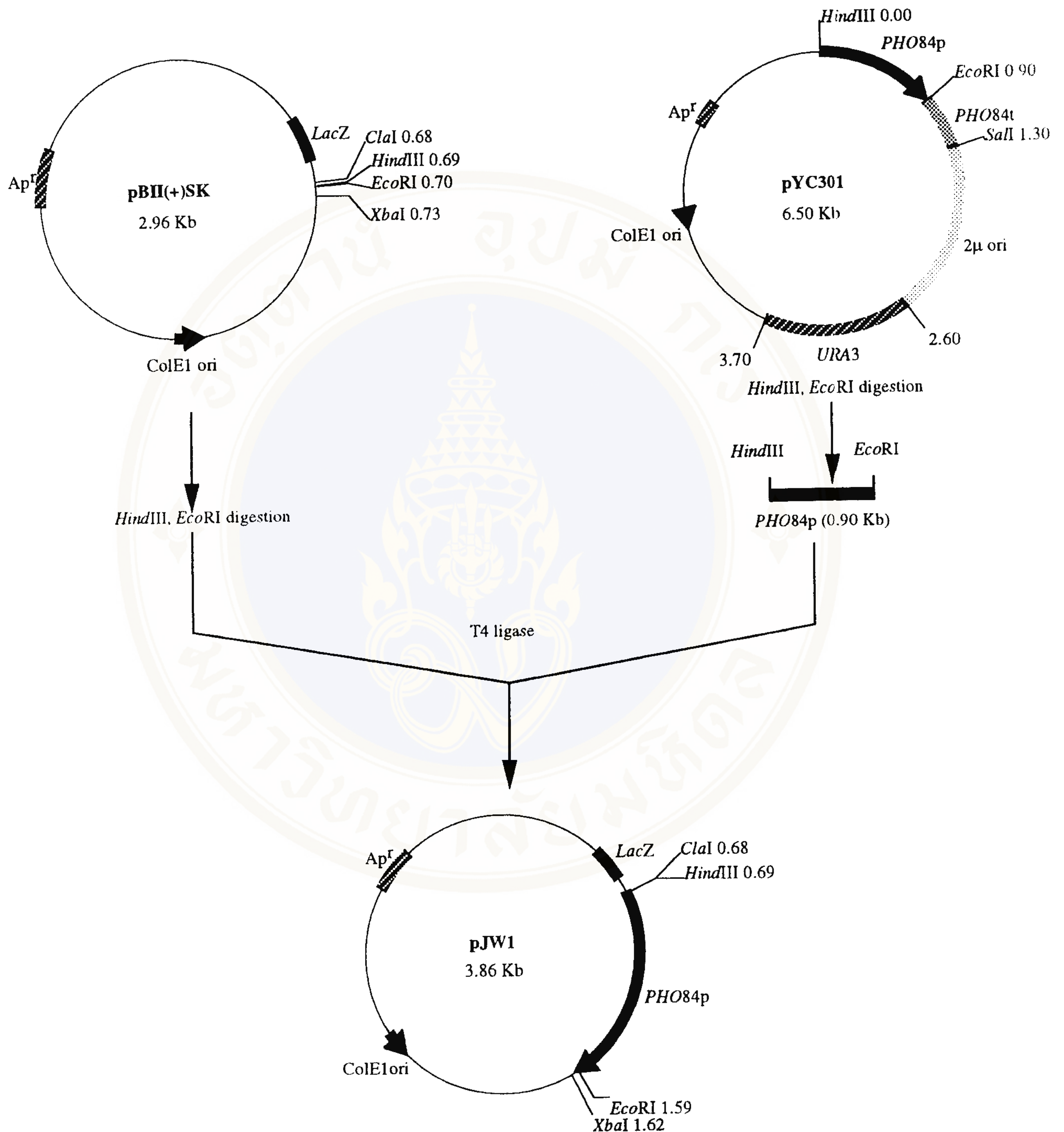

Figure 7. Construction diagram of pJW1 plasmid.

The 0.90 kb of the *PHO*84p gene fragment from plasmid pYC301 was inserted into *Hind*III and *Eco*RI sites of plasmid pBSII+SK to obtain plasmid pJW1.

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Table 10. The expected size of DNA bands of pYC301, pBSII+SK, pJW1, pTB326 and pJW2 plasmids cut with various enzymes.

Plasmid	Size (kb)	*Hind*III		*Hind*III + *Eco*RI		*Eco*RI + *Xba*I	
		No. of band	Expected size (kb)	No. of band	Expected size (kb)	No. of band	Expected size (kb)
pYC301	6.50	-	-	2	5.60, 0.90	-	-
pBSII+SK	2.96	-	-	2	2.95, 0.01	-	-
pJW1	3.86	-	-	2	2.96, 0.90	2	3.83, 0.03
pTB326	6.18	-	-	-	-	3	3.38,1.93, 0.86
pJW2	4.72	2	3.56, 1.16	-	-	2	3.86, 0.86

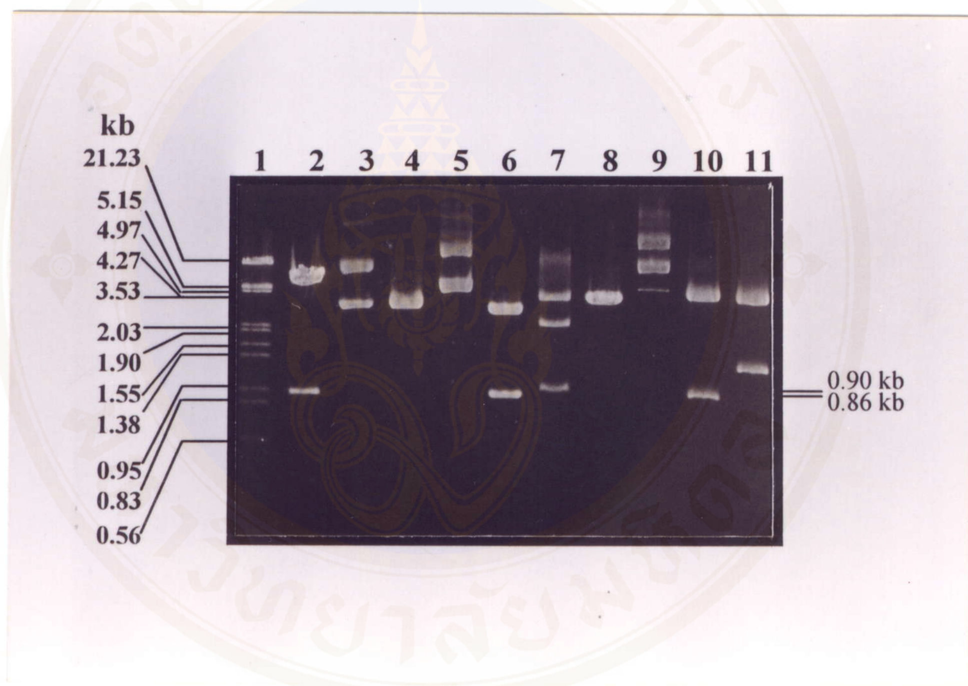

Figure 8. Restriction endonuclease analysis of pYC301, pBSII+SK, pJW1, pTB326 and pJW2 plasmids.

Lane 1. λ DNA cut with *Hind*III + *Eco*RI.
Lane 2. pYC301 cut with *Hind*III + *Eco*RI.
Lane 3. pBSII+SK uncut.
Lane 4. pBSII+SK cut with *Hind*III + *Eco*RI.
Lane 5. pJW1 uncut.
Lane 6. pJW1 cut with *Hind*III + *Eco*RI.
Lane 7. pTB326 cut with *Eco*RI + *Xba*I.
Lane 8. pJW1 cut with *Eco*RI + *Xba*I.
Lane 9. pJW2 uncut.
Lane 10. pJW2 cut with *Eco*RI + *Xba*I.
Lane 11. pJW2 cut with *Hind*III.

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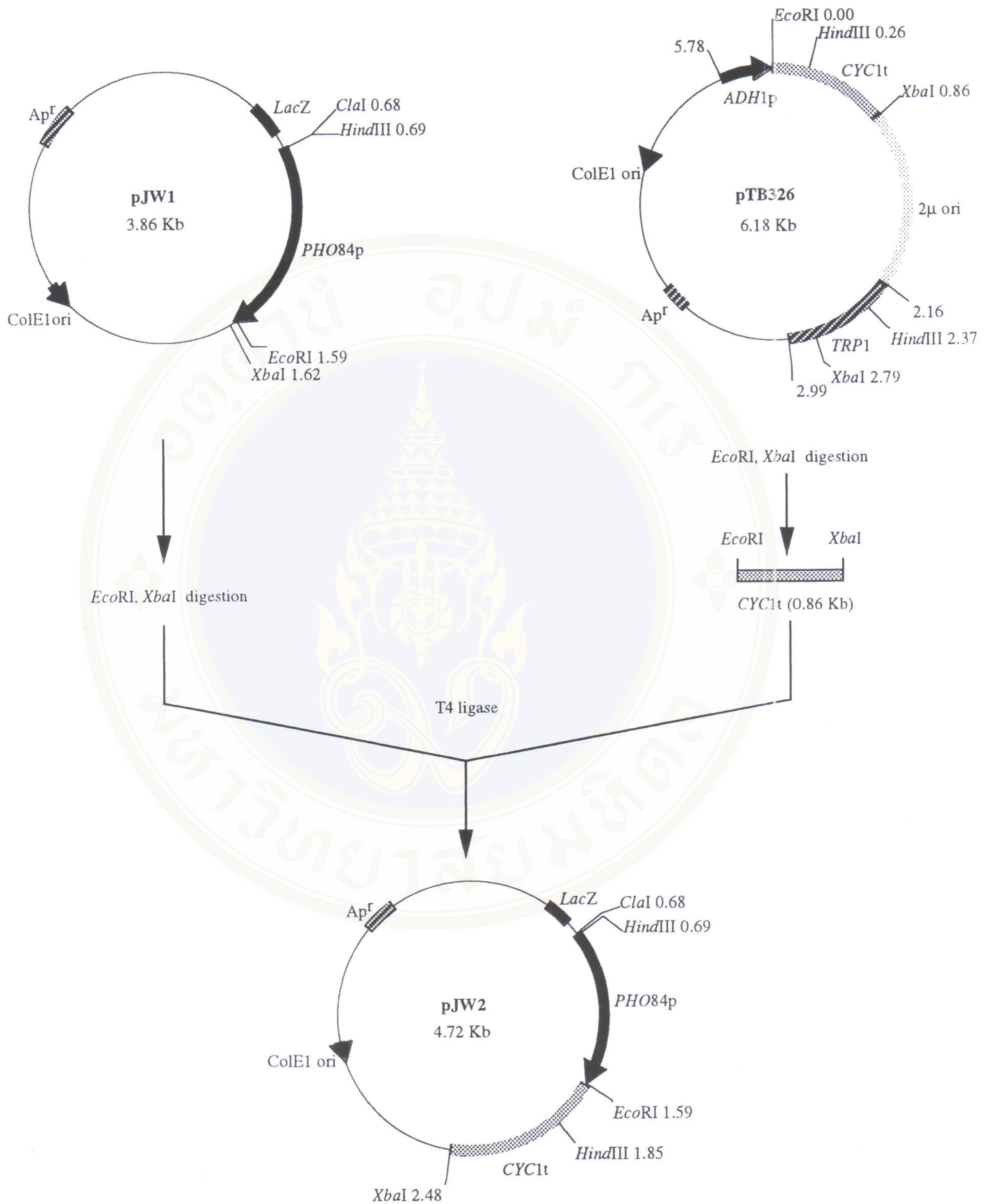

Figure 9. Construction diagram of pJW2 plasmid.

The 0.86 kb of the *CYC*1t gene fragment from plasmid pTB326 was inserted into *Eco*RI and *Xba*I sites at of plasmid pJW1 to obtain plasmid pJW2.

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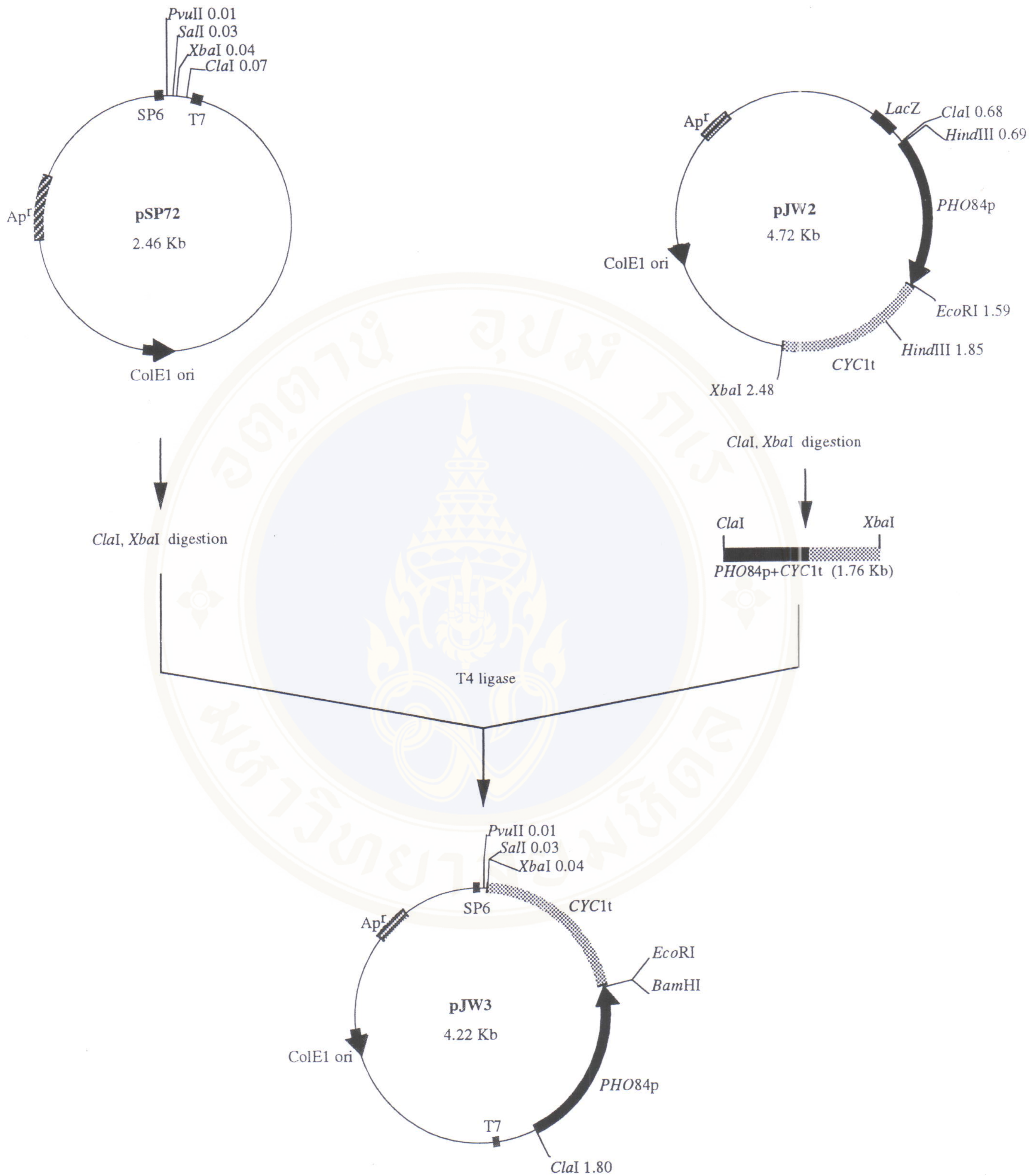

Figure 10. Construction diagram of pJW3 plasmid.

The 1.76 kb of the fragment containing the *PHO*84p and *CYC*1t gene from plasmid pJW2 was inserted into *Cla*I and *Xba*I sites of plasmid pSP72 to obtain plasmid pJW3.

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Table 11. The expected size of DNA bands of pSP72, pJW2 and pJW3 plasmids cut with various enzymes.

Plasmid	Size (kb)	*Cla*I + *Xba*I		*Eco*RI + *Xba*I		*Cla*I + *Sal*I		*Cla*I + *Pvu*II	
		No. of band	Expected size (kb)	No. of band	Expected size (kb)	No. of band	Expected size (kb)	No. of band	Expected size (kb)
pSP72	2.46	2	2.43, 0.03	-	-	-	-	-	-
pJW2	4.72	2	2.96, 1.76	-	-	-	-	-	-
pJW3	4.22	2	2.46, 1.76	2	3.36, 0.86	2	2.46, 1.76	2	2.44, 1.78

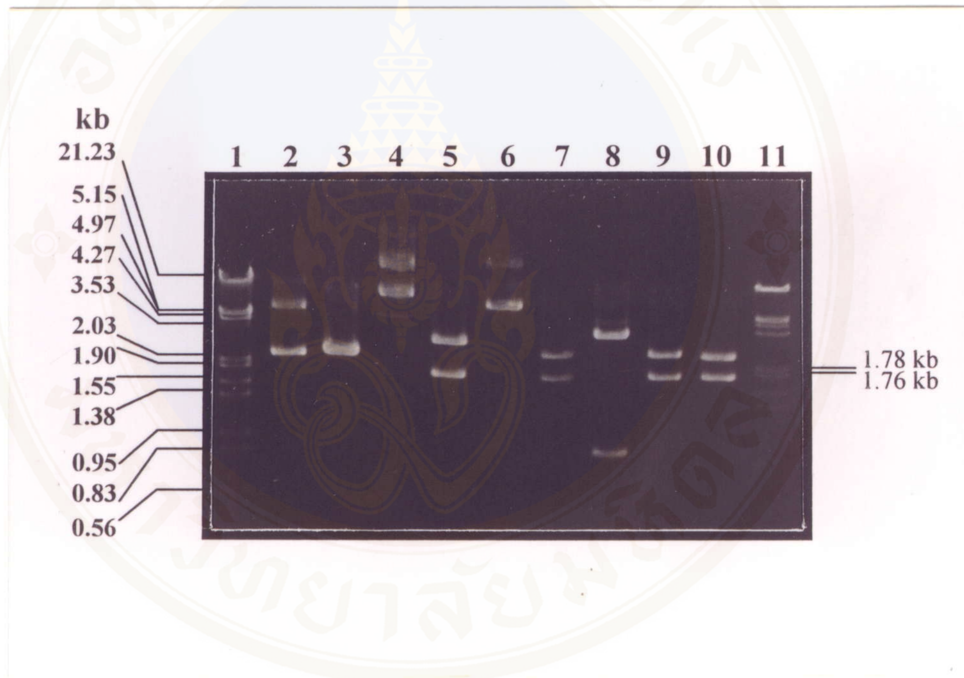

Figure 11. Restriction endonuclease analysis of pSP72, pJW2 and pJW3 plasmids.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pSP72 uncut.
Lane 3 pSP72 cut with *Cla*I + *Xba*I.
Lane 4 pJW2 uncut.
Lane 5 pJW2 cut with *Cla*I + *Xba*I.
Lane 6 pJW3 uncut.
Lane 7 pJW3 cut with *Cla*I + *Xba*I
Lane 8 pJW3 cut with *EcoRI* + *Xba*I.
Lane 9 pJW3 cut with *Cla*I + *Sal*I.
Lane 10 pJW3 cut with *Cla*I + *Pvu*II.
Lane 11 λ DNA cut with *Hind*III + *Eco*RI.

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Since the original plasmid pPM323 was rather large therefore the non-necessary part was eliminated. To construct the smaller plasmid, the *PHO*84p and *CYC*1t (1.78 kb) was prepared from pJW3 by *Cla*I and *Pvu*II double digestion. This fragment was inserted into plasmid pPM323 at corresponding sites to obtain vector pPM323-P3Δ which was also lacked part of *LEU*2d gene 1.26 kb. The construction diagram was shown in Figure 13.

Since the *LEU*2d gene in the plasmids pPM323-P1Δ and pPM323-P3Δ were not completed, therefore the *Cla*I fragment of *LEU*2d gene was religated to them at *Cla*I site to obtain plasmid pPM323-P1 and pPM323-P3, respectively. After religation, the new plasmids generated two bands when cleaved with *Cla*I. As shown in Figure 14 (lane 3 and 5), the upper bands about 7.49 and 6.07 kb were equivalent to pPM323P1Δ and pPM323-P3Δ, respectively. Whereas, the lower band about 1.26 kb was equivalent to the second band of the plasmid pPM323 cleaved with the same enzyme (lane 2). The orientation of *Cla*I fragment of *LEU*2d the two plasmids were analysed with *Kpn*I digestion, the right orientation gave the lower band about 2.08 kb as shown in Figure 15 (lane 2 and 4).

1.2.2 Construction of Plasmid pPM323-P2 and pPM323-P4

To obtain the expression plasmids with two selectable markers, the 1.10 kb fragment of *URA*3 gene generated from plasmid pPM323 by *Sal*I digestion was inserted into the vectors pPM323-P1 and pPM323-P3 at *Sal*I site to obtain plasmid pPM323-P2 and pPM323- P4. The construction diagrams were shown in Figure 16 and 17.

To confirm that the pPM323-P2 and pPM323-P4 plasmids contained the *URA*3 gene, they were cleaved with *Sal*I. As shown in Figure 18 (lane 4 and 6), the lower

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EcoRI 10.60
ClaI 0.02
EcoRI 9.83
KpnI 9.43
ClaI 9.34
ClaI 1.00
PGKp-PGKt
ClaI 1.75
2µ ori/LEU2d
pPM323
10.60 Kb
SalI 2.51
URA3
EcoRI 7.33
SalI 3.61
Ap^r ColE1 ori
PvuII 5.03

PvuII 0.01
SalI 0.03
XbaI 0.04
Ap^r
SP6
CYC1t
EcoRI
BamHI
pJW3
4.22 Kb
ColE1 ori
PHO84p
T7
ClaI 1.80

ClaI, SalI digestion
part of LEU2d (1.26 Kb) was deleted

ClaI, SalI digestion
ClaI SalI
PHO84p+CYC1t (1.76 Kb)

T4 ligase

pPM323-P1△ (7.49 Kb) + part of LEU2d (1.26 Kb)

ClaI digestion
T4 ligase

EcoRI 8.75
ClaI 0.02
EcoRI 7.98
PHO84p
EcoRI 0.90
KpnI 0.90
BamHI 0.90
KpnI 7.56
ClaI 7.45
CYC1t
XbaI 1.76
SalI 1.76
2µ ori/LEU2d
pPM323-P1
8.75 Kb
EcoRI 5.48
PvuII 3.18
ColE1 ori
Ap^r

Figure 12. Construction diagram of pPM323-P1 plasmid.

The 1.76 kb of the fragment containing the *PHO*84p and *CYC*1t gene from plasmid pJW3 was inserted into *Cla*I and *Sal*I sites of plasmid pPM323 to obtain plasmid pPM323-P1.

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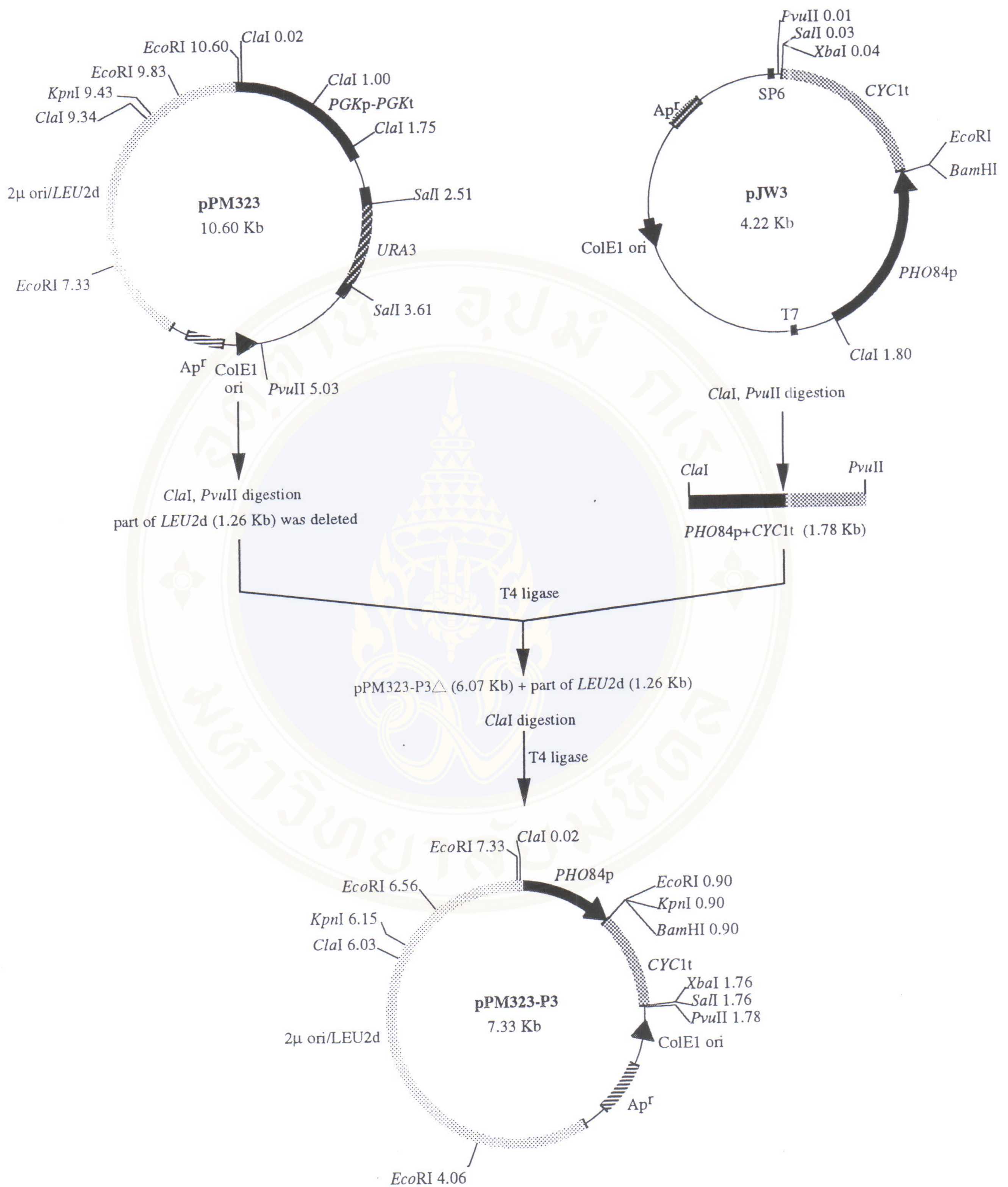

Figure 13. Construction diagram of pPM323-P3 plasmid.

The 1.78 kb of the fragment containing the *PHO*84p and *CYC*1t gene from plasmid pJW3 was inserted into *Cla*I and *Pvu*II sites of plasmid pPM323 to obtain plasmid pPM323-P3.

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Table 12. The expected size of DNA bands of pPM323, pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 plasmids cut with *Cla*I restriction enzyme.

Plasmid	Size (kb)	*Cla*I	
		No. of band	Expected size (kb)
pPM323	10.60	4	7.61, 1.26, 0.98, 0.75
pPM323-P1	8.75	2	7.49, 1.26
pPM323-P2	9.85	2	8.59, 1.26
pPM323-P3	7.33	2	6.07, 1.26
pPM323-P4	8.43	2	7.17, 1.26

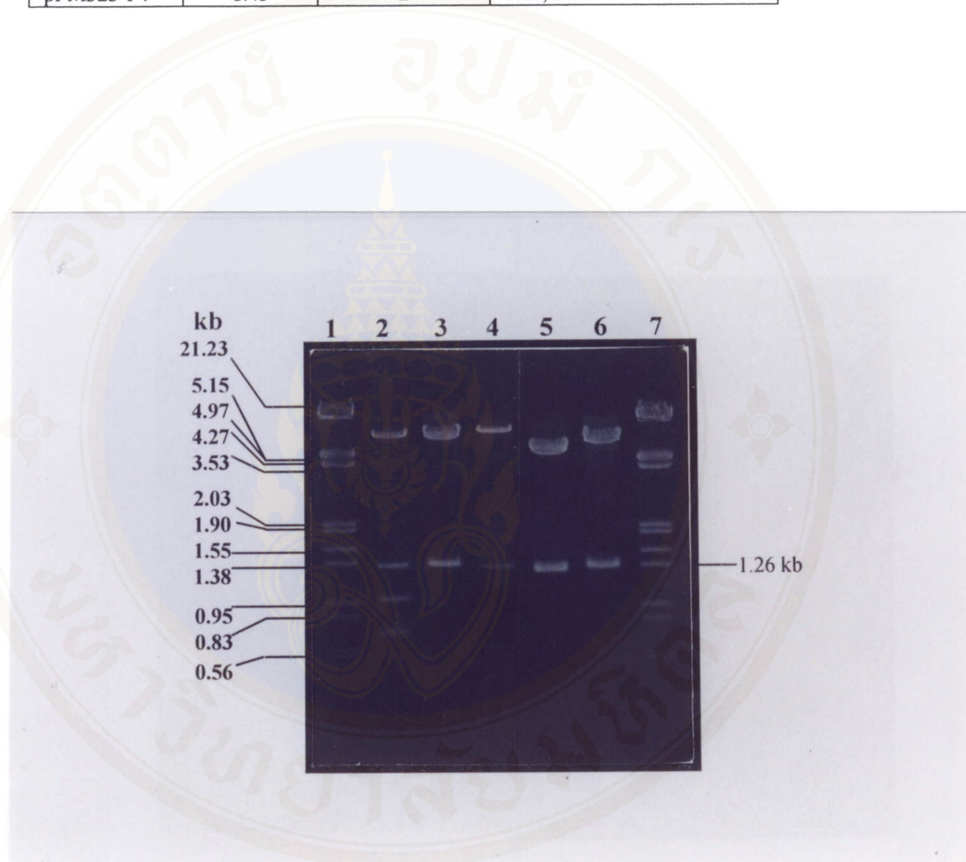

Figure 14. Restriction endonuclease analysis of pPM323, pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 plasmids.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pPM323 cut with *Cla*I.
Lane 3 pPM323-P1 cut with *Cla*I.
Lane 4 pPM323-P2 cut with *Cla*I.
Lane 5 pPM323-P3 cut with *Cla*I.
Lane 6 pPM323-P4 cut with *Cla*I.
Lane 7 λ DNA cut with *Hind*III + *Eco*RI.

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Table 13. The expected size of DNA bands of pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 plasmids cut with *Kpn*I restriction enzyme .

Plasmid	Size (kb)	*Kpn*I	
		No. of band	Expected size (kb)
pPM323-P1	8.75	2	6.67, 2.08
pPM323-P2	9.85	2	7.77, 2.08
pPM323-P3	7.33	2	5.25, 2.08
pPM323-P4	8.43	2	6.35, 2.08

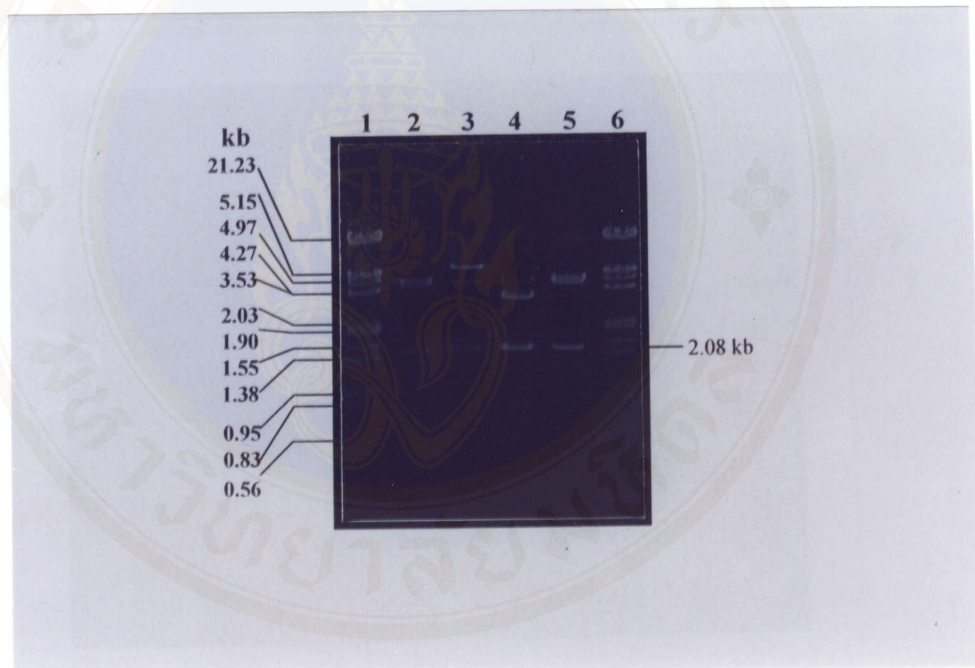

Figure 15. Restriction endonuclease analysis of pPM323, pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 plasmids.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pPM323-P1 cut with *Kpn*I.
Lane 3 pPM323-P2 cut with *Kpn*I.
Lane 4 pPM323-P3 cut with *Kpn*I.
Lane 5 pPM323-P4 cut with *Kpn*I.
Lane 6 λ DNA cut with *Hind*III + *Eco*RI.

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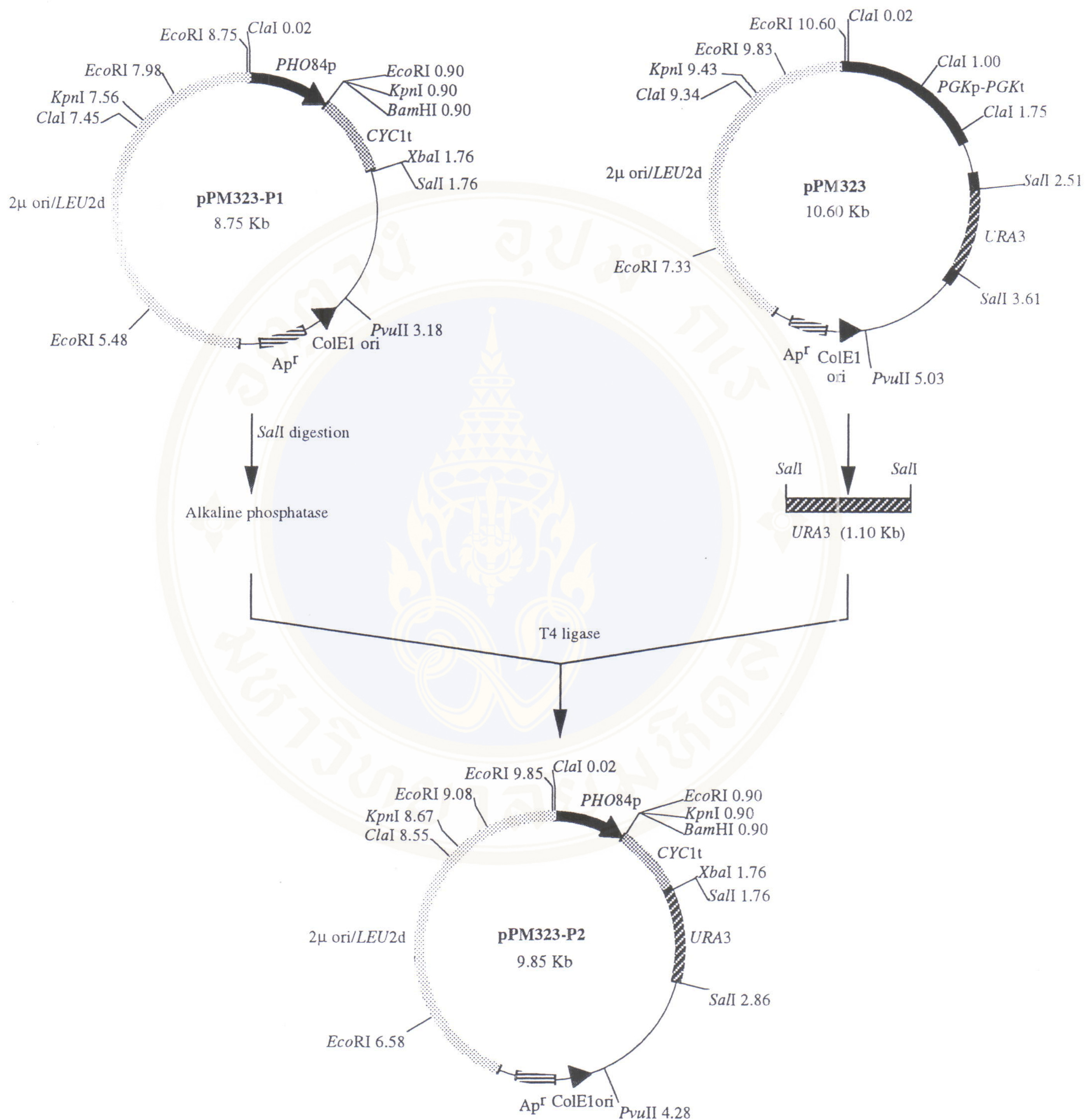

Figure 16. Construction diagram of pPM323-P2 plasmid.

The 1.10 kb of the *URA3* gene fragment from pPM323 was inserted into *Sal*I site of plasmid pPM323-P1 to obtain plasmid pPM323-P2.

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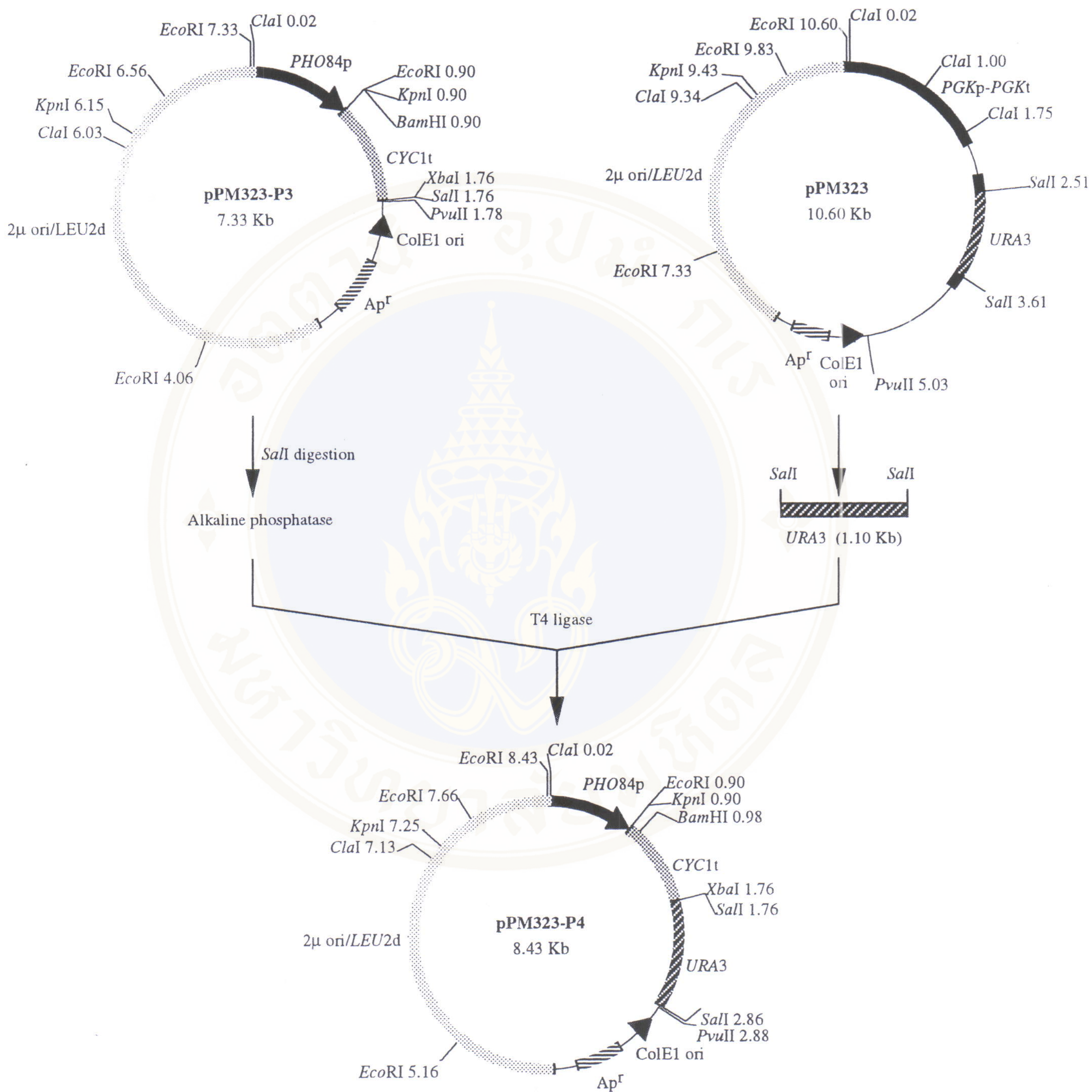

Figure 17. Construction diagram of pPM323-P4 plasmid.

The 1.10 kb of the *URA3* gene fragment from pPM323 was inserted into *Sal*I site of plasmid pPM323-P3 to obtain plasmid pPM323-P4.

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Table 14. The expected size of DNA bands of pPM323, pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 plasmids cut *Sal*I restriction enzyme.

Plasmid	Size (kb)	*Sal*I	
		No. of band	Expected size (kb)
pPM323	10.60	2	9.5, 1.10
pPM323-P1	8.75	1	8.75
pPM323-P2	9.85	2	8.75, 1.10
pPM323-P3	7.33	1	7.33
pPM323-P4	8.43	2	7.33, 1.10

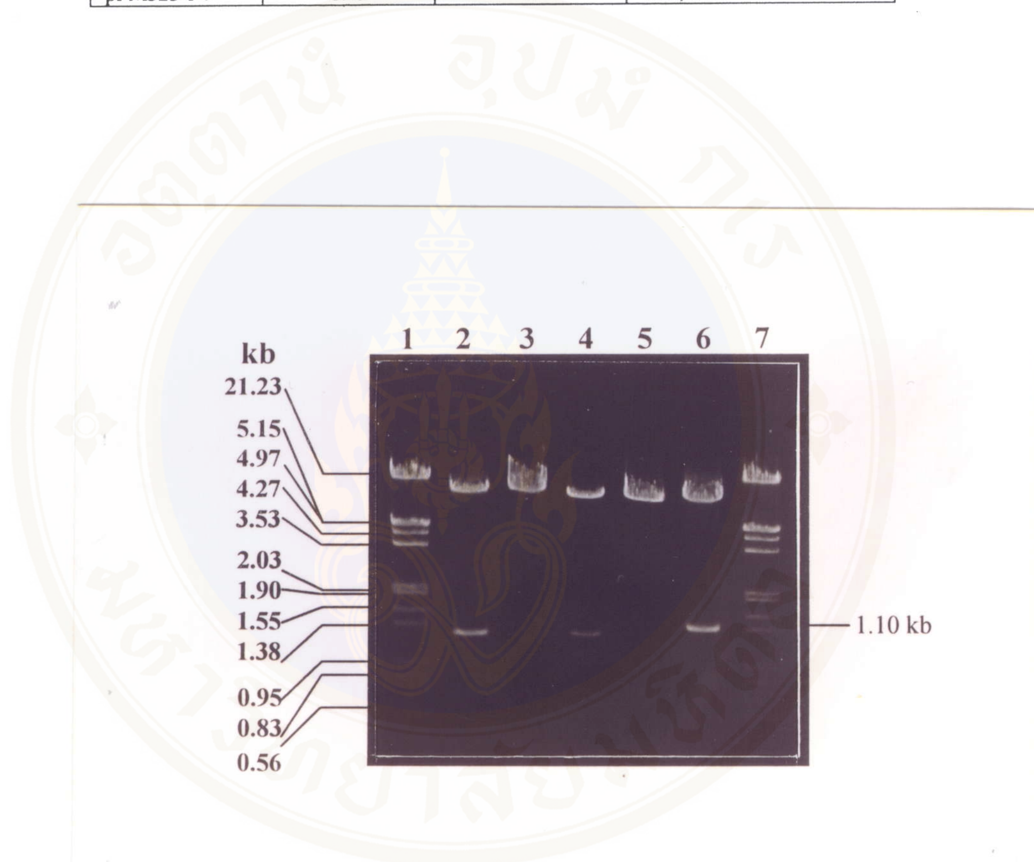

Figure 18. Restriction endonuclease analysis of pPM323, pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 plasmids.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pPM323 cut with *Sal*I.
Lane 3 pPM323-P1 cut with *Sal*I.
Lane 4 pPM323-P2 cut with *Sal*I.
Lane 5 pPM323-P3 cut with *Sal*I.
Lane 6 pPM323-P4 cut with *Sal*I.
Lane 7 λ DNA cut with *Hind*III + *Eco*RI.

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bands were equivalent to the lower band which was corresponding to *URA*3 gene of the plasmid pPM323 cleaved with the same enzyme (lane 2).

The construction diagram was shown in Figure 16 and 17. The presence of *LEU*2d gene fragment in the plasmid pEB-P2 and pEB-P4 were also confirmed by *Cla*I digestion, as shown in Figure 14 (lane 4 and 6), and *Kpn*I digestion as shown in Figure 15 (lane 3 and 5).

1.2.3 Confirmation of Plasmid pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4

The presence of the expression cassette containing *PHO*84p and *CYC*1t in the plasmid pPM323-P1 and pPM323-P2 were confirmed by *Cla*I and *Sal*I digestion. The pPM323-P1 generated three bands about 5.73, 1.76 and 1.26 kb as shown in Figure 19 (lane 8). The middle band was corresponded to the lower band of the plasmid pJW3 (lane 5) cleaved with the same enzymes.

For the pPM323-P2 plasmid, it generated four bands about 5.73, 1.76, 1.26 and 1.10 kb as shown in Figure 20 (lane 5). The second band from top (lane 4) was corresponded to the lower band of plasmid pJW3, containing *PHO*84-*CYC*1t (lane 2) digested with the same enzymes. The presence of the expression cassette in the plasmids pPM323-P3 and pPM323-P4 were confirmed by *Cla*I and *Pvu*II digestion. Plasmid pPM323-P3 generated three bands about 4.29, 1.78 and 1.26 kb, as shown in Figure 19 (lane 10) whereas, plasmid pPM323-P4 generated three bands about 4.29, 2.88 and 1.26 kb, as shown in Figure 20 (lane 7). The middle band was the combination of 1.78 kb from pJW3, containing *PHO*84-*CYC*1t and 1.10 kb *URA*3 fragment.

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Table 15. The expected size of DNA bands of pPM323, pJW3, pPM323-P1 and pPM323-P3 plasmids cut with various enzymes.

Plasmid	Size (kb)	*Eco*RI		*Cla*I + *Sal*I		*Cla*I + *Pvu*II	
		No. of band	Expected size (kb)	No. of band	Expected size (kb)	No. of band	Expected size (kb)
pPM323	10.60	3	7.33, 2.50, 0.77	5	5.76, 1.26, 1.10, 0.98, 0.75	5	4.33, 3.28, 1.26, 0.98, 0.75
pJW3	4.22	-	-	2	2.46, 1.76	2	2.44, 1.78
pPM323-P1	8.75	4	4.58, 2.50, 0.90, 0.77	3	5.73, 1.76, 1.26	-	-
pPM323-P3	7.33	4	3.16, 2.50, 0.90, 0.77	-	-	3	4.29, 1.78, 1.26

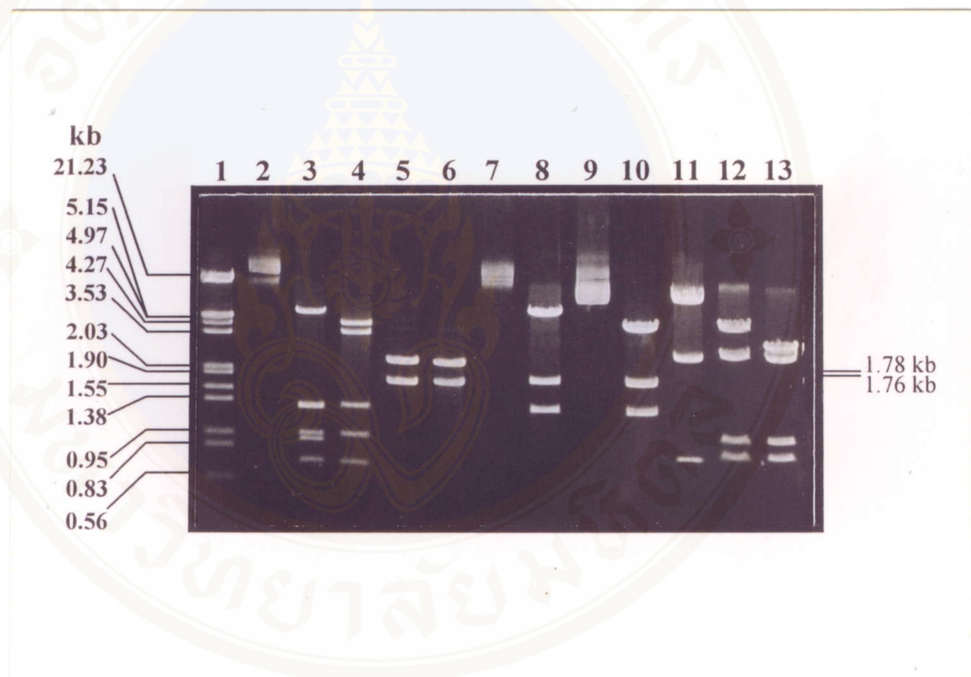

Figure 19. Restriction endonuclease analysis of pJW3, pPM323, pPM323-P1 and pPM323-P3 plasmids.

Lane 1λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pPM323 uncut.
Lane 3 pPM323 cut with *Cla*I + *Sal*I.
Lane 4 pPM323 cut with *Cla*I + *Pvu*II.
Lane 5 pJW3 cut with *Cla*I + *Sal*I.
Lane 6 pJW3 cut with *Cla*I + *Pvu*II.
Lane 7 pPM323-P1 uncut
Lane 8 pPM323-P1 *Cla*I + *Sal*I.
Lane 9 pPPM323-P3 uncut.
Lane 10 pPM323-P3 cut with *Cla*I + *Pvu*II.
Lane 11 pPM323 cut with *Eco*RI.
Lane 12 pPM323-P1 cut with *Eco*RI.
Lane 13 pPM323-P3 cut with *Eco*RI.

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Table 16. The expected size of DNA bands of pJW3, pPM323, pPM323-P2 and pPM323-P4 plasmids cut with various enzymes.

Plasmid	Size (kb)	*Eco*RI		*Cla*I + *Sal*I		*Cla*I + *Pvu*II	
		No. of band	Expected size (kb)	No. of band	Expected size (kb)	No. of band	Expected size (kb)
pPM323	10.60	3	7.33, 2.50, 0.77	-	-	-	-
pJW3	4.22	-	-	2	2.46, 1.76	2	2.44, 1.78
pPM323-P2	9.85	4	5.68, 2.50, 0.90, 0.77	4	5.73, 1.76, 1.26, 1.10	-	-
pPM323-P4	9.43	4	4.26, 2.50, 0.90, 0.77	-	-	3	4.29, 2.88, 1.26

Figure 20. Restriction endonuclease analysis of pJW3, pPM323, pPM323-P2 and pPM323-P4 plasmids.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pJW3 cut with *Cla*I + *Sal*I.
Lane 3 pJW3 cut with *Cla*I + *Pvu*II.
Lane 4 pPM323-P2 uncut.
Lane 5 pPM323-P2 cut with *Cla*I + *Sal*I.
Lane 6 pPM323-P4 uncut.
Lane 7 pPM323-P4 cut with *Cla*I + *Pvu*II.
Lane 8 pPM323 cut with *Eco*RI.
Lane 9 pPM323-P2 cut with *Eco*RI.
Lane 10 pPM323-P3 cut with *Eco*RI.
Lane 11 λ DNA cut with *Hind*III + *Eco*RI.

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Confirmation was further done by digestion with *Eco*RI. The pPM323-P1 and pPM323-P3 plasmids gave four bands about 4.58, 2.50, 0.90 and 0.77 kb and 3.16, 2.50, 0.90 and 0.77 kb, respectively as shown in Figure 19 (lane 12-13). The pPM323-P2 and pPM323-P4 plasmids gave four band about 5.68, 2.50, 0.98 and 0.77 kb, and 4.26, 2.5, 0.98 and 0.77 kb, respectively as shown in Figure 20 (lane 9-10). The results of restriction analysis were summarized in Table 15 and 16.

2. Construction of Plasmid for Expression of the PreS2d+S Gene in Yeast

To clone the PreS2d+S gene into the yeast expression plasmids, pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4, plasmid pSF5-1 was cleaved with *Bam*HI to isolate a 0.90 kb fragment of the PreS2d+S gene. The PreS2d+S gene fragment was inserted into the four plasmids at *Bam*HI site. The newly constructed plasmids were name pEB-P1, pEB-P2, pEB-P3 and pEB-P4, respectively. The construction diagrams of these new plasmids were illustrated in Figure 21, 22, 23 and 24, respectively.

To confirm that they contained the PreS2d+S gene and the insertion of the gene was in the right orientation to promoter, they were digested with *Bam*HI, and *Xba*I, respectively.

For the pEB-P1 plasmid, digestion with *Bam*HI gave two bands about 8.75 and 0.90 kb as shown in Figure 25 (lane 7). The upper band was corresponding to the plasmid pPM323-P1 (lane 2) and the lower band was corresponding to the lower band of plasmid pSF5-1 (lane 6) digested with the same enzymes.

For the pEB-P2 plasmid, digestion with *Bam*HI generated two bands about 9.85 and 0.90 kb as shown in Figure 25 (lane 8). The upper band was corresponding to the

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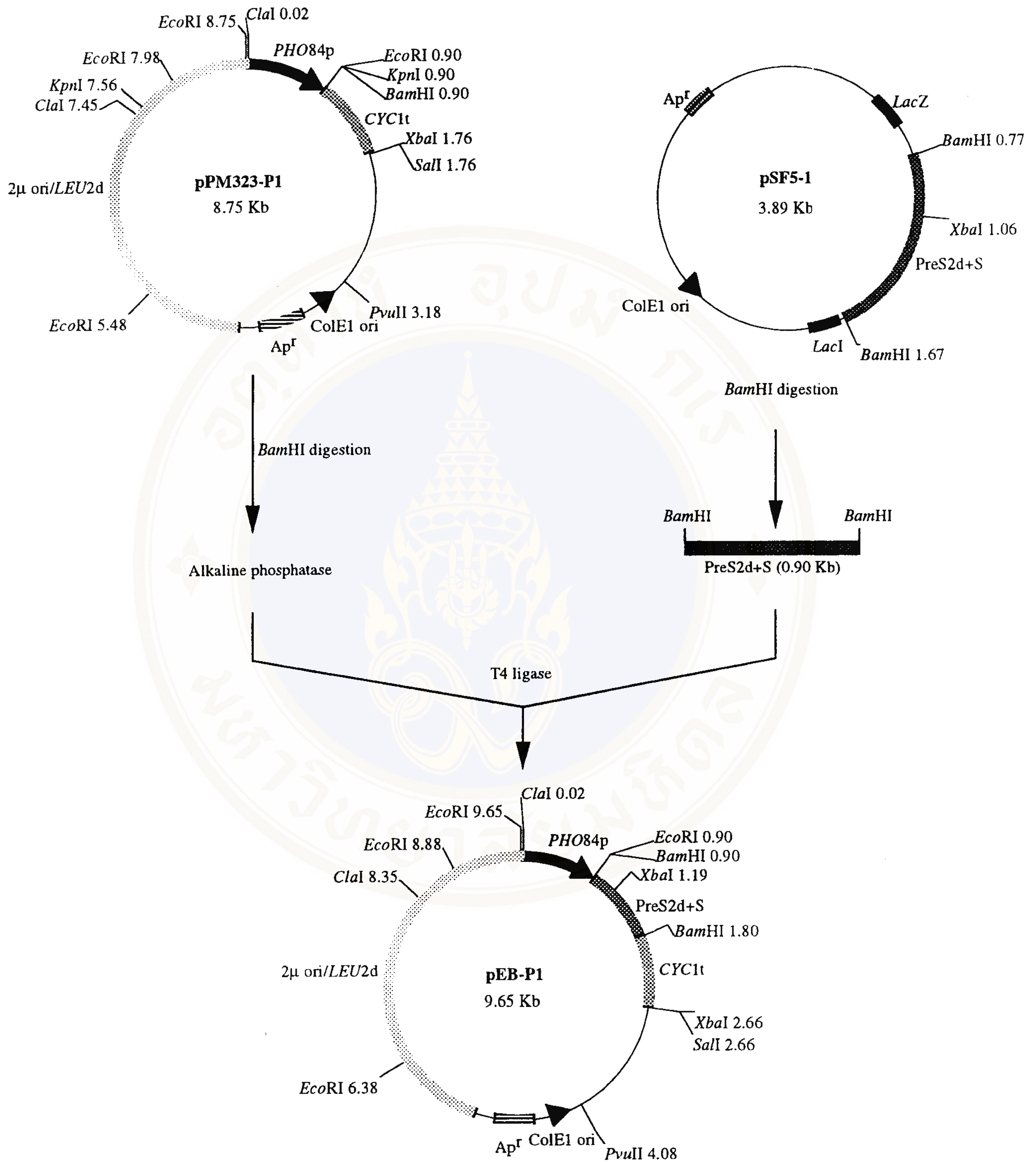

Figure 21. Construction diagram of pEB-P1 plasmid.

The 0.90 kb of the *Bam*HI-fragment containing the PreS2d+S gene from plasmid pSF5-1 was inserted into *Bam*HI site of plasmid pPM323-P1 to obtain plasmid pEB-P1.

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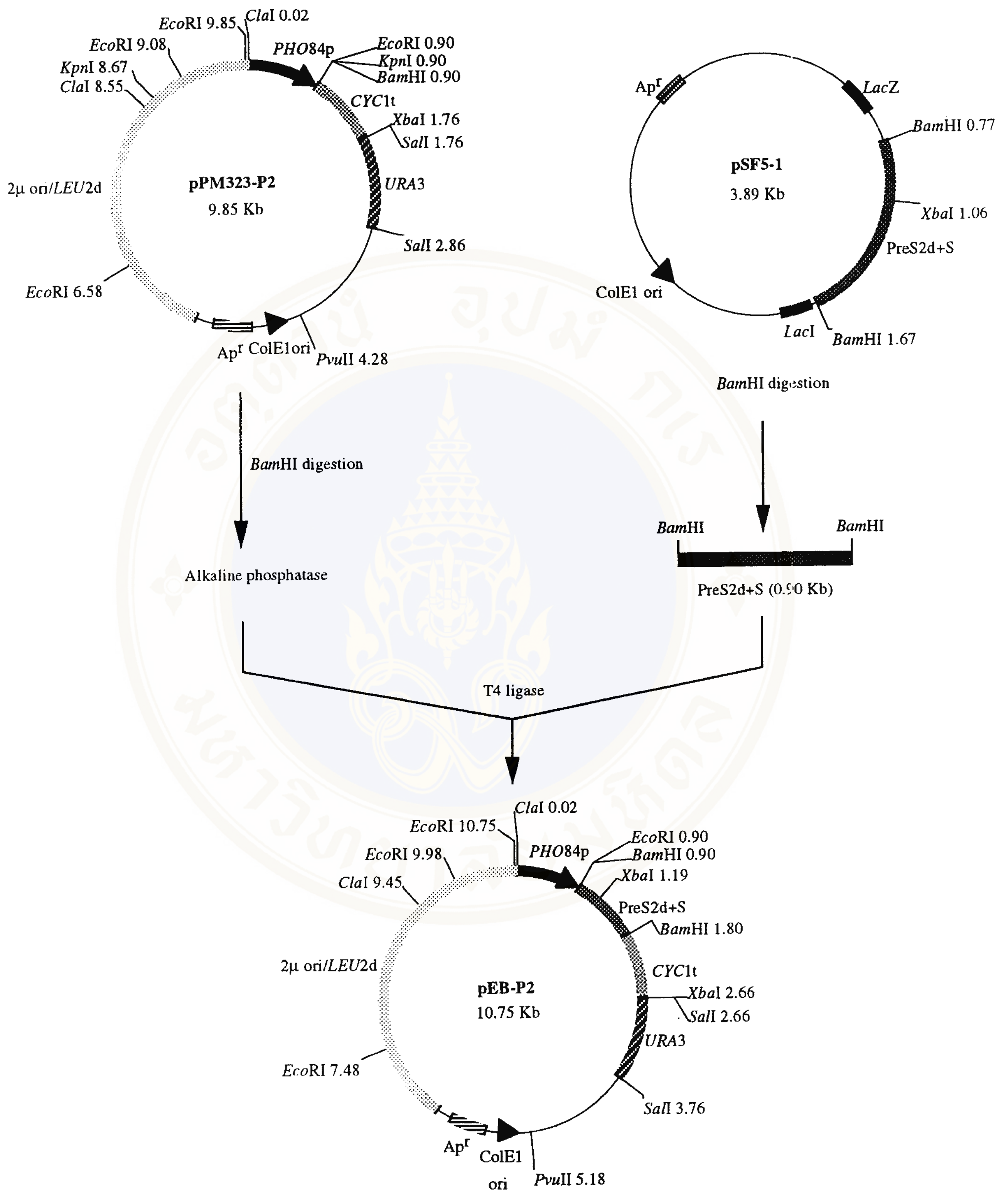

Figure 22. Construction diagram of pEB-P2 plasmid.

The 0.90 kb of the *Bam*HI-fragment containing the PreS2d+S gene from plasmid pSF5-1 was inserted into *Bam*HI site of plasmid pPM323-P2 to obtain plasmid pEB-P2.

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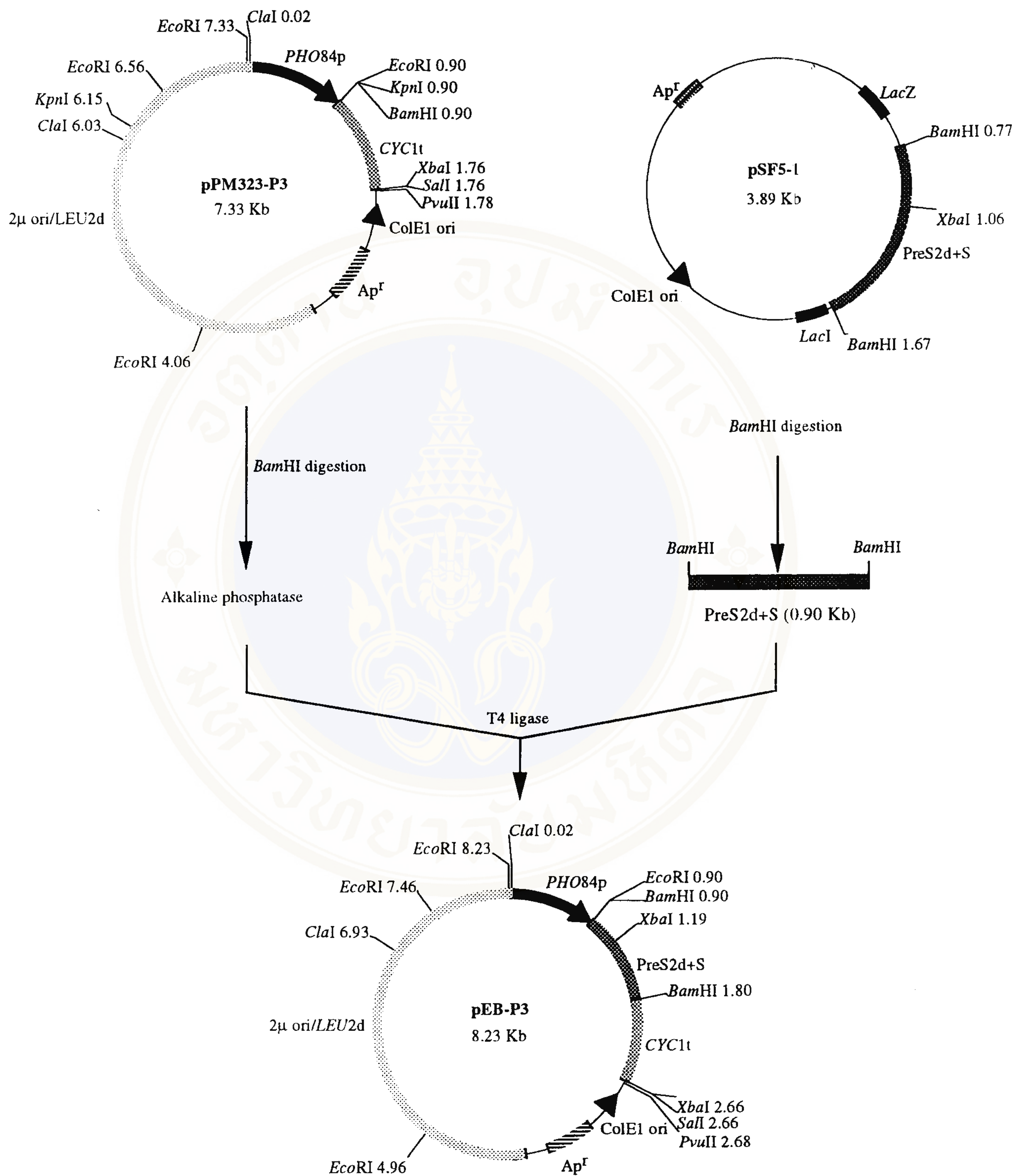

Figure 23. Construction diagram of pEB-P3 plasmid.

The 0.90 kb of the *Bam*HI-fragment containing the PreS2d+S gene from plasmid pSF5-1 was inserted into *Bam*HI site of plasmid pPM323-P3 to obtain plasmid pEB-P3.

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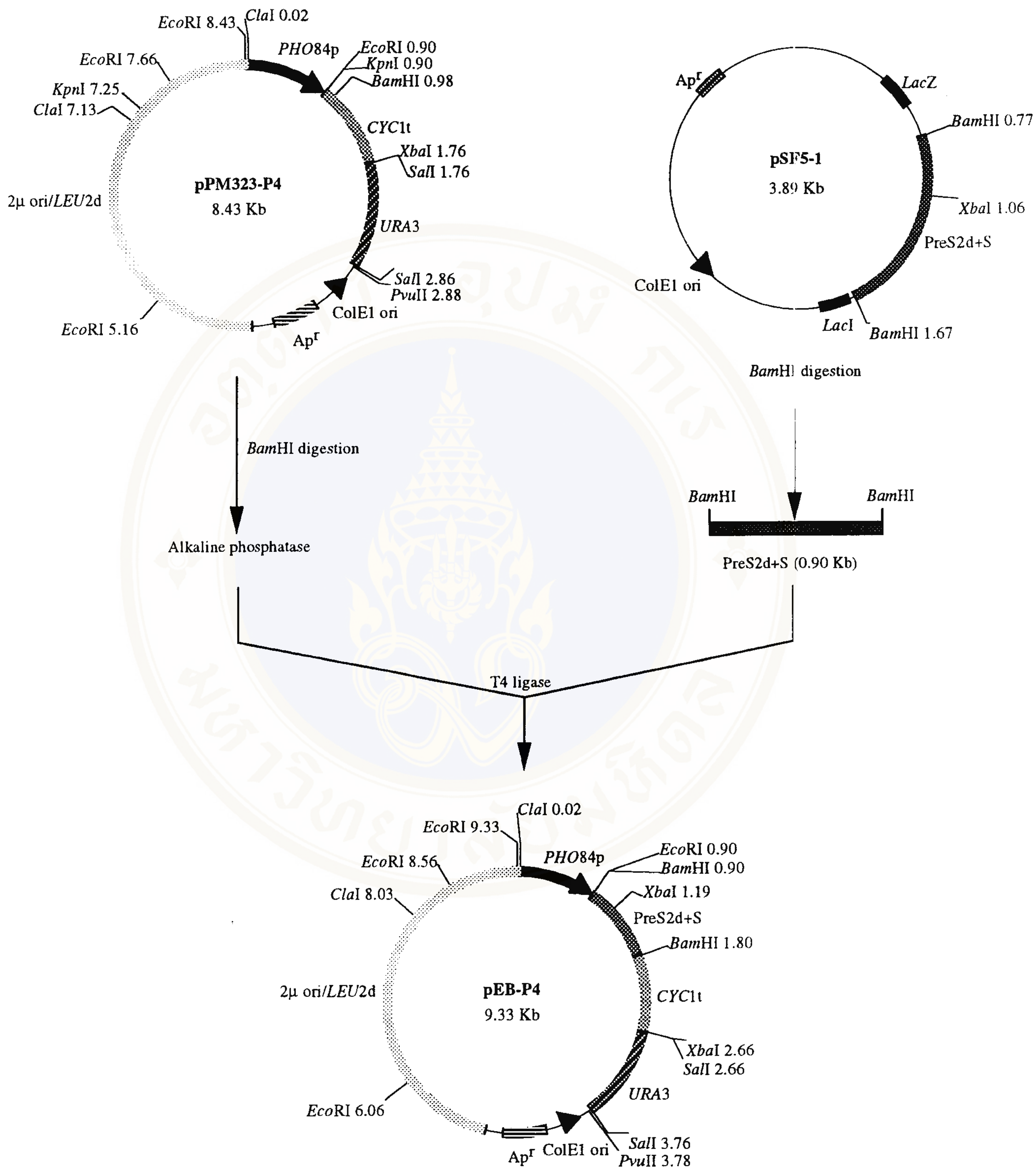

Figure 24. Construction diagram of pEB-P4 plasmid.

The 0.90 kb of the *Bam*HI-fragment containing the PreS2d+S gene from plasmid pSF5-1 was inserted into *Bam*HI site of plasmid pPM323-P4 to obtain plasmid pEB-P4.

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plasmid pPM323-P2 (lane 3) and the lower band was corresponding to the lower band of plasmid pSF5-1 (lane 6) digested with the same enzymes.

For the pEB-P3 plasmid, digestion with *Bam*HI obtained two bands about 7.33 and 0.9 kb as shown in Figure 25(lane 9). The upper band was corresponding to the plasmid pPM323-P3 (lane 4) and the lower band was corresponding to the lower band of plasmid pSF5-1 digested with the same enzymes (lane 6).

For the pEB-P4 plasmid, digestion with *Bam*HI gave two bands about 8.43 and 0.9 kb as shown in Figure 25 (lane 10). The upper band was corresponding to the plasmid pPM323-P4 (lane 5) and the lower band was corresponding to the lower band of plasmid pSF5-1 digested with the same enzymes (lane 6).

For the right orientation of the PreS2d+S gene in pEB-P1, pEB-P2, pEB-P3 and pEB-P4 plasmids, digestion the plasmids with *Xba*I generated two bands about 8.18 and 1.47 kb, 9.28 and 1.47 kb, 6.76 and 1.47 kb, 7.86 and 1.47 kb, respectively, as shown in Figure 26 (lane 3-6).

3. Construction of Recombinant Yeast Harboring the PreS2d+S

After the expression plasmids harboring the PreS2d+S gene, pEB-P1 (*LEU*2d marker), pEB-P2 (*LEU*2d and *URA*3 markers), pEB-P3 (*LEU*2d marker) and pEB-P4 (*LEU*2d and *URA*3 markers) were constructed in *E. coli*, the plasmid DNA was extracted by alkaline lysis method and transformed into *S. cerevisiae* by lithium acetate procedure. The host strain BJ5462 required leucine, trptophan and uracil as growth supplements, therefore the transformants harboring plasmids pEB-P1 and pEB-P3 were selected on SD agar supplemented with tryptophan and uracil (SD+trp+ura). Whereas the transformants harboring plasmids pEB-P2 and pEB-P4 were selected on SD agar

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Table 17. The expected size of DNA bands of pSF5-1, pPM323-P1, pPM323-P2, pPM323-P3, pPM323-P4, pEB-P1, pEB-P2, pEB-P3 and pEB-P4 plasmids cut with *Bam*HI restriction enzyme.

Plasmid	Size (kb)	*Bam*HI	
		No. of band	Expected size (kb)
pSF5-1	3.89	2	2.99, 0.90
pPM323-P1	8.75	1	8.75
pPM323-P2	9.85	1	9.85
pPM323-P3	7.33	1	7.33
pPM323-P4	8.43	1	8.43
pEB-P1	9.65	2	8.75, 0.90
pEB-P2	10.75	2	9.85, 0.90
pEB-P3	8.23	2	7.33, 0.90
pEB-P4	9.33	2	8.43, 0.90

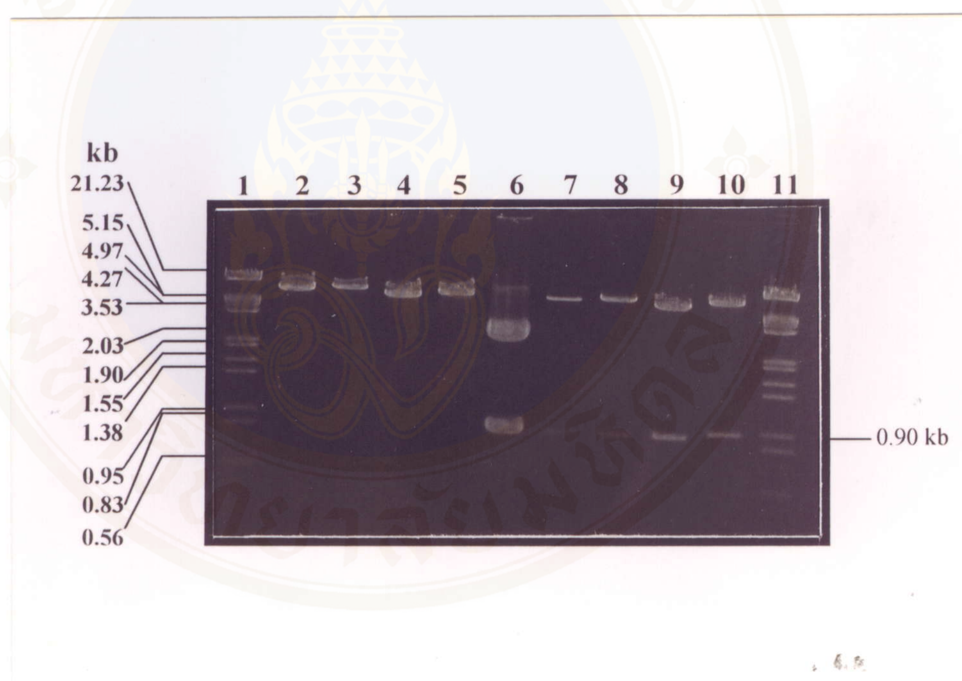

Figure 25. Restriction endonuclease analysis of pPM323-P1, pPM323-P2, pPM323-P3, pPM323-P4, pEB-P1, pEB-P2, pEB-P3 and pEB-P4 plasmids.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pPM323-P1 cut with *Bam*HI..
Lane 3 pPM323-P2 cut with *Bam*HI.
Lane 4 pPM323-P3 cut with *Bam*HI.
Lane 5 pPM323-P4 cut with *Bam*HI.
Lane 6 pSF5-1 cut with *Bam*HI
Lane 7 pEB-P1 cut with *Bam*HI.
Lane 8 pEB-P2 cut with *Bam*HI.
Lane 9 pEB-P3 cut with *Bam*HI.
Lane 10 pEB-P4 cut with *Bam*HI.
Lane 11 λ DNA cut with *Hind*III + *Eco*RI.

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Table 18. The expected size of DNA bands of pEB-P1, pEB-P2, pEB-P3 and pEB-P4 plasmids cut with *Xba*I restriction enzyme.

Plasmid	Size (kb)	*Xba*I	
		No. of band	Expected size (kb)
pEB-P1	9.65	2	8.18, 1.47
pEB-P2	10.75	2	9.28, 1.47
pEB-P3	8.23	2	6.76, 1.47
pEB-P4	9.33	2	7.86, 1.47

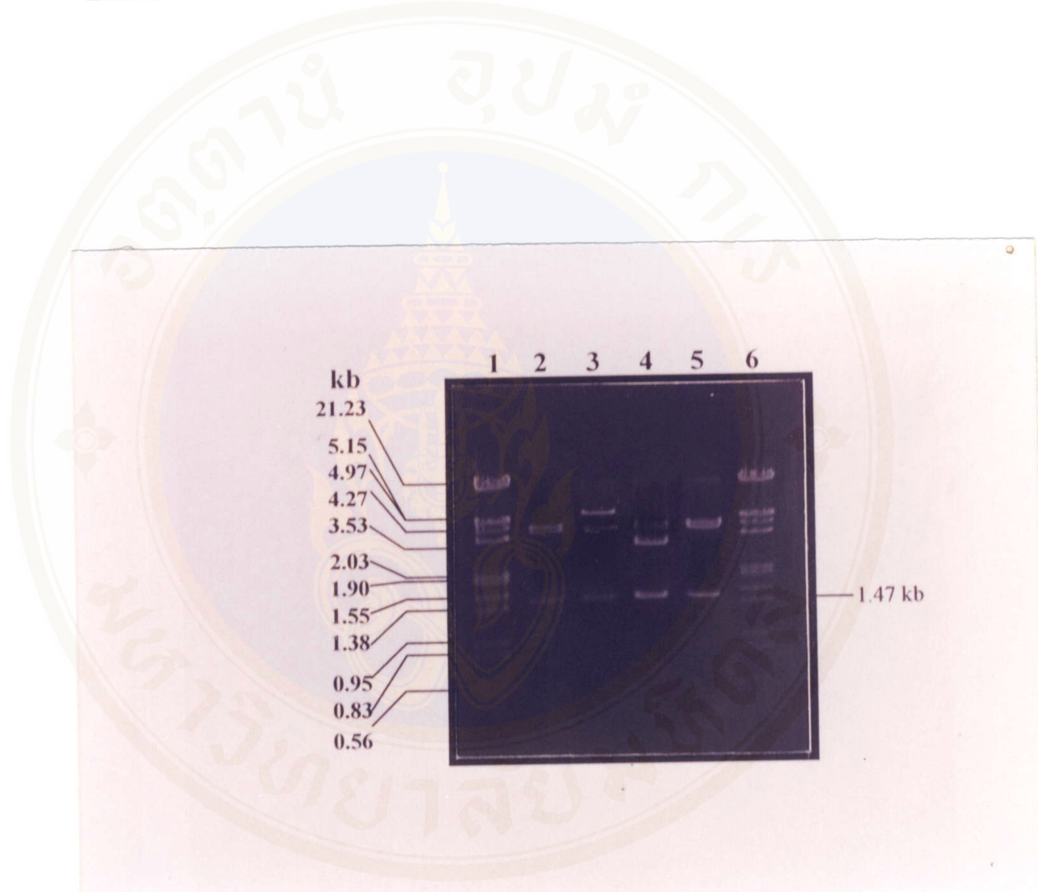

Figure 26. Restriction endonuclease analysis of pEB-P1, pEB-P2, pEB-P3 and pEB-P4 plasmids.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.
Lane 2 pEB-P1 cut with *Xba*I.
Lane 3 pEB-P2 cut with *Xba*I.
Lane 4 pEB-P3 cut with *Xba*I.
Lane 5 pEB-P4 cut with *Xbal*I.
Lane 6 λ DNA cut with *Hind*III + *Eco*RI.

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supplemented with only tryptophan (SD+trp). The corresponding plasmids, pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 were also transformed into the same yeast strain. Transformants were selected on SD agar plate as those for recombinant plasmids, pEB-P1, pEB-P2, pEB-P3 and pEB-P4, respectively. The yeast transformants showing big colony were selected after incubation for 3-4 days at 30 °C and confirmed for auxotrophic marker analysis by replica plating. At least ten transformants harboring each plasmid were selected for single colony isolation and further analysis.

The selection of transformants for further analysis was done by comparing the colony size of each transformants (data not shown). The recombinant yeasts with PreS2d+S gene of BJ5462 host with pEB-P series were designated as recombinant yeast P1B1 to P1B10 (pEB-P1), P2B1 to P2B2 (pEB-P2), P3B1 to P3B10 (pEB-P3) and P4B1 to P4B10 (pEB-P4). The recombinant yeast harboring vectors pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 were named PM1B1, PM2B1, PM3B1 and PM4B1, respectively.

4. Confirmation of Yeast Transformants

To confirm the presence of the PreS2d+S gene in the recombinant yeast, back transformation of the yeast DNA into *E. coli* was performed. Total yeast DNA was extracted by the yeast DNA minipreparation method and then transformed into *E. coli* strain DH5α. The *E. coli* transformants were selected on LB plate with ampicillin. The plasmids from *E. coli* were extracted by the rapid boiling method and the extracted DNA was analysed by restriction endonuclease digestion by *Bam*HI. As shown in Figure 27, 28, 29 and 30, the DNA extracts from recombinant yeasts series P1B, P2B,

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P3B and P4B (eight transformants each) gave the same DNA pattern as their origin expression plasmids, pEB-P1, pEB-P2, pEB-P3 and pEB-P4, respectively (lane 2). The DNA extract from the recombinant yeast gave two bands of about 8.75 and 0.90 kb, 9.85 and 0.90 kb, 7.33 and 0.90 kb and 8.43 and 0.90 kb (lane 3-10), respectively.

5. Screening of the Recombinant Yeast with High M HBsAg Production

To screen the recombinant yeast with high PreS2d+S gene expression, dot blot ELISA was performed. Fourty big colonies of the recombinant yeasts P1B, P2B, P3B and P4B series (10 colonies each) were screened. Single colony of recombinant yeast grown on SD agar supplemented with a requirement amino acid, at 30 °C for 3-4 days was employed as an inoculum in 10 ml High-Pi medium supplemented with required amino acids for cultivation of the preculture (Hiraoka *et al.*, 1994). The preculture was allowed to grow at 30 °C with vigorous shaking for 18-20 h, then diluted with 100 ml of the same medium to an OD_{660} of 0.1. The culture was allowed to shake at 30°C until an OD_{660} value reached the level of 0.5-1.0 (early logarithmic phase), prior to transfer to the same volume of Low-Pi medium supplemented with required amino acids. The culture was continuously shaked at 30 °C until an OD_{660} value reached the level of 5.0-6.0 (late logarithmic phase) as described in Materials and Methods. The cells were harvested at OD_{660} of 6.0 (mid log phase) and lysed with sterile glass beads in the presence of protease inhibitor mixture as described in Materials and Methods. The total proteins in the lysates were measured and the same amount of protein about 0.4 μg of total yeast protein of each recombinant yeast were blotted onto 2 sheets of nitrocellulose membrane. The membrane was subjected to dot detection of the HBsAg using anti-S monoclonal antibody.

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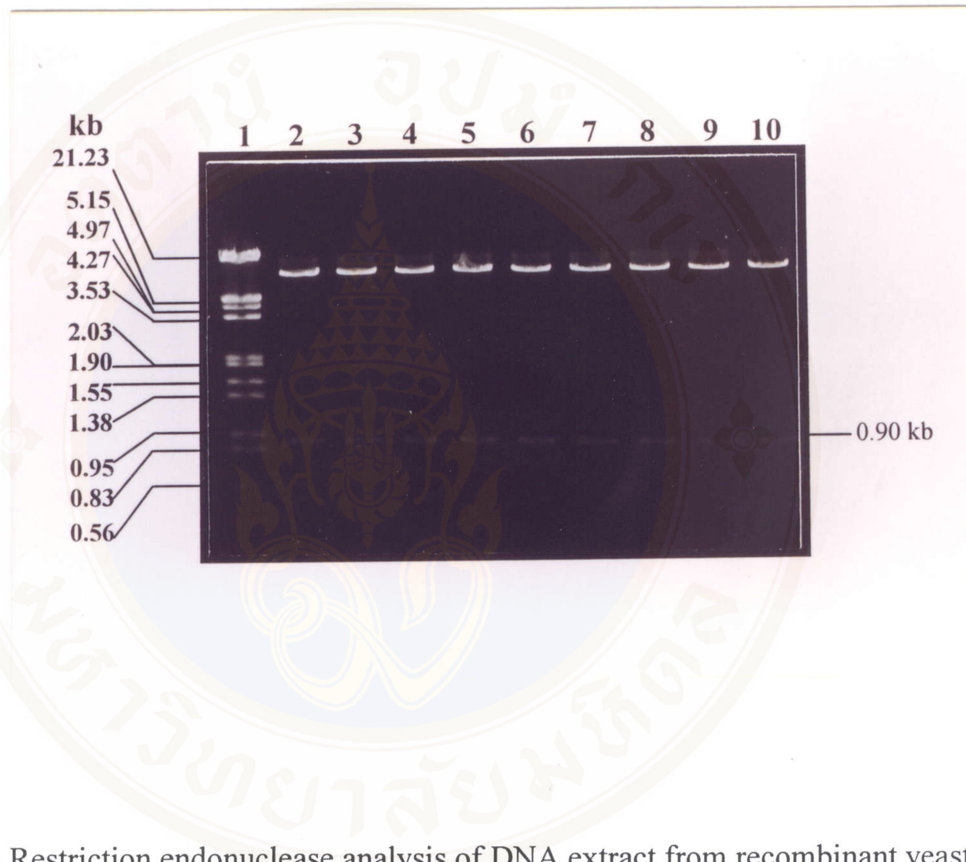

Figure 27. Restriction endonuclease analysis of DNA extract from recombinant yeasts P1B series.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.

Lane 2 pEB-P1 cut with *Bam*HI.

Lane 3-10 DNA extract from recombinant yeast P1B1 to P1B8 cut with *Bam*HI.

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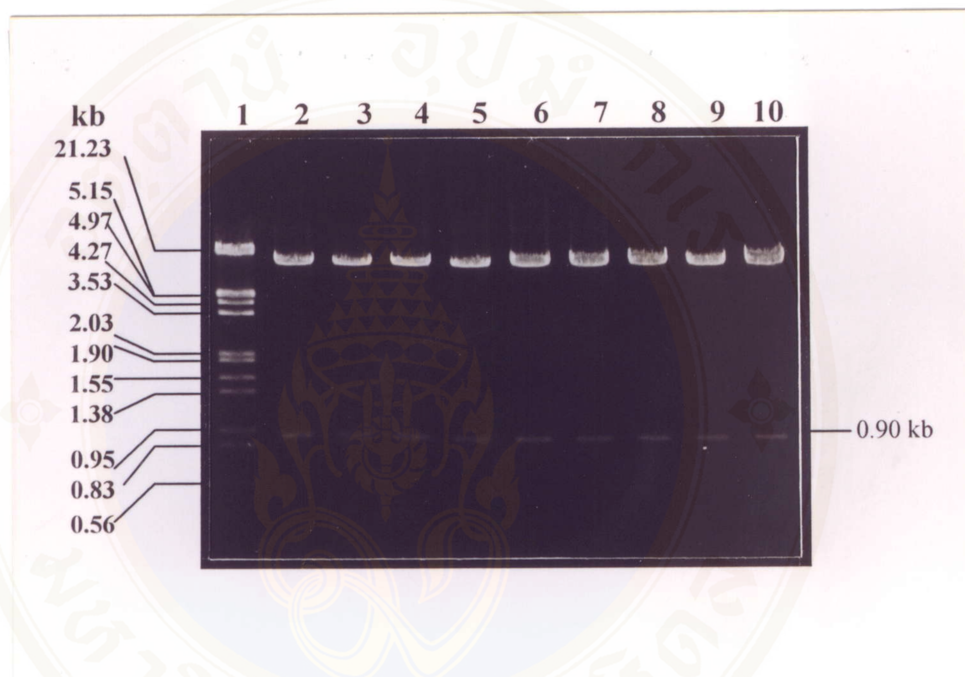

Figure 28. Restriction endonuclease analysis of DNA extract from recombinant yeasts P2B series.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.

Lane 2 pEB-P1 cut with *Bam*HI.

Lane 3-10 DNA extract from recombinant yeast P2B1 to P2B8 cut with *Bam*HI.

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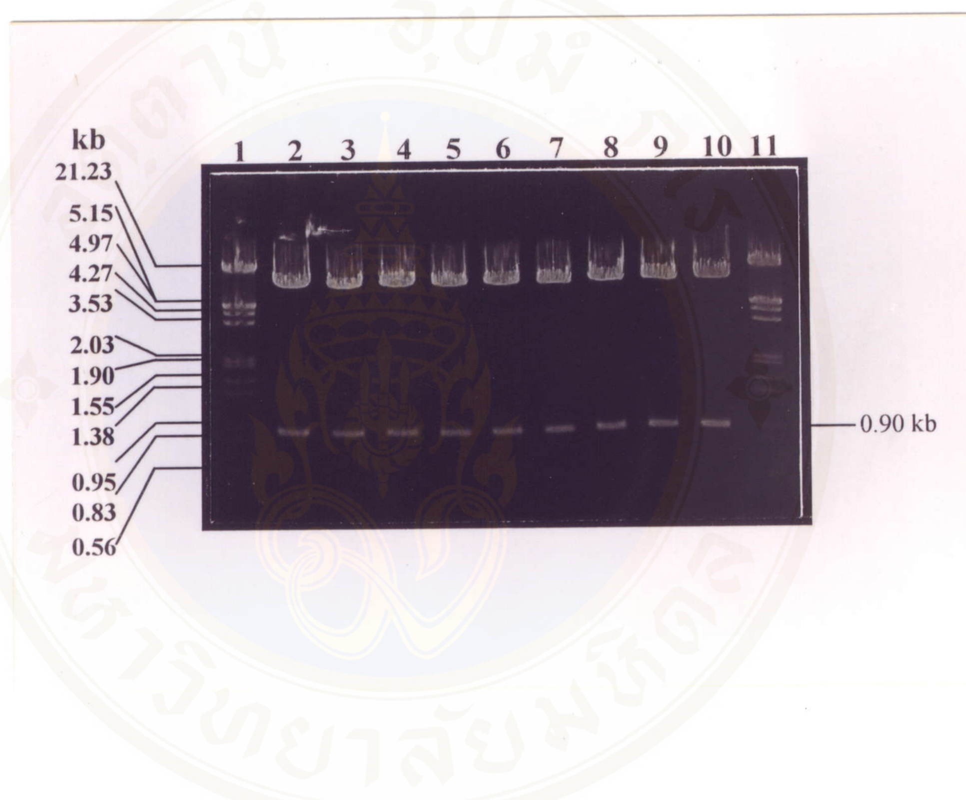

Figure 29. Restriction endonuclease analysis of DNA extract from recombinant yeasts P3B series.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.

Lane 2 pEB-P1 cut with *Bam*HI.

Lane 3-10 DNA extract from recombinant yeast P3B1 to P3B8 cut with *Bam*HI.

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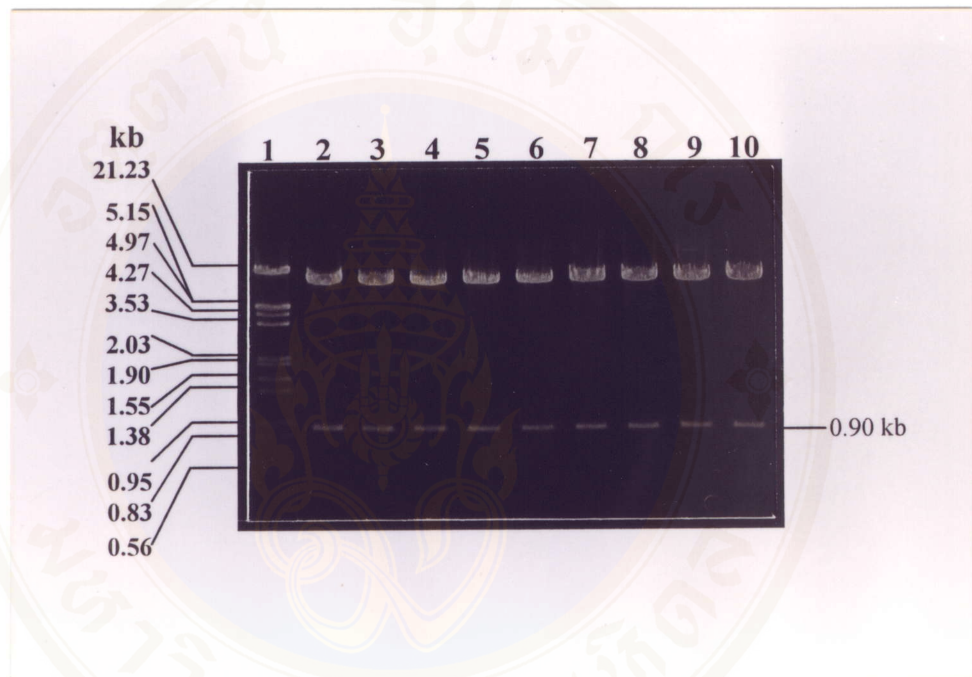

Figure 30. Restriction endonuclease analysis of DNA extract from recombinant yeasts P4B series.

Lane 1 λ DNA cut with *Hind*III + *Eco*RI.

Lane 2 pEB-P4 cut with *Bam*HI.

Lane 3-10 DNA extract from recombinant yeasts P4B1 to P4B8 cut with *Bam*HI.

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Figure 31 demonstrated the screening of recombinant yeast with high PreS2d+S gene product using anti-S monoclonal antibody. All the extracts (column 1-8; row A-E) and the purified HBsAg from HBV carriers (column 1 and 8; row F) gave positive result. Whereas the extracts from the recombinant harboring plasmid pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4 gave negative result as compared to the dot intensity of the recombinant yeast (column 2,3,6 and 7; row F). Five recombinant yeast of each series giving the highest intensity of the dots, the recombinant P1B1; P1B2; P1B3; P1B4 and P1B5 (pEB-P1), P2B3; P2B4; P2B7; P2B8 and P2B9 (pEB-P2), P3B2; P3B3; P3B8; P3B9 and P3B10 (pEB-P3) and P4B2; P4B6; P4B7; P4B9 and P4B10 (pEB-P4) were selected for further analysis.

6. Quantification of the M HBsAg

Protein extracts of the five selected recombinant yeast of each series were subjected to quantification of the M HBsAg by ELISA method using the Sorin biomedica ET1-MAK3 kit and human HBsAg which mainly contains S gene product (S protein), from Auszyme Monoclonal diagnostic as a standard. Therefore, the quantification of the M HBsAg was measured as HBsAg. As demonstrated in Table 19 the product on of M HBsAg was varied in each recombinant yeast series. Two high value of each recombinant yeasts series were selected to compared the productivity.

It was shown that the recombinant yeast harboring plasmid with one selectable marker (pEB-P1 and pEB-p3) produced M HBsAg higher than those with two selectable markers (pEB-P2 and pEB-P4). In addition, the recombinant yeast harboring plasmid with *LEU2*d selectable marker having small size (pEB-P3) produced M

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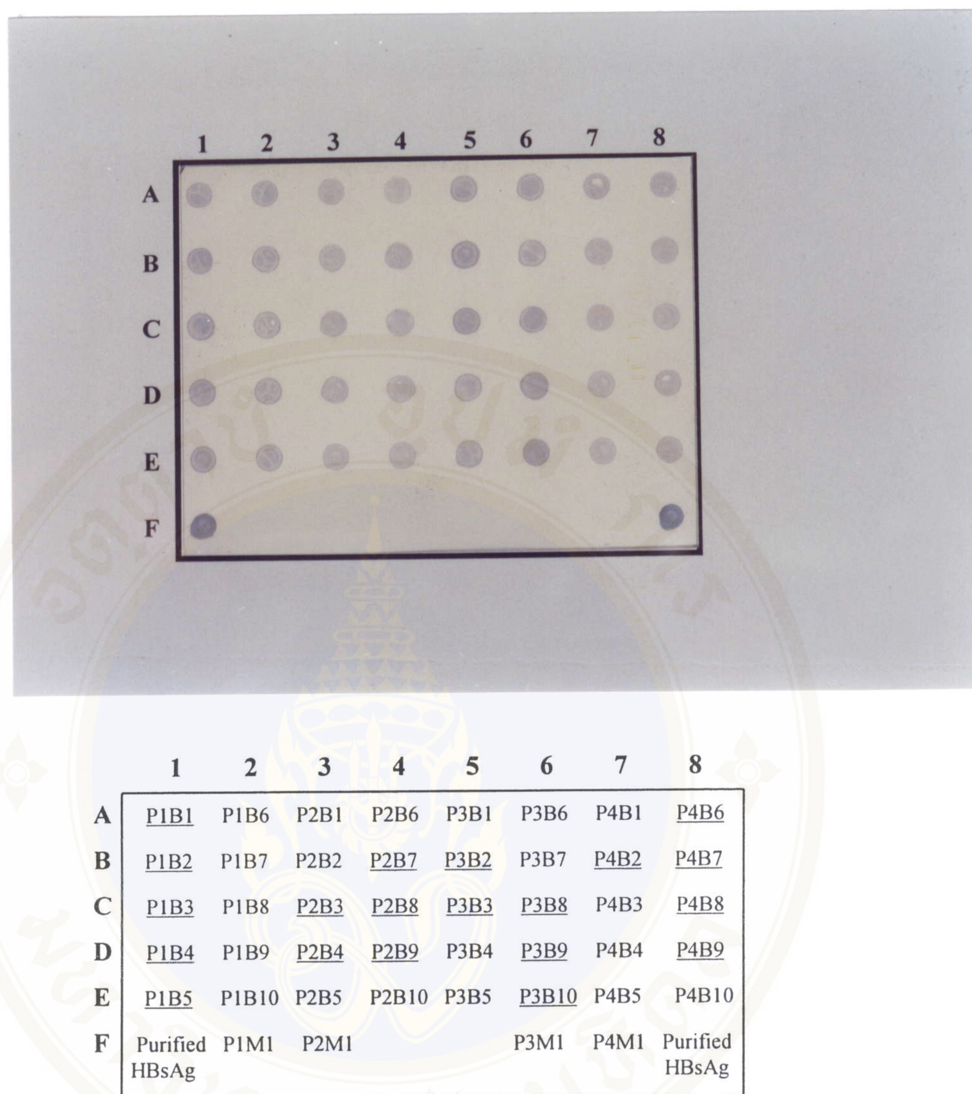

	1	2	3	4	5	6	7	8
A	P1B1	P1B6	P2B1	P2B6	P3B1	P3B6	P4B1	P4B6
B	P1B2	P1B7	P2B2	P2B7	P3B2	P3B7	P4B2	P4B7
C	P1B3	P1B8	P2B3	P2B8	P3B3	P3B8	P4B3	P4B8
D	P1B4	P1B9	P2B4	P2B9	P3B4	P3B9	P4B4	P4B9
E	P1B5	P1B10	P2B5	P2B10	P3B5	P3B10	P4B5	P4B10
F	Purified HBsAg	P1M1	P2M1			P3M1	P4M1	Purified HBsAg

Figure 31. Screening of recombinant yeast with M HBsAg production by dot blot ELISA using anti-S monoclonal antibody.

Total protein of each dot was 0.4 μg. The selected recombinant yeasts were underlined.

Column 1-2 (rowA-E) Protein extract of recombinant yeast P1B1 to P1B10.

Column 3-4 (row A-E) Protein extract of recombinant yeast P2B1 to P2B10.

Column 5-6 (row A-E) Protein extract of recombinant yeast P3B1 to P3B10.

Column 7-8 (row A-E) Protein extract of recombinant yeast P4B1 to P4B10.

Column 2,3,6,7 (row F) Protein extract of recombinant yeast PM1B1, PM2B1, PM3B1 and PM4B1.

Column 1,8 (row F) Purified HBsAg from HBV carriers.

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HBsAg higher than that big size (pEB-P1). From this result, the recombinant yeast P3B8 harboring plasmid pEB-P3 produced highest M HBsAg among those of the four plasmids. It was selected for further studies.

Table 19. Quantification of the M HBsAg from recombinant yeast.

Plasmid	Size (kb)	Recombinant yeast	HBsAg production (ng/mg protein)	Average* (ng/mg protein)
pPM323-P1	8.75	M1B1	0.00	
pEB-P1	9.65	P1B1	61.47 ± 6.83*	51.48 ± 14.13
		P1B2	16.88 ± 0.59	
		P1B3	27.24 ± 0.57	
		P1B4	41.49 ± 15.6*	
		P1B5	17.32 ± 1.32	
pPM323-P2	9.85	M2B1	0.00	
pEB-P2	10.75	P2B3	25.19 ± 2.86*	21.50 ± 5.21
		P2B4	7.88 ± 4.05	
		P2B7	17.82 ± 0.96*	
		P2B8	9.66 ± 1.24	
		P2B9	11.07 ± 0.20	
pPM323-P3	7.33	PM3B1	0.00	
pEB-P3	8.23	P3B2	24.77 ± 5.29	63.18 ± 1.61
		P3B3	28.10 ± 3.81	
		P3B8	64.32 ± 9.13*	
		P3B9	62.04 ± 6.79*	
		P3B10	24.32 ± 9.10	
pPM323-P4	8.43	PM4B1	0.00	
pEB-P4	9.33	P4B2	16.81 ± 0.35	29.47 ± 8.42
		P4B6	21.92 ± 0.99	
		P4B7	35.42 ± 7.58*	
		P4B9	23.51 ± 11.3*	
		P4B10	20.88 ± 11.0	
pEB-M5-2	10.26	M5B1	49.05 ± 6.44	

Note: The values were obtained from two independent experiments, duplicated each.
The M HBsAg was expressed as HBsAg as assayed by Sorin Biomedica ETI-MAK-3 kit using human HBsAg as a standard.
*Average of the two high values of each recombinant yeast series.

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7. Characterization of the M HBsAg Protein

To investigate the size of the M HBsAg which expressed in the recombinant yeast, the protein extract from recombinant yeast, P1B1, P2B3, P3B8, P4B7, negative control recombinant yeast, PM1P1, PM2P1, PM3P1, PM4P1 and positive control (purified HBsAg from HBV carriers), were loaded on two sets of 15% SDS-PAGE gel. One gel was used for investigation of protein profile by Comassie Brilliant Blue^{R} and the other was subjected to Western bot analysis. The mouse anti-PreS2 monoclonal antibody was employed for detection of M HBsAg. As shown in Figure 32a, the protein profile of recombinant yeast, P1B1, P2B3, P3B8 and P4B7 (lane 2-5) were not different from that of negative control PM1P1, PM2P1, PM3P1 and PM4P1 (lane 6-9). However, by Western blot analysis using mouse anti-PreS2 monoclonal antibody, two close bands were detected in the protein extract from the four recombinant yeasts, but not those from negative control, as shown in Figure 32b, (lane 2-5 as compared to lane 6-9). The molecular weights of these bands were calculated to be 34 kDa and 37 kDa as major and minor proteins of M HBsAg, respectively.

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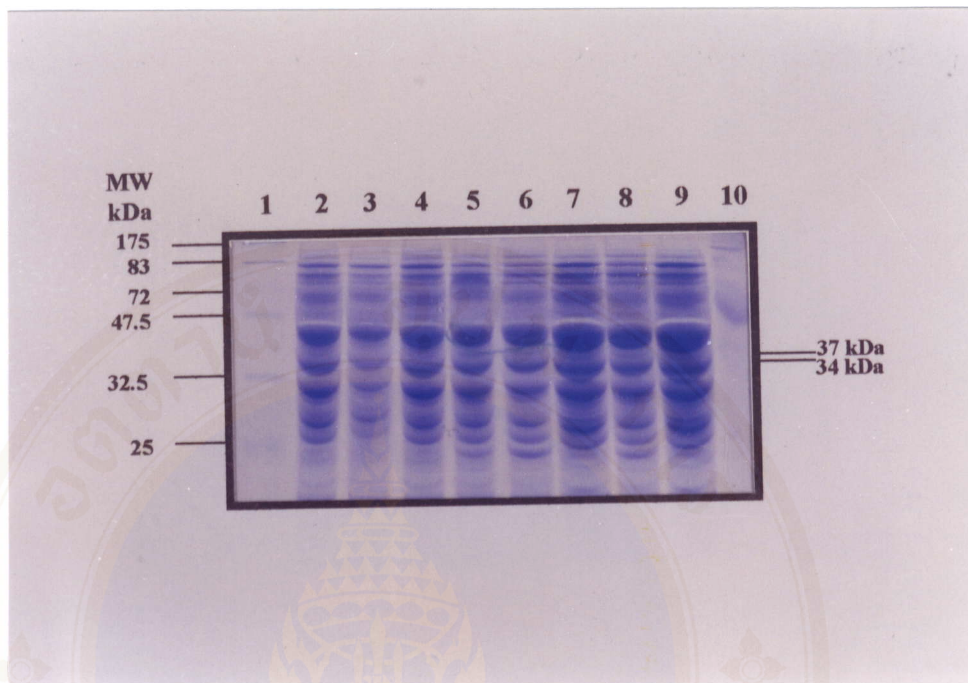
a



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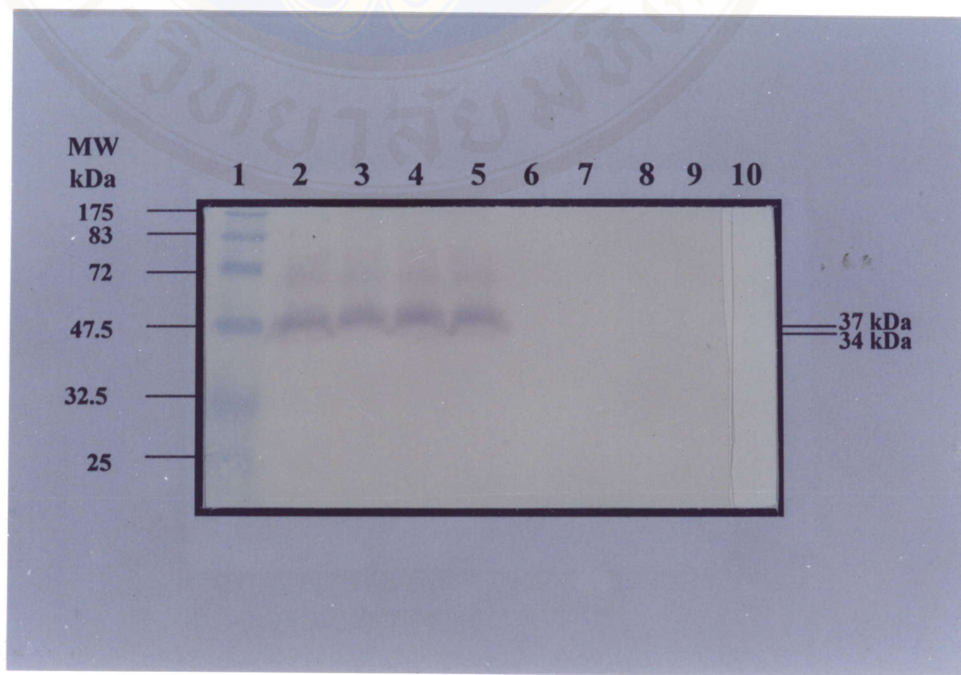

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Figure 32. Protein profile and Western blot analysis of the protein extract from recombinant yeast, P1B1, P2B3, P3B8, P4B7, PM1B1, PM2B1, PM3B1 and PM4B1.

a) Protein profile stained with Coomassie Brilliant Blue[R].

b) Western blot analysis detected with mouse anti-PreS2 monoclonal antibodies.

Lane 1 prestained protein molecular weight marker.

Lane 2 protein extract of recombinant yeast P1B1.

Lane 3 protein extract of recombinant yeast P2B3.

Lane 4 protein extract of recombinant yeast P3B8.

Lane 5 protein extract of recombinant yeast P4B7.

Lane 6 protein extract of recombinant yeast PM1B1.

Lane 7 protein extract of recombinant yeast PM2B1.

Lane 8 protein extract of recombinant yeast PM3B1.

Lane 9 protein extract of recombinant yeast PM4B1.

Lane 10 purified HBsAg from HBV carriers.

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8. Growth Kinetics of Recombinant Yeast

It was found in Table 19 that, the productivity of the M HBsAg by plasmid pEB-P3 was highest among the four plasmids. To examine whether cell growth affected the productivity, growth kinetics of all recombinant yeasts were studied, and doubling time and specific growth rate were calculated. The recombinant yeast, P1B1, P2B3, P3B8, P4B7, PM1B1, PM2B1, PM3B1 and PM4B1 were cultivated in High-Pi medium until OD_{660} was 0.5 (early log phase) then the cells were transferred to Low-Pi medium for induction of the gene expression. The growth was measured at OD_{660} every 3 h until the culture reached stationary phase. As shown in Figure 33, the doubling time (td) of the recombinant yeast P1B1, P2B3, P3B8 and P4B7 were 3.38, 4.68, 3.25, and 4.03 h, respectively whereas those of PM1B1, PM2B1, PM3B1 and PM4B1 were 2.92, 3.67, 2.89, and 3.47, respectively. The specific growth rate of the recombinant yeast P1B1, P2B3, P3B8 and P4B7 were 0.205, 0.149, 0.213 and 0.172 h^{-1}, respectively and those of PM1B1, PM2B1, PM3B1 and PM4B1 were 0.237, 0.189, 0.240, and 0.200 h^{-1}, respectively. The result demonstrated that the specific growth rate were in agreement with the M HBsAg productivity, i.e., the recombinant yeast harboring one selectable marker (P1B1 and P3B8) grew faster than those with two selectable markers (P2B3 and P4B7), and the recombinant yeast harboring small plasmid (P3B8 and P4B7) grew faster than those harboring large plasmid (P1B1 and P2B3).

9. Determination of Plasmid Copy Number of Recombinant Yeast

In order to examine that the different level of the M HBsAg productivity of four plasmids would be due to the plasmid copy number in the recombinant yeast, Southern blot analysis was performed. The amount of a plasmid-borne gene (PreS2d+S) relative

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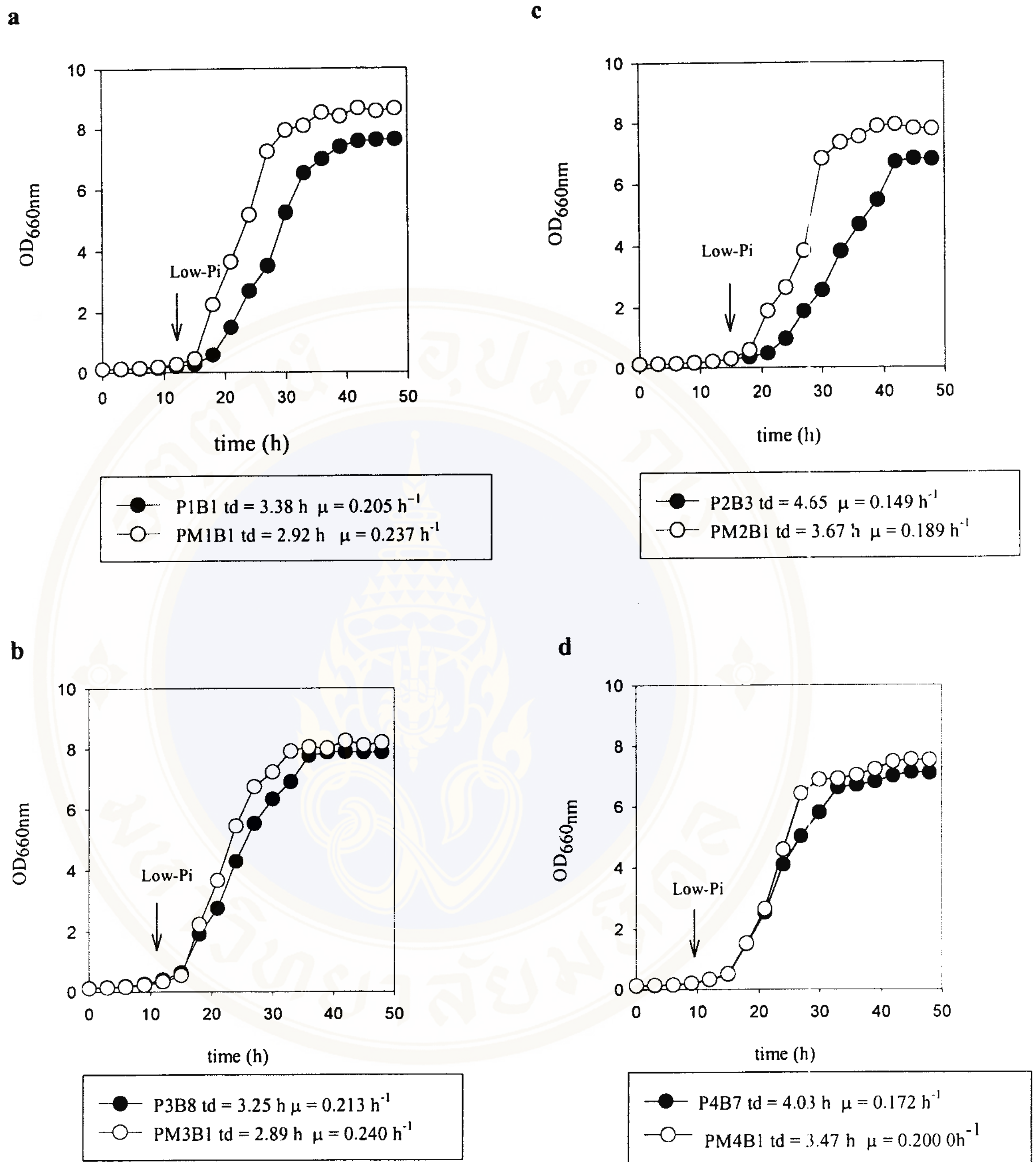

Figure 33. Growth kinetics of recombinant yeast for M HBsAg production.

(a) P1B1 (9.65 kb) and PM1B1 (8.75 kb) (c) P2B3 (10.75 kb) and PM2B1 (9.85 kb),
(b) P3B8 (8.23 kb) and PM3B1 (7.33 kb) (d) P4B7 (9.33 kb) and PM4B1 (8.43 kb).

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to the single copy of chromosomal gene (*TRP*1) of the four recombinant yeast, which represent the recombinant plasmid copy number, were determined. Total DNA was isolated from recombinant yeast P1B1, P2B3, P3B8 and P4B7 grown in selective condition and then restricted with *Eco*RI that cleaved the DNA on both sides of the plasmid-borne gene (PreS2d+S) (see Figure 21-24) and also cleaved chromosomal genes. This digestion generated fragment containing the PreS2d+S gene of plasmids pEB-P1, pEB-P2, pEB-P3 and pEB-P4 about 5.48, 6.58, 4.06 and 5.16 kb, respectively, as shown in Figure 34a (lane 2-5 as compared to lane 6). After agarose gel electrophoresis, the DNA fragments were transferred to a sheet of nylon membrane and hybridized with the DIG labeled 0.90 kb *Bam*HI fragment containing PreS2d+S gene derived from pSF5-1 plasmid (Figure 25), and 0.42 kb *Hind*III-*Xba*I fragment containing *TRP*1 gene derived from pTB326 plasmid (Figure 9). These two DIG labeling probes were hybridized to the PreS2d+S gene from the plasmid and the single copy chromosomal fragment *TRP*1, respectively. The result of DNA hybridization was shown in Figure 34b. Two differently sized bands were detected in each lane (lane 2-5) on the nylon membrane whereas the same sized bands were detected in all lanes. The size of upper bands (lane 2-5) were the same as the fragment containing PreS2d+S gene obtained by *Eco*RI cleaved plasmids pEB-P1, pEB-P2, pEB-P3 and pEB-P4 (Figure 34a, lane 2-5). The sizes of two lower bands (lane 2-5) were about 4.00 and 0.95 kb which was the same size as the *Eco*RI digested genomic DNA of the host BJ5462 (lane 6). The 0.95 kb would correspond to the *TRP*1 gene on the yeast chromosome since the *TRP*1 gene in plasmid pTB326 was about 0.83 kb (Figure 8).

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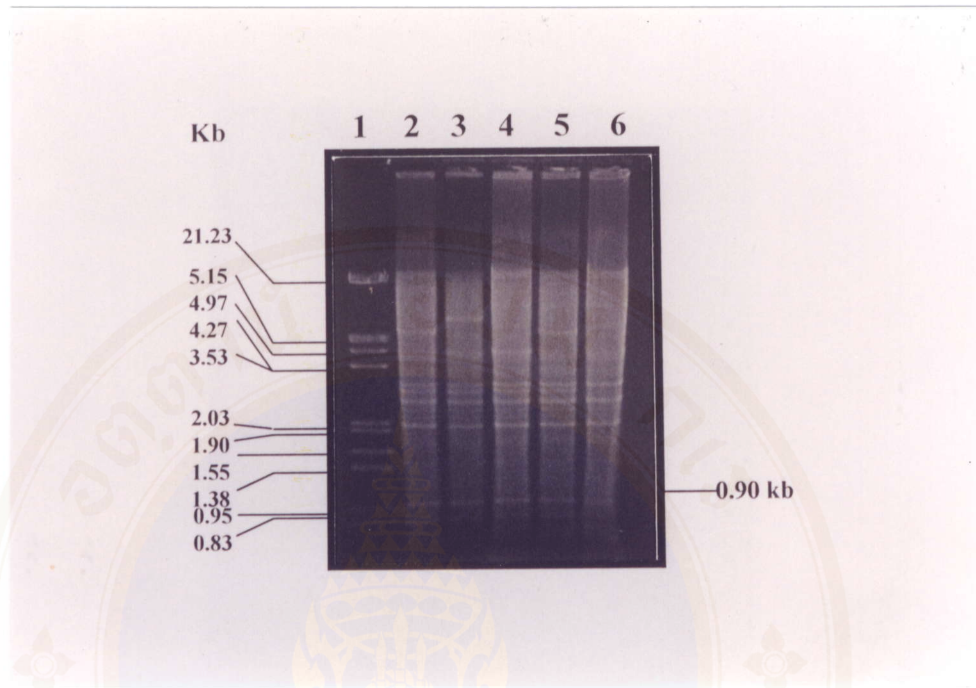

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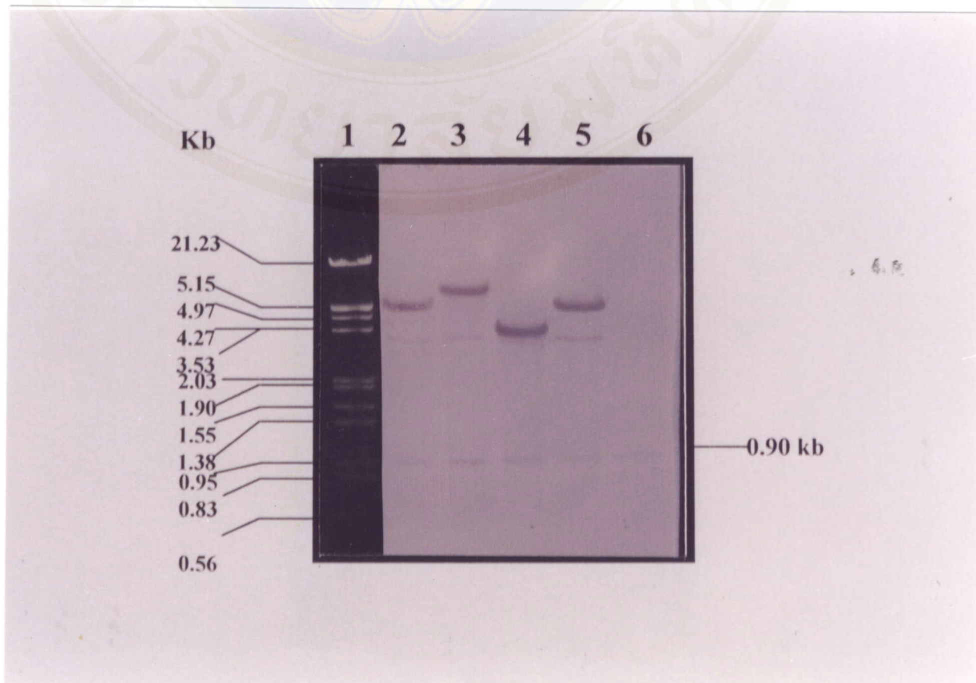

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Figure 34. Restriction endonuclease analysis and Southern blot analysis of recombinant yeast, P1B1, P2B3, P3B8, P4B7 and host BJ5462 DNA.

a) Restriction endonuclease analysis.

b) Southern blot analysis hybridize to PreS2d+S and *TRP*1 DIG-labeled DNA probes.

Lane 1 λ marker cut with *Hind*III and *Eco*RI.

Lane 2 DNA extract from recombinant yeast B1P1 cut with *Eco*RI.

Lane 3 DNA extract from recombinant yeast B2P3 cut with *Eco*RI.

Lane 4 DNA extract from recombinant yeast B3P8 cut with *Eco*RI.

Lane 5 DNA extract from recombinant yeast B4P7 cut with *Eco*RI.

Lane 6 DNA extract from host BJ5462 cut with *Eco*RI.

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The upper and lower bands on the nylon membrane were scanned using the same size of area and the ratio of the intensity of the two bands was calculated. It was shown in Table 20 that the recombinant yeast P1B1, P2B3, P3B8 and P4B7 showed plasmid copy number 68.33, 61.00, 72.00 and 66.67, respectively. It was indicated that the plasmid copy number of the plasmid pEB-P3 in the recombinant yeast P3B8 was highest among those of the four plasmids.

Table 20. Plasmid copy number of recombinant yeast.

Recombinant yeast	Plasmid (kb)	Plasmid copy number
P1B1	pEB-P1 (9.65)	68.33 ± 6.50
P2B3	pEB-P2 (10.75)	61.00 ± 4.36
P3B8	pEB-P3 (8.23)	72.00 ± 3.46
P4B7	pEB-P4 (9.33)	66.67 ± 10.2

Note: The plasmids copy number were determined from three independent experiments.

10. Determination of Plasmid Stability in the Recombinant Yeast

To examine whether the plasmid size difference would affect the plasmid stability which in turn result in the level of M HBsAg productivity, % plasmid stability was determined. The recombinant yeasts P1B1, P2B3, P3B8 and P4B7 were grown under non-selective condition in complex medium broth (YPD) at 30 °C for 24 h with shaking and then plated on YPD for single colony isolation after incubation at 30 °C for 2 days, hundred colonies were spotted on YPD and replica plated on SD supplemented with required amino acids. The colonies that lost the plasmid were not able to grow on SD plate. The culture in YPD broth were transferred continuously into YPD broth with 10% inoculum, then plated on YPD as described above. This method was repeated for five cycles and % plasmid stability was calculated. It was shown in Table 21 that the % plasmid stability of recombinant yeast was in agreement with the M HBsAg productivity and that of P3B8 was highest.

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Table 21. % Plasmid stability of recombinant yeast.

Recombinant yeast	Plasmid (kb)	% Plasmid stability					
		1st Cycle	2nd Cycle	3rd Cycle	4th Cycle	5th Cycle	Average
P1B1	pEB-P1 (9.65)	100.00	99.50	99.00	99.50	99.00	99.4 ± 0.42
P2B3	pEB-P2 (10.75)	97.50	98.00	98.50	98.50	98.00	98.1 ± 0.42
P3B8	pEB-P3 (8.23)	100.00	99.50	99.50	99.50	99.50	99.6 ± 0.22
P4B7	pEB-P4 (9.33)	99.00	98.50	99.00	99.00	98.50	98.8 ± 0.27

11. Optimization of Induction Phase for High M HBsAg Production by Recombinant Yeast

In the screening of recombinant yeast with high M HBsAg production in part 5, the induction was performed by cultivation in High-Pi medium until an OD_{660} value reached the level of 0.5-1.0 (early logarithmic phase), and then transferred to the Low-Pi medium and continuously grown until an OD_{660} value reached the level of 5.0-6.0 (late logarithmic phase) as described by Hiraoka *et al.*, 1994. In order to optimize the induction phase to obtain the maximum productivity, the recombinant yeast P3B8 was induced at different growth phase. It was firstly grown in High-Pi medium until cell density reached the level of OD_{660} 0.5, 1.5, 3.0 and 6.0, which were in the early to mid log phase, then the cells were transferred to Low-Pi medium for induction. Samples were taken after induction for measurement of growth by OD_{660} and investigation of M HBsAg production by dot blot ELISA and ELISA assay.

Comparison of the dot intensity of the M HBsAg demonstrated that the cells induce at OD_{660} 0.5 of all cultivation time gave the highest intensity of the dots as shown in Figure 35. As shown in Figure 35, the specific growth rates of the P3B8 among four induction phases were not different, μ=0.229 h^{-1}, whereas its cell density when induced at OD_{660} 6.0 was highest and followed by OD_{660} 3.0, 1.5 and 0.5, respectively.

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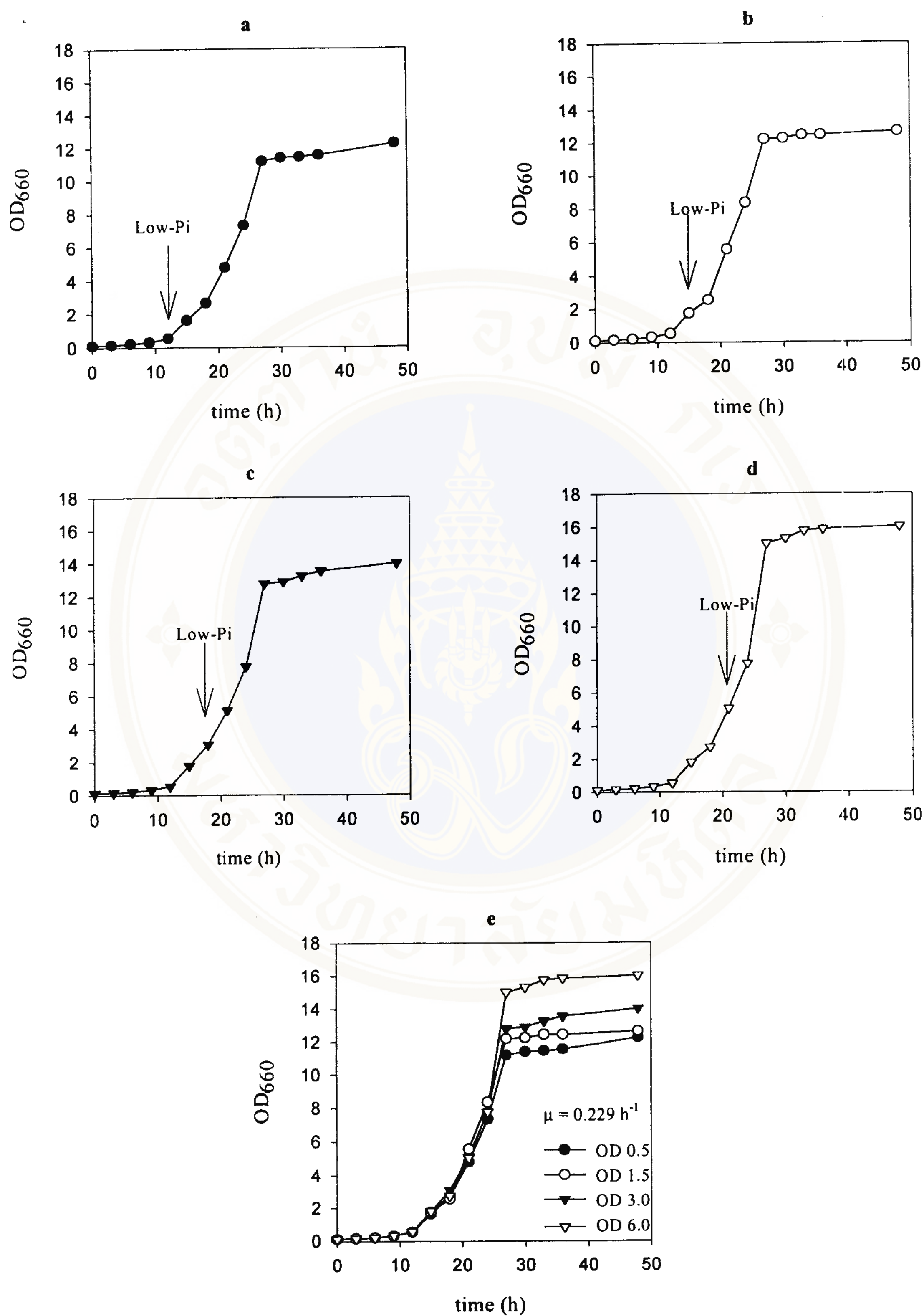

Figure 35. Growth kinetics of recombinant yeast P3B8 grown in High-Pi and transferred to Low-Pi at various growth phase.
(a) OD_{660} 0.5 (b) OD_{660} 1.5 (c) OD_{660} 3.0
(d) OD_{660} 6.0 (e) OD_{660} 0.5, 1.5, 3.0 and 6.0 (combination a-d)

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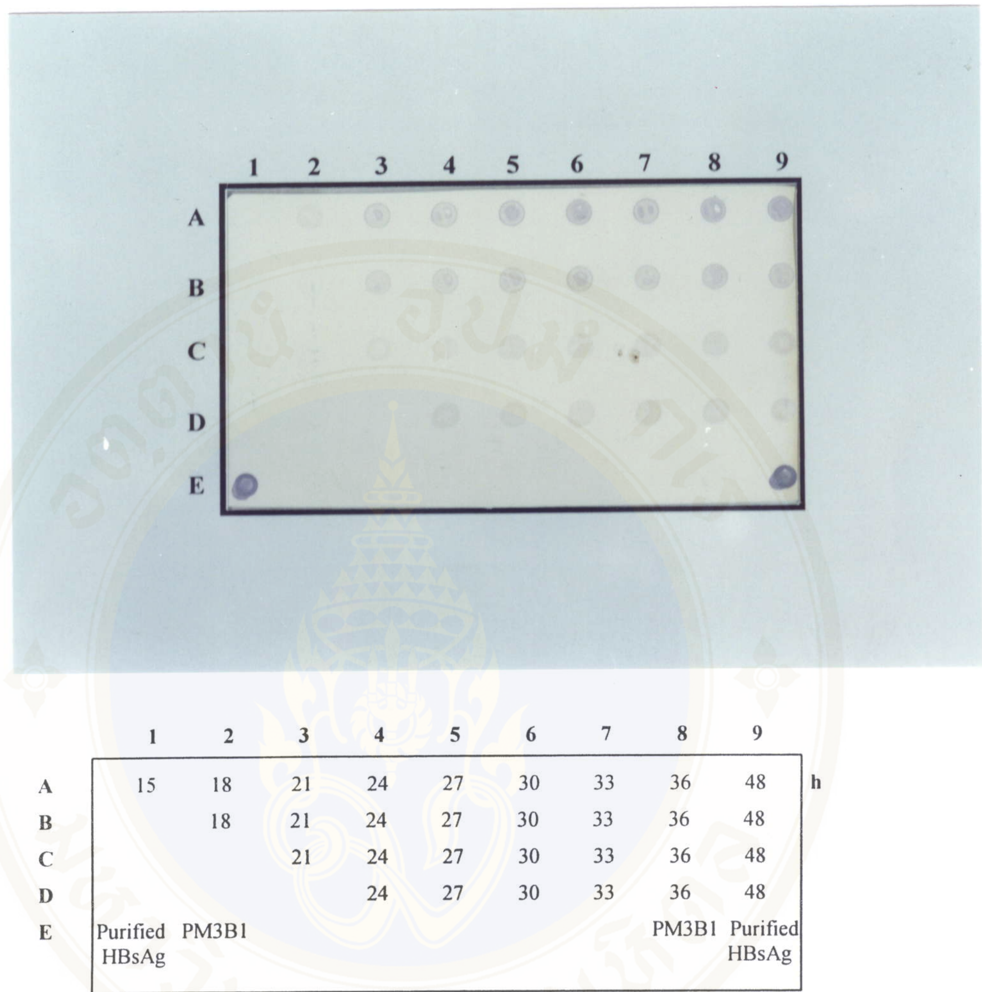

	1	2	3	4	5	6	7	8	9	
A	15	18	21	24	27	30	33	36	48	h
B		18	21	24	27	30	33	36	48	
C			21	24	27	30	33	36	48	
D				24	27	30	33	36	48	
E	Purified HBsAg	PM3B1						PM3B1	Purified HBsAg	

Figure 36. Dot blot ELISA of protein extract of recombinant yeast P3B8 grown in High-Pi and transferred to Low-Pi at various growth phase.

Total protein of each dot was 0.4 μg and anti-S monoclonal antibody was used for detection.

Row A (column 1-9)	Induced at OD_{660} 0.5, protein extract at 15, 18, 21, 24, 27, 30, 33, 36 and 48 h of cultivation time.
Row B (column 2-9)	Induced at OD_{660} 1.5, protein extract at 18, 21, 24, 27, 30, 33, 36 and 48 h of cultivation time.
Row C (column 3-9)	Induced at OD_{660} 3.0, protein extract at 21, 24, 27, 30, 33, 36 and 48 h of cultivation time .
Row D (column 4-9)	Induced at OD_{660} 6.0, protein extract at 24, 27, 30, 33, 36 and 48 h of cultivation time .
Row E (column 2 and 8)	Protein extract of recombinant yeast PM3B1.
(column 1 and 9)	Purified HBsAg from HBV carriers.

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To confirm that the induction at OD_{660} 0.5 gave the highest M HBsAg, M HBsAg produced at 27 h of cultivation time of all induction phase were quantified. All the cultures were at the beginning of stationary phase as shown in Figure 36e. As shown in Table 22, the M HBsAg production at OD_{660} 0.5 was highest. Therefore, the induction of the M HBsAg production at OD_{660} 0.5 or at early log phase was selected to induce the PreS2d+S gene expression in further study.

Table 22. Quantification of the M HBsAg produced by recombinant yeast P3B8 induced with Low-Pi at various induction phase (OD_{660}), and cultivated for 27 h.

Induction Phase (OD_{660})	HBsAg (ng/mg protein)	HBsAg (μg/l culture)
0.5	75.00 ± 3.40	105.00 ± 4.76
1.5	42.27 ± 0.26	74.39 ± 0.43
3.0	15.89 ± 1.80	41.94 ± 4.76
6.0	20.01 ± 4.69	55.41 ± 2.16

Note: The value were obtained from two independent experiments, duplicated each.
The M HBsAg was expressed as HBsAg as assayed by Sorin Biomedica ETI-MAK-3 kit using human HBsAg as a standard.

12. Comparison of the M HBsAg Production of Recombinant Yeast Grown in Low-Pi and High-Pi Induced with Low-Pi

Above study showed that the induction of the PreS2d+S gene expression was high in the culture with Low-Pi in the early growth phase. In order to investigate whether the induction by cultivation in Low-Pi medium from the start without transferring from High-Pi to Low-Pi could give high level of expression. The specific growth rate and the M HBsAg productivity of the recombinant yeast P3B8 cultivation in Low-Pi medium was studied to compare with that induced with Low-Pi at OD_{660} 0.5. As shown in Figure 37, the cell density of the recombinant yeast, when grown in Low-Pi alone, was lower than the cells grown in High-Pi and induced in Low-Pi at OD_{660}

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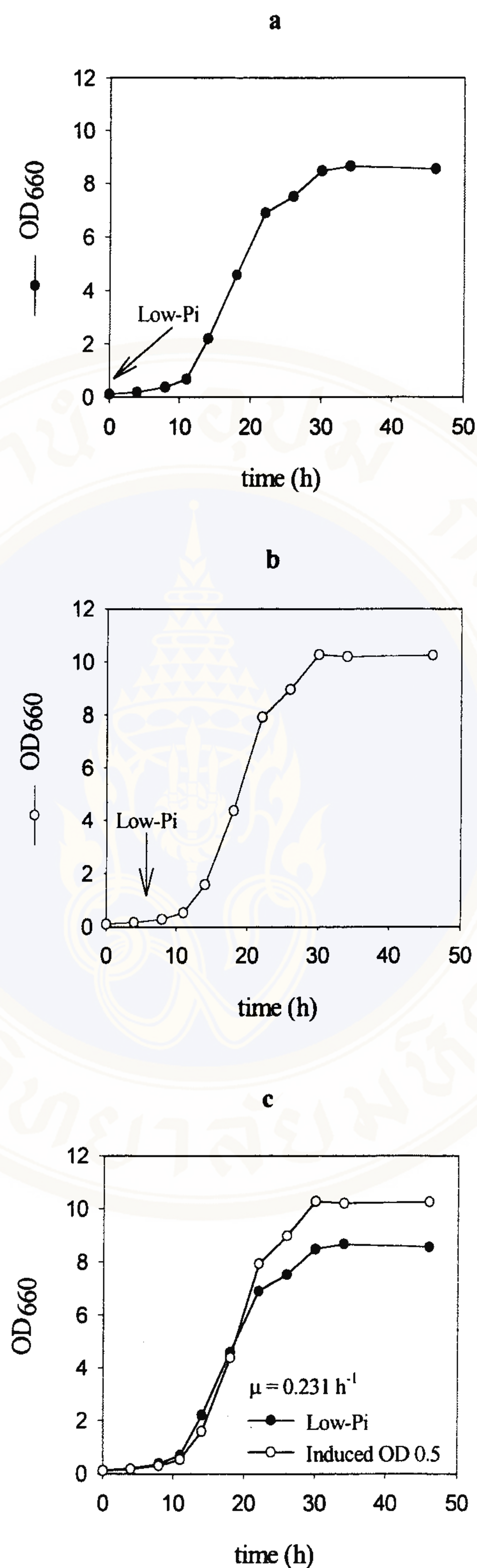

Figure 37. Growth kinetics of recombinant yeast P3B8 grown in Low-Pi, and in High-Pi induced with Low-Pi at OD_{660} 0.5.
(a) Low-Pi medium (b) induced at OD_{660} 0.5
(c) Low-Pi medium and induced at OD_{660} 0.5 (combination a-b)

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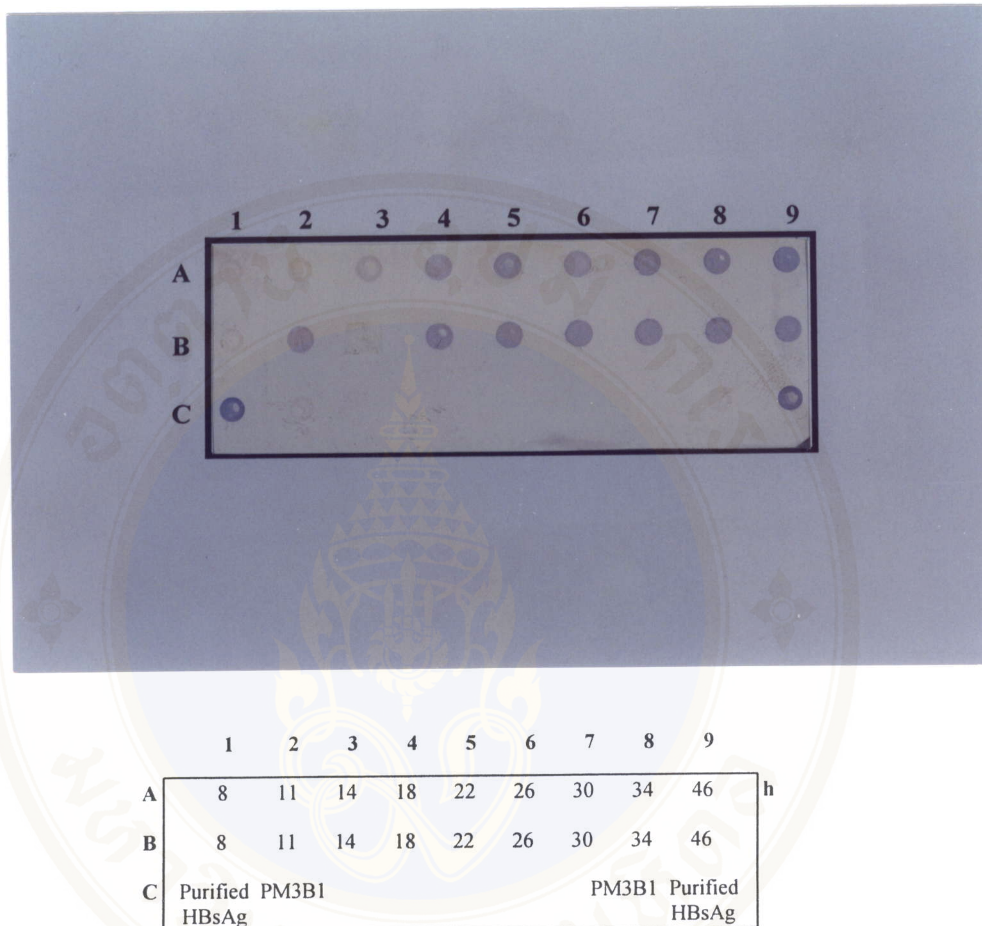

	1	2	3	4	5	6	7	8	9	
A	8	11	14	18	22	26	30	34	46	h
B	8	11	14	18	22	26	30	34	46	
C	Purified HBsAg	PM3B1						PM3B1	Purified HBsAg	

Figure 38. Dot blot ELISA of protein extract of recombinant yeast P3B8 grown in Low-Pi, and in High-Pi induced with Low-Pi at OD_{660} 0.5.

Total protein of each dot was 0.4 µg and anti-S monoclonal antibody was used for detection.

Row A (column 1-9) Protein extract of culture grow in Low-Pi medium at 8, 11, 14, 18, 22, 26, 30, 34 and 46 h of cultivation time.

Row B (column 2-9) Protein extract of culture grow in High-Pi and induced with Low-Pi medium at 8, 11, 14, 18, 22, 26, 30, 34, and 46 h of cultivation time.

Row C (column 2 and 8) Protein extract of recombinant yeast PM3B1.

(column 1 and 9) Purified HBsAg from HBV carrie.

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0.5, after the mid log phase. Nevertheless, the M HBsAg from the culture grown with Low-Pi induction, was earlier detectable, by dot blot ELISA, as shown in Figure 38 (Row A and B column 2) than that grown in Low-Pi from the start of cultivation. The dot intensity of M HBsAg in protein extract from 18-46 h of cultivation were not different (Row A and B column 4-9). Therefore the M HBsAg production among two conditions were further quantified by ELISA technique. As shown in Table 23, the M HBsAg production of the recombinant yeast P3B8 grown in Low-Pi was lower, than then that of cells grown in induction condition at the same period of the cultivation time. This result confirmed that the induction at OD_{660} 0.5 was appropriated for induction of the PreS2d+S gene expression.

Table 23. Comparison of the M HBsAg production by recombinant yeast P3B8 grown in Low-Pi, and in High-Pi induced with Low-Pi at OD_{660} 0.5.

Cultivation time (h)	HBsAg (ng/mg protein)		HBsAg (μg/l culture)	
	Low-Pi	High-Pi Induced with Low-Pi	Low-Pi	High-Pi Induced with Low-Pi
8	0.43 ± 8.56	1.12 ± 0.04	0.14 ± 2.76	0.39 ± 0.01
11	1.59 ± 0.32	8.62 ± 3.21	0.27 ± 5.52	7.55 ± 0.87
14	2.56 ± 1.41	21.72 ± 3.02	2.45 ± 0.14	26.84 ± 3.34
18	29.33 ± 2.03	29.65 ± 0.53	13.66 ± 12.54	31.36 ± 1.11
22	36.78 ± 5.67	50.95 ± 0.48	34.03 ± 5.24	68.97 ± 15.83
26	51.90 ± 0.55	61.21 ± 5.22	39.35 ± 4.10	85.21 ± 5.23
30	60.07 ± 6.26	74.89 ± 0.19	65.0 ± 0.68	91.89 ± 5.23
34	66.62 ± 2.35	77.64 ± 3.10	95.97 ± 3.42	103.36 ± 3.14
46	87.11 ± 1.36	92.63 ± 11.13	100.64 ± 10.94	118.06 ± 8.91

Note: The value were obtained from two independent experiments, duplicated each.
The M HBsAg was expressed as HBsAg as assayed by Sorin Biomedica ETI-MAK-3 kit using human HBsAg as a standard.

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13. Time-Course of Cell Growth and Production of M HBsAg

The time-course profile of cell growth and M HBsAg production in the recombinant yeast P3B8 was examined in order to know the correlation of the production of the M HBsAg at various growth. The recombinant yeast P3B8 was grown in High-Pi and induced with Low-Pi as described above. Cell samples were collected at time interval for measuring the OD_{660} and quantification of the M HBsAg by ELISA.

As shown in Figure 39, the recombinant yeast P3B8 reached the higest OD_{660} of 10.8 at 90 h. After induction with Low-Pi for 3 h, the M HBsAg was 1.13 ng/mg protein. Afterward, the production was increase continuously and reached maximum level of 100.09 ng/mg protien (146.92 μg/l) at stationary phase (90h). Subsequently, the level of product was gradually decrease. Through out at the induction in the stationary phase, the cell viability was stable (Figure 39c) and decreased after 90 h of cultivation. The results indicated that the M HBsAg was production under the control of phosphate, was growth associated and highest at stationary phase. This observation was in parallel to the M HBsAg production level.

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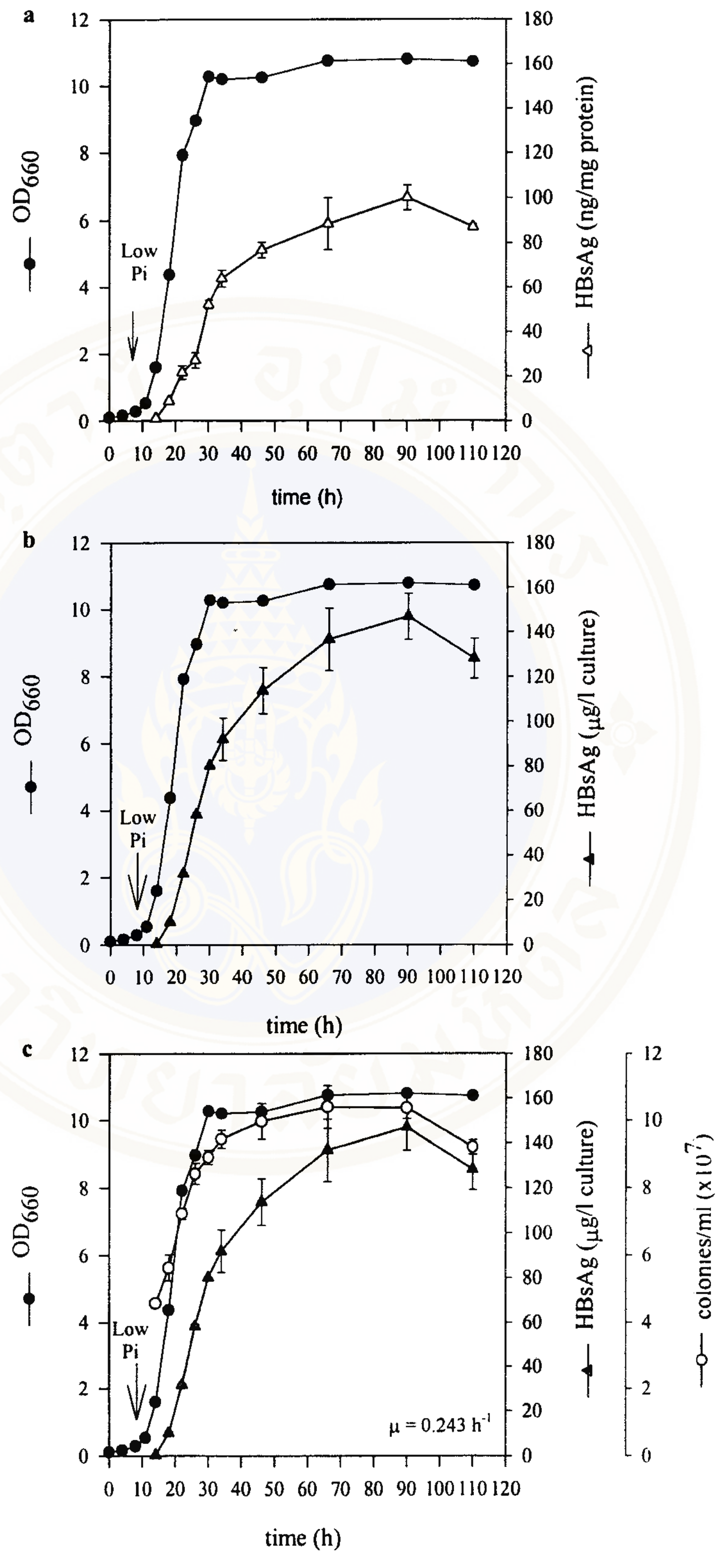

Figure 39. Time-course of the M HBsAg production in recombinant yeast P3B8.

(a) HBsAg (ng/mg protein)

(b) HBsAg (μg/l culture)

(c) HBsAg (μg/l culture) and cell viability (colonies/ml)

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14. Stability of the M HBsAg Protein at Various Growth Phase

Western Blot analysis was performed to confirm that the M HBsAg production at log phase and stationary phase was stable. The recombinant yeast P3B8 was grown as in Part 13. The cells were collected at equal cell density (OD_{660} 10.0) for 26, 30, 46, 66, 90 and 110 h. The protein samples were prepared from the cell samples, which were adjusted to the same cell density (OD_{660} = 10.0 in 2 ml), by boiling with 1x SDS sample buffer followed with centrifugation at 4 °C for 10 min. The crude protein extract from P3B8 and negative control were loaded on two sets of 15% SDS-PAGE gel, for Coomassie Blue staining and Western blotting using anti-S and anti-PreS2 monoclonal antibody as primary antibody. As shown in Figure 40, lane 2-6, the M HBsAg protein from recombinant yeast was still intact throughout the cultivation time when detected with anti-PreS2 monoclonal antibody as well as anti-S monoclonal antibody (data not shown).

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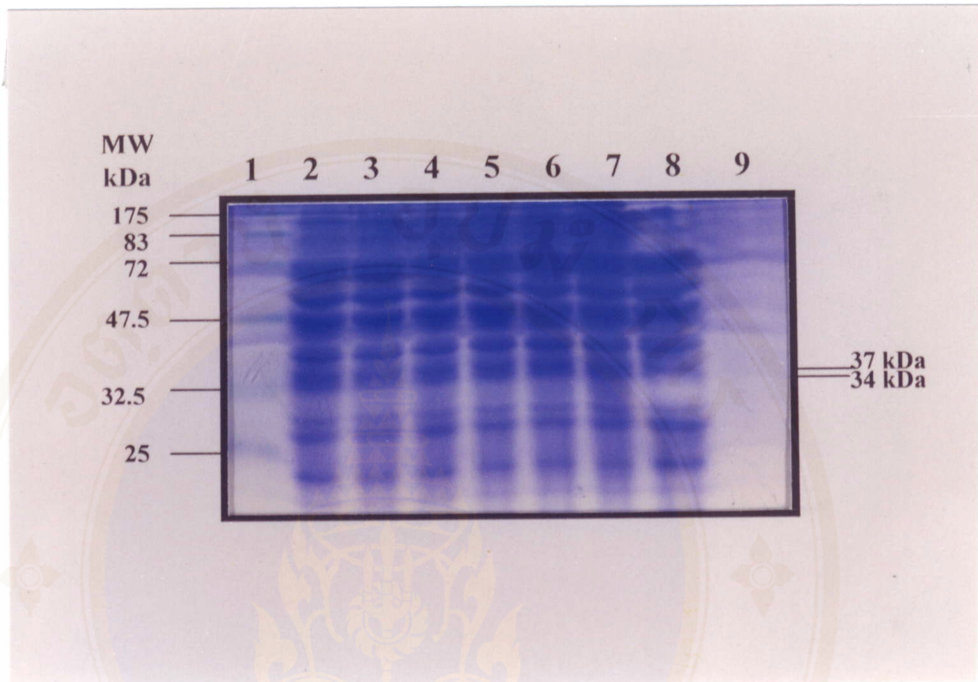

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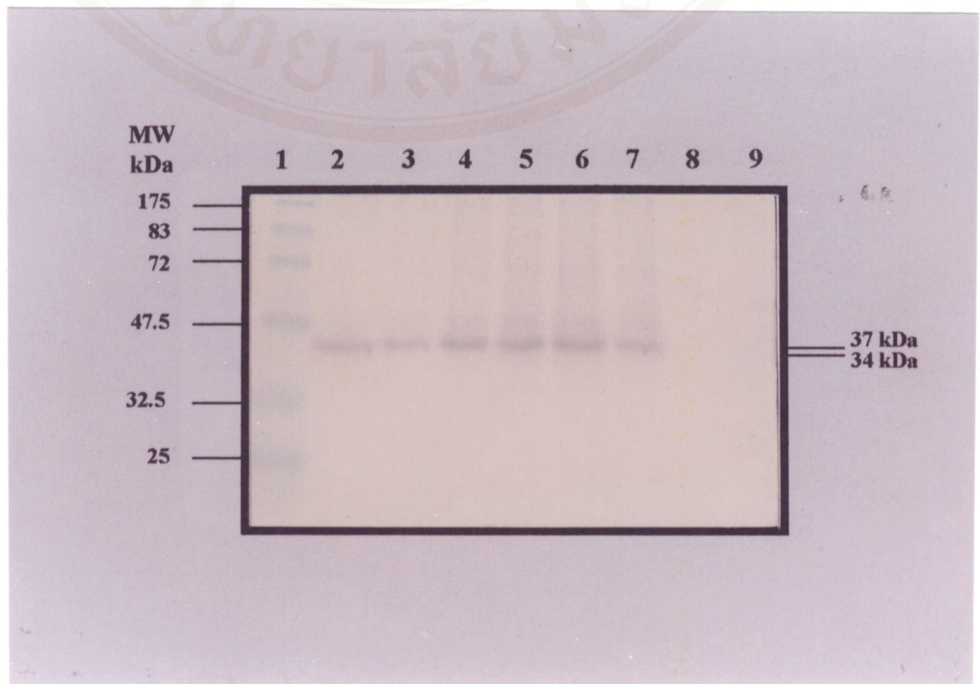

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Figure 40. Protein profile and Western blot analysis of the protein extract from recombinant yeast P3B8 at 26, 30, 46, 66, 90, and 110 h of cultivation.

Protein profile stained with Coomassie Brilliant Blue^{R}.

c) Western blot analysis detected with mouse anti-PreS2 monoclonal antibodies.

Lane 1 prestained protein molecular weight marker.

Lane 2 protein extract of recombinant yeast at 26 h.

Lane 3 protein extract of recombinant yeast at 30 h.

Lane 4 protein extract of recombinant yeast at 46 h.

Lane 5 protein extract of recombinant yeast at 60 h.

Lane 6 protein extract of recombinant yeast at 90 h.

Lane 7 protein extract of recombinant yeast at 110 h.

Lane 8 protein extract of recombinant yeast PM3B1 (negative control).

Lane 9 Purified HBsAg from HBV carriers.

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CHAPTER V

DISCUSSION

Several controllable promoter systems are known in *Saccharomyces cerevisiae*, such as regulatory system for galactose pathway enzymes, repressible phosphatases, and other proteins. In these systems, the cells can be grown to high density before the promoters are activated, by increasing the galactose or decreasing inorganic phosphate (Pi) concentration, respectively, of the cultivation medium. However, control of promoter activity by decreasing Pi concentration of the cultivation medium would be more convenient and economical. There are reports of attempts to produce HBsAg expressed by acid phosphatase promoter, *PHO*5p, which was activated by such methods (Itoh *et al*., 1986). In this study, the expression of the M HBsAg gene was regulated by the *PHO*84 promoter. The *PHO*84 gene encodes a high-affinity Pi transporter and is derepressed by Pi starvation. The expression of HBsAg would be, therefore, activate by this promoter in decreasing Pi concentration. The expression of the M HBsAg by *PHO*84p has never been reported therefore it is interesting to investigate it.

1. Construction of the Recombinant Yeast Strain which Could Express the M HBsAg by the Inducible *PHO*84 Promoter

The construction of the recombinant yeast strain that could express the M HBsAg included the following steps : 1) construction of the yeast expression plasmid with *PHO*84p and *CYC*1t, 2) construction of plasmid for the PreS2d+S gene in yeast, 3) construction of recombinant yeast harboring the PreS2d+S gene, 4) screening of the

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recombinant yeast with high M HBsAg production, 5) quantification of the M HBsAg and 6) characterization of the M HBs protein.

The host strain used in this work was BJ5462 (*MAT*α, *ura*3-5, *trp*1*leu*2Δ1, *his*3Δ 200, *pep*4::*HIS*3, *prb*1Δ1.6R *can*1 *GAL*). This strain is protease deficient which would minimize proteolytic degradation of the M HBsAg protein. In order to transform recombinant plasmid harboring PreS2d+S gene, the plasmid with *PHO*84p and suitable selectable markers were constructed. The pPM323 was used as the starting plasmid construction of the yeast expression plasmid, because this plasmid contains two selectable markers, *LEU*2d and *URA*3. The aim to use the *LEU*2d as one selectable marker was to maintain the recombinant plasmid in high copy number. Since the *LEU*2d gene has a deletion of the 5'-end sequence starting ATG initiation codon, which results in very poor expression (Erhart and Hollenberg, 1983), and its exppression is less than 5% of wild type gene. Therefore, when the *leu*2 host is transformed with the plasmid harboring the defective *LEU*2 gene, the plasmid has to be maintained in high copy number to overcome the efficient amount of β-isopropylmalate dehydrogenase (the enzyme encoded by *LEU*2) for synthesis of leucine when cell grown in selective condition. For another selectable marker, *URA*3, the aim to include this gene in the plasmid was to reduce the addition uracil as growth supplement in the medium.

Some part of DNA in the plasmid pPM323 which was not required for plasmid function both in *E. coli* and *S. cerevisiae*, was also deleted in order to reduce the size of plasmids. Since the construction of plasmid with desired characteristics was done by several steps. The restriction analysis in Figure 19 and 20 confirmed the presence of the expression cassette containing *PHO*84p and *CYC*1t, couplet *LEU*2d and *URA*3 gene

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in the expression plasmid pPM323-P1 (*LEU*2d+*URA*3), pPM323-P2 (*URA*3), pPM323-P3 (*LEU*2d+*URA*3) and pPM323-P4 (*URA*3).

All four plasmids had unique *Bam*HI site between the promoter and terminator therefore the PreS2d+S gene was inserted at corresponding site to obtain yeast expression plasmids pEB-P1 (*LEU*2d+*URA*3), pEB-P2 (*URA*3), pEB-P3 (*LEU*2d+*URA*3) and pEB-P4 (*URA*3) (Figure 25). The insertion of the PreS2d+S gene could occur in two orientations which 5' end of the gene was next to the promoter (+ orientation) or the terminator (-orientation). As there was one *Xba*I site in the fragment, which was about 0.25 kb from 5' end of the PreS2d+S gene, and the plasmids also contained one *Xba*I site located down stream of the *Bam*HI cloning site (Figure 26). The PreS2d+S gene orientation was therefore, analyzed with *Xba*I digestion. The PreS2d+S gene, which the protease sensitive site (Arg48-Thr49) was removed, was used in this study because the product, modified M HBsAg, was more stable to yeast proteolysis than wild type M HBsAg (Foo-trakoon, 2000). It was expected that the stable M HBsAg to proteolysis would result in high yield. As reported by Itoh and Fujisawa, (1986a) and Imamura *et al.* (1987), the yield of the modified M HBsAg was higher than the wild type M HBsAg about 1,000 times, when the same host strain was used.

To obtain the recombinant yeast harboring the expression plasmid with PreS2d+S gene, the four constructed plasmids were transformed into yeast BJ5462. The transformants were selected on SD agar plate supplemented with trp+ura (pEB-P1, pEB-P3) and trp (pEB-P2, pEB-P4). The primary screening was done by selection of the transformants with big colony size. The transformants harboring plasmids pEB-P1, pEB-P2, pEB-P3 and pEB-P4 showed similar colony size as those harboring plasmids

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pPM323-P1, pPM323-P2, pPM323-P3 and pPM323-P4, respectively (data not shown). This result demonstrated that the growth rate of transformants harboring recombinant plasmids were not different from their corresponding transformants harboring only vectors. The normal growth is necessary for cultivation of recombinant yeast when preinduction growth stage was required before heterologous gene expression.

The presence of transformed plasmids in the transformants were confirmed by checking the amino acid markers (data not shown) and back transformation into *E. coli*. The restriction analysis with *Bam*HI of plasmids extracted from *E. coli* transformed with DNA form yeast transformants showed the same pattern as that from the original plasmids (Figure 27-30). These results would indicated that the selected transformants or recombinant yeasts harbored the expression plasmid with PreS2+Sd gene fragment.

For the screening of recombinant yeast with high M HBsAg production, phosphate starvation induction was carried out by growing the recombinant yeast in High-Pi medium (11.0 mM) until reach OD_{660} of 0.5, the cells were then harvested, washed, and transferred to Low-Pi medium (0.22 mM) for induction (Hiraoka *et al*, 1994). As the expression levels of the PreS2d+S gene might be varied among individual transformants harbored each recombinant plasmid, ten tranformants of each plasmids were selected. Dot blot ELISA was employed to screen numbers of recombinant which expressed high amount of the M HBsAg by using mouse anti-S monoclonal antibody Figure 31 showed that the dot intensity of M HBsAg from different transformants or recombinant yeasts were different. However this screening was roughly, five recombinants of each recombinant yeast series P1B (pEB-P1), P2B (pEB-P2), P3B (pEB-P3), P4B (pEB-P4) were further subjected to quantification of the M HBsAg based on the ELISA assay using humans HBsAg as standard. The result in

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Table 19, demonstrated that the M HBsAg production in each recombinant yeast series were varied. It was probably due to the efficiency of gene expression and the plasmid copy number.

Western blot analysis was performed to characterize the size of the M HBsAg expressed in yeast strain BJ5462. Since, the high titer of mouse anti-S monoclonal antibody for detection of S epitope on the Western blot was not available, only the mouse anti-PreS2 monoclonal antibody was used. The bands of 34 kDa (major) and 37 kDa (minor) were observed in the crude protein extract from recombinant yeasts P1B1, P2B3, P3B8 and P4B7 whereas the bands were not detected in that from PM1B1, PM2B1, PM3B1 and PM4B1 (Figure 32b). The anti-PreS2 monoclonal antibody used in this work was prepared against amino acid 17-26 (Gerlich, personal communication). These amino acids were identified to be PreS2 epitope within the immunodominant regions (Meisel *et al.*, 1994).

This result indicates that the recombinant yeast could express the M HBsAg protein having correct PreS2 epitope. The size of the protein, 34 and 37 kDa observed in this study were the same as those reported by Kobayashi *et al.*, 1988, which purposed to be glycosylated form in yeast.

2. Comparison of the Expression of the M HBsAg by Yeast Expression Plasmid Containing One and Two Selectable Markers

Since the quantification of the M HBsAg by ELISA assay demonstrated that the M HBsAg production by the plasmids with one selectable (pEB-P1 and pEB-P3) marker was higher than those with two selectable markers about 2.1-2.4 folds (pEB-P1 was compared with pEB-P3 and pEB-P2 was compared with pEB-P4) (Table 19). It

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might result from the burden of cells to express high amount of β-isopropylmalate dehydrogenase (*LEU*2d), orotidine-5'-phosphate uptake (*URA*3) and M HBsAg from the recombinant plasmid. This effect might lead to the loss of some copy number of plasmid in recombinant yeast culture and inturn gave lower yield of M HBsAg. Furthermore, comparison of the M HBsAg production in each pair of plasmid harboring the same selectable marker, showed that the production by small plasmids (pEB-P3 and pEB-P4) were slightly higher than the large ones (pEB-P1 and pEB-P2) about 1.2-1.4 folds. This result was probably due to the burden of the cells to maintain the large plasmid. The growth kinetic of the four recombinant yeasts were investigated in order to confirm that the different in plasmid structure influenced the cell growth and could affect the productivity. Indeed, the doubling time and specific growth rate of recombinant yeast grow in High-Pi and induce with Low-Pi at early log phase were in agreement with its the productivity of each recombinant yeast series (Figure 33), i.e., the recombinant yeast harboring one selectable marker (P1B1 and P3B8) grew faster than those with two selectable (P2B and P4B) markers, and the recombinant yeast harboring small plasmid (pEB-P3 and pEB-P4) grew faster than those with the large plasmid (pEB-P1 and pEB-P2).

The plasmid copy number in the four recombinant yeasts was also determined in order to support that the variation in M HBsAg productivity depent on plasmid copy number. It was found that the copy number was slightly different (Table 20) and in agreement with the level of productivity. The plasmid copy number containing *LEU*2d gene determine in this work was rather high (68.33, 61.00, 72.00 and 66.67 copies) as compared to some report (Erhart and Hollenberg, 1983). It was reported that the copy number of plasmid with *LEU*2d gene was 35. However the results were not

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comparable because the plasmid size, host strain and technique for determination of copy number were different. The speculation that the plasmid stability would result in variation of the variation in the M HBsAg productivity, could be ruled out because all the four plasmids were stable as shown in Table 21. Despite that the recombinants yeast were cultivated in non-selective condition for five cycles, about 8 generations in each cycle, only 0.5-2% of plasmid were lost. The result was in agreement with Erhart and Hollenberg, (1983).

In conclusion, the expression of the M HBsAg by the recombinant harboring one selectable marker and small size, plasmid pEB-P3, was highest among the four plasmids. However, the production was still low (63.18 ng/mg protein) as compared to previous reports (Table 19).

3. Optimization of the Induction Phase for High M HBsAg Production by Recombinant Yeast

Cultivation and induction of recombinant yeast for heterologous gene expression by *PHO*84p could be done by cultivation in High-Pi medium (11.0 mM) for 6 h (OD_{660} 0.5), then transferred to Low-Pi medium (0.22mM) for induction (Hiraoka *et al*., 1994). The aim of this work was to optimize only the suitable induction phase to achieve the highest M HBsAg production but not to optimize the Pi concentration. The work was done by varying in the OD_{660} before induction. The selected recombinant harboring pEB-P3, P3B8 was cultivated in High-Pi medium until growth reached various OD_{660} and then transferred to Low-Pi medium for induction. Comparison of the M HBsAg production at various induction phases by dot blot ELISA (Figure 36), would indicate that the production by induction OD_{660} of 0.5 was highest. The result was confirmed by

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quantification by ELISA assay. The amount of the M HBsAg at various induction phases from the same period of cultivation were compared (Table 22). It was found that the amount of M HBsAg at induction OD_{660} 0.5 was highest among four induction phases.

The different in productivity was not likely the result from difference growth since the growth rate of recombinant yeast in all induction condition were the same (Figure 35). The decrease in production when the cells were induced late along log phase of growth would be due to the fact that the expression of *PHO*84p, increase along the log phase and reach its maximum in mid to late log phase (Martinez *et al*., 1998). Therefore, induction of M HBsAg production in early log phase (OD_{660} 0.5) was highest. Attempt to induce the PreS2d+S gene expression by cultivation in Low-Pi from the start of cultivation to obtain higher M HBsAg production was not successed (Table 23). The M HBsAg production of recombinant yeast grown with induction was higher along the period of growth and the production was earlier detected than that of cells grown in Low-Pi (Table 23). It was probably due to the metabolic burden of cell to express M HBsAg in Low-Pi at the beginning of cultivation because the *PHO*84 was expressed only in Low-Pi condition (Bun-ya *et al*., 1991). Surprisingly, the growth rate in both conditions was the same (Figure 37). This result could be explained that the level of expression was not too high to interfere the cell growth. Nevertheless, when the cultivation was prolong, the growth yield in Low-Pi was higher. The lower growth yield in the optimization experiment of induction phase was also observed when the expression of M HBsAg was prolong after induction (Figure 35e). It could be explained that in Low-Pi condition the plasmid containing cells was unstable because there was asymmetric plasmid segregation upon the production of M HBsAg. As a

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result, the plasmid free cells occurred but they could retain for the ability to grow in selective medium for some time before stop growing (Srienc *et al.*, 1985). However, the production in Low-Pi condition was not much lower than in induction condition.

From the study, it can be concluded that the M HBsAg production achieved highest level when the recombinant yeast was induced in Low-Pi at early log phase (OD_{660} 0.5). The production in Low-Pi condition was also achieved, although the production level was slightly lower than the induction condituon.

4. Time-Course of Cell Growth and the Production of M HBsAg

The objective of this work was to correlate the M HBsAg production by induction condition along the growth phase. The M HBsAg production was observed immediately when the cells were transferred to Low-Pi medium at OD_{660} 0.5 (Figure 39). The maximum production was obtained when the cells were in stationary phase or after 90 h of cultivation and slightly decreased afterward. The production of M HBsAg level was in the same trend as the cell viability, which would indicate that the production was growth associated. The result was in agreement with the production of human lysozyme using the *PHO*84p (Hiraoka *et al.,* 1994). It was observed that the maximum level of lysozyme production was in stationary phase. Despite the fact that the expression of *PHO*84 was maximum at late log phase (Martinez *et al.*, 1998). However it could not rule out that the M HBsAg was stable because the gene PreS2d+S, in which the protease sensitive site was removed, was used (Foo-trakoon, 2000). Furthermore, the host strain was protease deficient strain. This observation was different from Foo-trakoon, (2000) in that the expression of the PreS2d+S by *GAL*10p in host strain JEL-1 was maximum in late log phase and declined immediately upon

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stationary phase. The stability of the M HBsAg promoter at various growth phase was investigated by Western blot analysis (Figure 40). It was found that the bands were still intact. The result would support that the M HBsAg expression in the BJ5462 strain was stable. It can be concluded from this study that the M HBsAg production was highest in stationary phase and growth associated. In addition, the M HBsAg produced in stationary phase was stable

Regarding to the yield of M HBsAg produced by *PHO*84p, it was found that the production was much lower (146 µg/l culture) than previous report (1.19 g/l), using *GAP*p (Itoh and Fujisawa, 1986a, Kuroda *et al.*, 1993). Although, the comparison of the production yield is depend on several factors such as yeast strain background, plasmid composition, size of plasmid and assay method, the promoter is the most important factor. It was clearly demonstrated that the promoter *PHO*84p was not strong enough to achieve high M HBsAg product.

The expression by *PHO*84p could be improved by construction of the hybrid promoter by fusion the regulatory region of *PHO*84p with the strong promoter of other gene such as *GAP* (Cousens *et al*, 1987, Hinnen *et al.*, 1989).

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CHAPTER VI

CONCLUSION

1. Four yeast expression plasmids, pPM323-P1 (*LEU2*d), pPM323-P2 (*LEU2*d+*URA3*), pPM323-P3 (*LEU2*d) and pPM323-P4 (*LEU2*d+*URA3*) with expression cassette harboring *PHO84*p and *CYC1*t were constructed.
2. Four yeast expression plasmids harboring PreS2d+S gene were constructed: pEB-P1 (*LEU2*d), pEB-P2 (*LEU2*d+*URA3*), pEB-P3 (*LEU2*d) and pEB-P4 (*LEU2*d+*URA3*).
3. Four recombinant yeasts strain BJ5462 harboring the four expression plasmids for PreS2d+S gene were constructed.
4. The M HBsAg production by the four recombinant yeasts harboring pEB-P1, pEB-P2, pEB-P3, and pEB-P4 were quantified. They were 51.48, 21.50, 63.18 and 29.47 ng/mg protein, respectively.
5. Western blot analysis of the crude proteins from the four recombinant yeasts using anti-PreS2 mAb demonstrated that all yeasts produced M HBsAg protein bands of 33 and 36 kDa.
6. Growth kinetics of the four recombinant yeasts were studied. The specific growth rates of the recombinant yeasts harboring plasmid pEB-P1, pEB-P2, pEB-P3 and pEB-P4 were 0.205, 0.143, 0.213 and 0.172 h^{-1}, respectively.
7. The plasmid copy number and plasmid stability in the four recombinant yeasts were analysed.

   7.1 The plasmid copy number were 68.33, 61.00, 72.00 and 66.67, respectively.

   7.2 The plasmid stability were 99.4, 98.1, 99.5, 98.8 %, respectively.

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8. The recombinant yeast harboring plasmid pEB-P3, showing highest M HBsAg production, specific growth rate, plasmid copy number and plasmid stability, was chosen for the study of M HBsAg production.
9. The M HBsAg production by induction with Low-Pi at various growth phase was studied.
   - 9.1 The production was 75.00, 42.27, 15.89 and 20.01 ng/mg protein, when induced at OD_{660} 0.5, 1.5, 3.0 and 6.0, respectively.
   - 9.2 The production was 92.63 ng/mg protein when induced at the beginning of cultivation.
10. The induction condition with Low-Pi at OD_{660} 0.5 was chosen for study of the M HBsAg production in small batch.
    - 10.1 The maximum production was 100.09 ng/mg protein (146.92 μg/l) at stationary phase or 90 h of incubation time.
    - 10.2 The production was slightly decreased when the cells entered stationary phase at 110 h of cultivation time.
    - 10.3 The production was growth associated.
11. Western blot analysis of the crude protein of the cells in logarithmic phase and stationary phase using anti-S mAb and anti-PreS2 mAb demonstrated that the molecular mass of M HBsAg protein bands were the same.

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APPENDIX A

1. Stock solution for High-Pi and Low-Pi media.

1.1 +P and –P stock solution (per litre)

Chemical	Final concentration	
	+P solution	-P solution
KH_2PO_4	6 g	-
KCl	-	6 g
$MgSO_47H_2O$	2 g	2 g
$CaCl_22H_2O$	1.32 g	1.32 g
KI solution (0.1 g/l)	4 ml	4 ml
Trace element (1000X)	4 ml	4 ml

Note : The solution could be stored at room temperature.

1.1 1000X Trace element solution (per litre)

$FeSO_47H_2O$	1.00	g
$ZnSO_47H_2O$	8.80	g
$CuSO_45H_2O$	0.40	g
$MnSO_44H_2O$	0.15	g
$Na_2B_4O_710H_2O$	0.10	g
$(NH_4)_6Mo_7O_{24}4H_2O$	0.05	g

HCl was used to dissolve the crystal before adjust to final volume and store at 4 °C.

1.2 1000X Vitamin mix (per litre)

Nicotinic acid	0.20	g
Pyridoxine	0.20	g
Thiamine	0.20	g
Pantothenic acid	0.20	g

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Inositol 10.0 g

Biotin 0.02 g

Filter sterile and store at 4 °C.

2. Stock solution for amino acids for yeast culture.

Amino acid	Stock (g/100 ml)	Final concentration in medium (mg/l)	Volume stock used (ml/l)	Volume of stock to spread on plate (ml)
L-Leucine	1	100	10	0.1
Tryptophan	1	20	2	0.1
Uracil	0.2	20	10	0.2

Note : store at room temperature.

3. Sterilization

3.1 Generally, all medium and solutions were autoclaved at 15 lbs/sq.inch. at 121 °C for 15 min, YNB, High-Pi, Low-Pi media and amino acids were autoclaved at 110 °C for 10 min.

3.2 Filter solution through sterile 0.45 μm nitrocellulose membrane.

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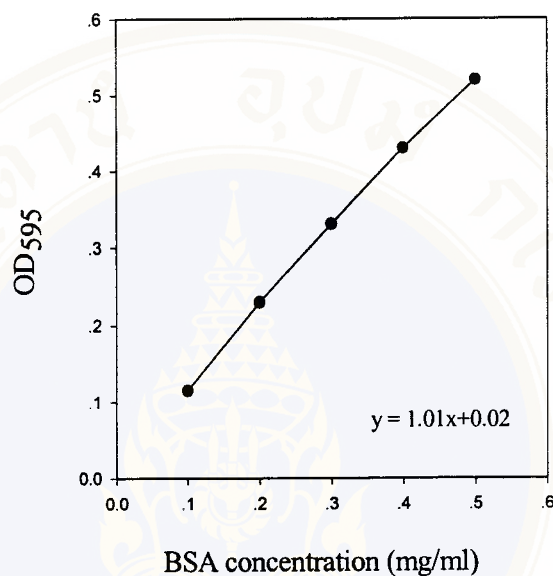
APPENDIX B



Standard curve for the Protein assay

Bovine serum albumin (BSA) was diluted to 0.1-0.5 mg/ml and determined the concentration with Bradford analysis. The linearity of curve is between OD_{595} 0.16-0.52.

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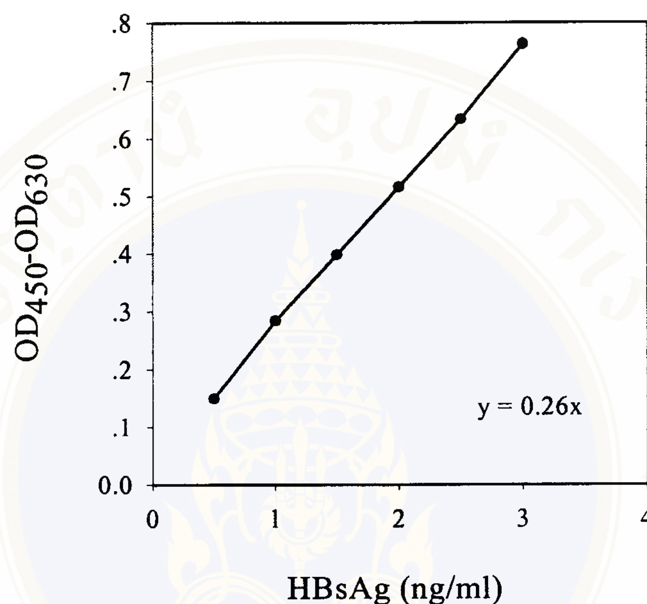
APPENDIX C



Standard curve for the quantification of M HBsAg production

Human HBsAg concentration of 9 ng/ml was diluted to 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ng/ml. It was assayed with Sorin Biomedica EIT-MAK-3 kit. The linearity of the curve is between $OD_{450-630}$ 0.149-0.763.

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APPENDIX D

1. The growth studied of recombinant yeast in High-Pi and induced with Low-Pi at OD_{660} 0.5.

Time (h)	OD_{660nm}							
	pPM323-P1 (PM1B1)	pEB-P1 (P1B1)	pPM323-P2 (PM2B1)	pEB-P2 (P2B3)	pPM323-P3 (PM3B1)	pEB-P3 (P3B8)	pPM323-P4 (PM4B1)	pEB-P4 (P4B7)
0	0.107	0.108	0.117	0.118	0.114	0.125	0.127	0.121
3	0.122	0.118	0.130	0.130	0.133	0.148	0.140	0.135
6	0.144	0.125	0.143	0.145	0.159	0.186	0.150	0.149
9	0.184	0.148	0.163	0.173	0.219	0.265	0.206	0.193
12	0.271	0.198	0.209	0.207	0.330	0.400	0.326	0.315
15	0.443	0.269	0.299	0.288	0.538	0.634	0.515	0.502
18	2.250	0.569	0.579	0.353	2.230	1.910	1.530	1.510
21	3.650	1.490	1.880	0.473	3.660	2.750	2.660	2.530
24	5.170	2.700	2.650	0.955	5.420	4.290	4.580	4.090
27	7.240	3.520	3.830	1.860	6.750	5.540	6.430	5.020
30	7.940	5.240	6.820	2.550	7.240	6.340	6.890	5.800
33	8.090	6.520	7.350	3.810	7.920	6.910	6.920	6.630
36	8.520	6.980	7.540	4.680	8.060	7.780	7.040	6.710
39	8.390	7.380	7.880	5.460	8.010	7.880	7.230	6.820
42	8.660	7.560	7.930	6.690	8.260	7.900	7.480	7.010
45	8.560	7.610	7.800	6.810	8.120	7.880	7.550	7.130
48	8.630	7.630	7.790	6.790	8.210	7.890	7.530	7.110

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2. Time-course of cell growth of M HBsAg production in recombinant yeast P3B8.

Time (h)	OD_{660}	Viable cell (colonies/ml x10^7)	HBsAg (ng/mg protein)	HBsAg (μg/l culture)
0	0.109			
4	0.166			
8	0.289			
11	0.533			
14	1.590	4.580	1.13 ± 0.203	0.40 ± 0.00
18	4.370	5.630	8.82 ± 0.29	10.01 ± 0.51
22	7.920	7.240	21.72 ± 3.03	31.58 ± 0.55
26	8.960	8.420	27.16 ± 3.52	58.06 ± 0.92
30	10.280	8.900	52.25 ± 1.84	80.01 ± 0.22
34	10.200	9.400	63.93 ± 3.84	91.89 ± 9.45
46	10.250	9.980	76.76 ± 3.57	113.78 ± 10.33
66	10.750	10.400	88.46 ± 11.53	136.64 ± 13.90
90	10.800	10.380	100.09 ± 5.53	146.92 ± 10.33
110	10.730	9.200	87.11 ± 1.36	128.07 ± 8.91

Note: The recombinant yeast was cultivated in High-Pi and induced with Low-Pi at OD_{660} 0.5. The cells were harvested at 14 h of cultivation time.

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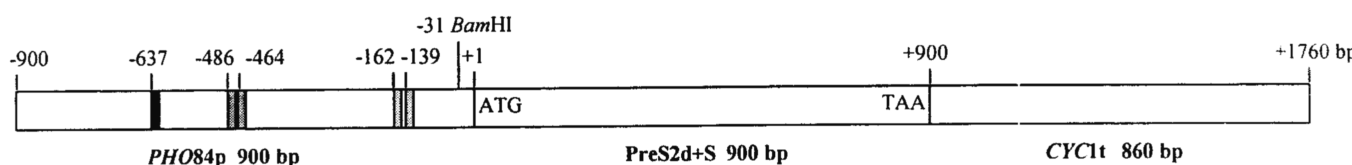
APPENDIX E

1. *PHO*84p sequence with PHO4 protein-binding motif 3'CACGTG 5' or 3'CACGTT 5', TATA box and ATG start codon of PreS2d+S gene are underlined (Bun-ya *et al.*, 1991).

5'cacgttggtg ctgttatagg cgccctatac gtgcagcatt tgctcgtaag
ggccctttca actcatctag cggctatgaa gaaaatgttg cccggctgaa
aaacacccgt tcctctcact gccgcaccgc ccgatgccaa tttaatagtt
ccacgtggac gtgttatttc cagcacgtgg ggcggaaatt agcgacggca
attgattatg gttcgccgca gtccarcgaa atcagtgaga tcggtgcagt
tatgcaccaa atgtcgtgtg aaaggctttc cttatccctc ttctcccgtt
ttgcctgctt attagctaga ttaaaaacgt gcgtattact cattaattaa
ccgacctcat ctatgagcta attattattc cttttggcca gcatgatgca
accacattgc acaccggtaa acaccggtaa tgccaactta gatccactta
ctattgtggc tcgtatacgt atatatataa gctcatcctc atctcttgta
taaagtaaag ttctaagttc acttctaaat tttatctttc ctcatctcgt
*Eco*RI
agatcaccag ggcacacaac aaacaaaact ccacgaagaa ttcgagctcg
*Kpn*I *Bam*HI
gtacccgggg atcctaagag cagtcatcct caggccatg 3'

2. DNA boundary of *PHO*84p, PreS2d+S and *CYC*1t.



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APPENDIX F

Gene Designation	Definition/Gene product
*ADH*1	Alcoholdehydrogenase I
*ADH*2	Alcoholdehydrogenase II
*AOX*1	Alcohol oxidase I
BRF	Component of RNA polymerase III transcription initiation factor
*CYC*1	Cytochrome-c-isoform 1
GAP/GLD	Glyceraldehyde phosphate dehydrogenase
GLN	Glutamine
HCS	G1/S-specific cyclin
H2K	Major histocompatibility complex
*LEU*2	β-isopropylmalate dehydrogenase
*MF*α	Mating factor α gene
MOX	Methanol oxidase
MT	Metallothionein
PGK	Phosphoglycerate kinase
*PHO2/BAS2/GRF*10	Homeodomain protein
*PHO*3	Acid phosphatase
*PHO*4	Basic helix-loop-helix transcription factor
*PHO*6	Zinc-finger regulatory protein
*PHO*7	Protein required for *PHO*3 expression
*PHO*8	Vacuolar alkaline phosphatase

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APPENDIX F

(continued)

Gene Designation	Definition/Gene Product
*PHO*9/*PEP*4	Proteinase A
*PHO*10	Acid phosphatase
*PHO*11	Acid phosphatase
*PHO*13	4-Nitrophenylphosphatase
*PHO*80	Cyclin
*PHO*81	Cyclin-dependent kinase
*PHO*82	Constitutive mutation of *PHO*4
*PHO*84	High-affinity phosphate transporter
*PHO*85	Cyclin-dependent kinase
*PHO*86	Protein associated with phosphate transport Complex
*PHO*87	Member of phosphate permease family
*PHO*88	Membrane protein
*PHO*89	High-affinity Na^+-dependent phosphate Transporter
*TDH*3	Gyceraldehyde-3-phosphate dehydrogenase3
*TRP*1	Phosphoribosylanthranilate isomerase
*U*2	Small nuclear RNAs (snRNAs)
*URA*3	Orotidine-5'-phosphate decarboxylase
*XPR*2	Protease-encoding gene PreS2 region

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