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Characterising Yeast Isolates from Malaysia towards the Development of Alternative Heterologous Protein Expression Systems

(Pencirian Pencilan Yis dari Malaysia untuk Pembangunan Sistem Pengekspresan Protein Heterolog Alternatif)

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ABSTRACT

Yeasts with GRAS (Generally Regarded as Safe) status are commonly used as hosts for heterologous protein production. Yeasts are suitable expression hosts as they have been extensively characterised genetically. The objective of this project was to isolate yeasts from Malaysian food sources and subsequently to develop these as alternative hosts for heterologous protein production. Yeasts were isolated from Malaysian traditional fermented food namely 'tapai', 'tuak' and 'ragi'. A total of 23 isolates were obtained and subjected to molecular identification by amplification and sequencing of the universally conserved ribosomal internal transcribed spacer (ITS), 26S rDNA and 18S rDNA sequences. We identified three species of yeasts, Saccharomyces cerevisiae, Hanseniaspora guilliermondii *and* Pichia anomala, *which have a long tradition of usage in food production and have no adverse effects on humans. To test the feasibility of the yeasts as heterologous expression hosts, we have constructed an integrative vector, p1926Zeo containing the yeast 26S rDNA and Zeocin® resistance cassette. The p1926Zeo vector was linearised and transformed into both* P. anomala *and* H. guilliermondii *isolates via electroporation. Both hosts were successfully transformed at a relatively high efficiency. The transformants obtained had a growth profile similar to the respective wild type, indicating that integration of the plasmids into the host chromosome did not affect growth. These transformants were stable as they exhibited resistance to Zeocin even after 20 generations. Thus, both* P. anomala *and* H. guilliermondii *isolates exhibited the potential to be further developed as alternative heterologous protein expression hosts.*

Keywords: Hanseniaspora guilliermondii*;* heterologous protein*;* Pichia anomala*; yeast expression systems*

ABSTRAK

Yis berstatus GRAS (Generally Regarded as Safe) merupakan hos yang kerap digunakan dalam penghasilan protein heterolog. Yis merupakan hos pengekspresan yang sesuai kerana maklumat genetiknya telah difahami secara mendalam. Objektif projek ini adalah untuk memencilkan yis daripada sumber makanan di Malaysia dan seterusnya digunakan dalam pembangunan hos alternatif bagi penghasilan protein heterolog. Yis dipencilkan daripada makanan terfermentasi seperti tapai, ragi dan tuak. Sebanyak 23 pencilan yis telah dikenal pasti berdasarkan pada pengecaman molekul yang melibatkan amplifikasi dan penjujukan kawasan yang terpulihara iaitu penjarak jujukan dalaman (ITS), jujukan 26S rDNA serta jujukan 18S rDNA. Tiga pencilan yis telah dikenalpasti mempunyai potensi untuk dijadikan hos alternatif bagi penghasilan protein heterolog iaitu Saccharomyces cerevisiae, Hanseniaspora guiiliermondii *dan* Pichia anomala *yang telah lama digunakan dalam proses penghasilan makanan dan tidak mendatangkan kemudaratan kepada manusia. Untuk menguji kesesuaian yis tersebut sebagai hos pengekspresan, kami telah membina vektor terintergrasi p1926Zeo. Vektor p1926Zeo mengandungi jujukan 26SrDNA yis dan kaset keringtangan terhadap antibiotik Zeocin®. Vektor p1926Zeo telah dilinearkan dan di transformasikan ke dalam kedua-dua pencilan* P. anomala *dan* H. guilliermondii *melalui kaedah elektroporasi. Kedua-dua hos tersebut berjaya ditransformasi-kan pada kecekapan yang agak tinggi. Profil pertumbuhan transforman mempunyai kesamaan dengan profil pertumbuhan yis asal tersebut. Ini menunjukkan intergrasi plasmid ke dalam kromosom hos tidak menjejaskan pertumbuhan yis tersebut. Transforman ini juga menunjukkan kerintangan terhadap Zeocin walaupun selepas 20 generasi. Oleh itu, kedua-dua pencilan* P. anomala *dan* H. guilliermondii *mempunyai potensi untuk dibangunkan sebagai hos pengekspresan protein heterlog alternatif.*

Kata kunci: Hanseniaspora guiiliermondii*;* Pichia anomala*;* protein heterolog*; sistem pengekspresan yis*

INTRODUCTION

Approximately 50 years after the discovery of the double helix deoxyribonucleic acid (DNA) structure by Watson and Crick (1953), new developments have revolutionised the protein production industry via the introduction of heterologous recombinant protein production in the

pharmaceutical industry. Since the demonstration of insulin mass-production heterologously in *Escherichia coli* by protein recombinant technology (Murray 1971), a range of alternative expression systems employing bacteria, yeasts and mammalian cells has been developed for the production of a wide variety of heterologous recombinant proteins (Gellisen 2005). From the onset of gene technology, yeasts have been amongst the commonly used host cells for the production of heterologous proteins. Several yeasts derived from traditional foods such as *Saccharomyces cerevisiae, Kluyveromyces lactis* and *Yarrowia lypolytica* have obtained the GRAS (Generally Regarded as Safe) status. Yeast genetics has been extensively characterized in comparison to other eukaryotes (Guerra et al. 2006), and in addition, are simply organized ubiquitous eukaryotes able to adapt rapidly to alterations of environmental conditions. Being unicellular organisms, they retain the advantage of bacteria in ease of manipulation and growth capacity (Madzak et al. 2004).

Based on earlier studies, yeasts are able to perform many higher eukaryote specific post-translation modifications such as glycosylation, disulfide bridge formation, folding and proteolytic processing (Miller et al. 2007). In addition, they are capable of producing large amounts of specific proteins in shorter periods of time in comparison to mammalian cells. Thus, yeasts can be used as a donor for genes encoding products of interest or employed as excellent hosts for the production of recombinant proteins (Tamas & Shewry 2006).

The earliest studies in adapting yeasts as hosts for heterologous protein expression was carried out on the baker's yeast *S. cerevisiae* (Carlson et al. 1983). From that time onwards a range of alternative yeast hosts have been developed for heterologous protein expression namely the methylotrophic yeasts such as *Hansenula polymorpha* (Gellisen & Melber 1996), *Pichia pastoris, Pichia methanolica* (Cregg 1999), *Candida boidinii* (Gellisen 2000), the budding yeasts *Kluyveromyces lactis* (Bergkamp et al. 1992) and the dimorphic yeasts, *Yarrowia lipolytica* and *Arxula adeninivoransi* (Gellisen 2005).

Transformation vector plasmids consist of DNA hybrid sequences that are derived from both eukaryotes and prokaryotes. Sequences that are derived from prokaryotes usually consist of the origin of replication (*ori*) and antibiotic resistance genes that play an important role in multiplication and screening while in the bacterial host cell. Sequences originating from eukaryotes are designed to allow integration into the yeast host, where these elements will play selective roles during the transformation. The sequences chosen for designing the vector selective elements are those that are not found in the yeast host. For example *HIS4* and *Sh ble* used in *P. pastoris* (Cregg 1999), *ADE* in *P. methanolica, LEU2* and *URA3* in *H. polymorpha* (Gellisen et al. 1995).

Vectors used in yeasts for recombinant protein production are designed to integrate into the host genome either by random or homologous integration, in which for the latter, the target is the DNA at a specific locus of the yeast host genome. For example, in *P. pastoris* and *P. methanolica*, the integration target is at either one of the two alcohol oxidase (*AOX*) genes or at the Histone 4 locus (*HIS4*) (Ilgen et al. 2005). Whereas in *H. polymorpha*, it is either by specific or random integration at certain loci of the genome by substitution and disruption of the *TRP3* locus (Agaphonov et al. 1995), at *ARS* sequences (Sohn et al. 1996), at *LEU2* (Brito et al. 1999) or the rDNA regions (Cox et al. 2000).

Nevertheless, every expression host has its own shortcomings. For example *P. pastoris* is one of the most used hosts in production of recombinant proteins, however it has only been used at industrial scale for producing the Hepatitis B vaccine. This is because the *P. pastoris* expression system utilises methanol as its carbon source and inducer, and methanol can be hazardous to human health if it is used extensively in recombinant protein production for commercial purposes (Hellwig et al. 2005).

Thus, it is not surprising that researchers all over the world are currently investigating the possibilities of discovering new yeasts or modifying available yeast hosts as alternative hosts for recombinant protein production. The ultimate aim of this project is to develop yeast expression systems from local resources as alternatives to those covered by patents. Yeasts that will be used as expression hosts in this research were isolated from Malaysian traditional fermented foods, namely *tapai, tempe* and *ragi*. This will enable us to obtain yeast strains that are potentially Generally Recognized as Safe (GRAS) and those that are Food-Additive compatible according to the Food and Drug Administration (FDA).

Materials and Methods

Identification of Yeasts Isolates

The yeast isolates were identified based on the universal conserved ribosomal DNA (rDNA) sequences. The conserved ribosomal DNA regions were amplified using the following primers: for Internal Transcribed Spacer (ITS) amplification; ITS Forward: 5' GTCTCCGTTGTTGGACCAGC 3' and ITS Reverse: 5' ATATGCTTAAGTTCAGCG GGT 3' (White et al. 1990), for 18S rRNA amplification; 18S Forward: 5' AACCT GGTTGATCCTGCCAGT 3' and 18S Reverse: 5' TGATCCTTCTGCAGGTT CACCTAC 3' (Medlin et al. 1988), and for 26S rRNA amplification; 26S Forward: 5' GCATATCAATAAGCGGAGGAAAAG 3' and 26S Reverse: 5' GGTCCGTGTTTC AAGACG 3'. The amplified products were cloned into pGEMT Easy vector (Promega, USA) and were sequenced.

Construction of P1926ZEO

For the construction of p1926Zeo, the 26S rDNA integration fragment and Zeocin resistance cassette were ligated into the backbone of pUC19. The 26S rDNA integration fragment was flanked by *Nde*1 sites using

the primers, 26SNDE1Forward (5'CGATTA**CATATG**G CAGAAATCA 3', *Nde*1 restriction site in bold letters and underlined) and 26SNDE1Reverse (5' AGTGATTGT**CATATG**ACACGGACC A 3', *Nde*1 restriction site in bold letters and underlined) via PCR. The resulting amplicons were digested with *Nde*1, gel purified and ligated into pUC19 digested with *Nde*1. The Zeocin resistance cassette fragment was flanked by *BsRD*1 and *Sca*1 using the primers ZeoBsrd1Forward (5' CCTT**GCAATG**TGCGGATCCC 3', *BsrD*1 restriction site in bold letters and underlined) and ZeoSca1Reverse (5'CACATGTTG**TCATGA**AG CTTGCAAA 3'), *Sca*1 restriction site in bold letters and underlined), also via PCR. The resulting amplicon was digested with *BsrD*1 and *Sca*1, gel purified and ligated into pUC19 digested with *BsrD*1 and *Sca*1.

TRANSFORMATION OF *H. guilliermondii* AND *P. anomala* WITH p1926SZeo

The plasmid p1926Zeo was amplified in *Escherichia coli*, prepared using the EndoFree™ Plasmid Midi Kit protocol and reagents (Qiagen, USA), linearised with *Bgl*II restriction enzyme and measured by spectroscopy at 260 nm. The following transformation procedure was employed. Both the yeasts *H. guilliermondii* and *P. anomala* were grown overnight in Yeast-Peptone-Dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose). Twenty microlitres of yeast culture were transferred into 100 mL of YPD medium and incubated at 30°C until the cells reached log phase at an absorbance reading of 1.5-2.0 at 600 nm (A_{600nm}). Both the yeast (*H. guilliermondii* and *P. anomala*) cells were harvested by centrifugation. For each transformation, 8×10^{10} cells were suspended at room temperature for 30 minutes in 8 mL of 100 mM LiAc, 10 mM DTT, 0.6 M sorbitol and 10 mM Tris-HCL, pH 7.5. The cells were then pelleted, resuspended in 1.5 mL of ice cooled 1 M sorbitol at a final concentration of about 10^{10} cells/mL. The cells were mixed with 100 ng of linearised p1926SZeo, transferred to a 0.2 cm gap vial, and incubated 5 minutes on ice. The electroporating pulse was applied at 1.5 kV, 25 μF, 200 Ω using Gene Pulser Xcell (Biorad, USA). The electroporated cells were immediately diluted in 1 mL of ice-cold 1 M sorbitol, and 50 to 200 μl aliquots were spread on plates containing 100 μg/mL Zeocin in YPD medium agar. The absence of a yeast origin of replication in the plasmids assured that the transformed yeasts carried the plasmid integrated into the yeast host. Transformants grew on YPD agar medium supplemented with Zeocin, between 2 to 3 days at 30°C. Untransformed *P. anomala* and *H. guilliermondii* failed to grow in this medium.

GROWTH RATE PROFILING FOR *P. anomala* AND *H. guilliermondii* TRANSFORMANTS

Positive transformants of both *H. guilliermondii* and *P. anomala* that had resistance toward Zeocin up to 1500 μg/mL were cultured in 5 mL YPD broth. Spectometry absorbance reading A_{600nm} was performed every four hours over 48 hours.

STABILITY OF TRANSFORMANTS CARRYING RESISTANCE TO ZEOCIN

To test the stability of the transformants carrying p1926Zeo, clones were grown in liquid YPD medium, without selective pressure, for 40 generations for *H. guilliermondii* and 24 generations for *P. anomala*. In brief a cloned cell was initially inoculated in 5 mL YPD liquid medium (1×10^5 cells/mL) and incubated for 24 h at 28°C with aeration. The cells were diluted and plated onto YPD agar media to get approximately 100 colonies per plate and sub-cultivated in fresh 5 mL YPD medium. After incubation, the number of colonies presenting resistance towards Zeocin (100 μg/mL) was counted (Guerra et al. 2006).

RESULTS AND DISCUSSION

IDENTIFICATION OF YEAST ISOLATES

A total of 23 yeast isolates were obtained and subjected to molecular identification. Molecular identification involves the amplification and sequencing of ribosomal DNA (rDNA), a highly conserved DNA region universally found in living cells. Based on internal transcribed spacer (ITS) sequences, we identified numerous species of yeasts, namely, *Saccharomyces* sp., *Hanseniaspora* sp., *Candida* sp. and *Pichia* sp., isolated from Malaysian traditional fermented food. The yeast species identified and their origins are listed in Table 1. Three yeast species identified, for example *Pichia anomala, Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii*, are routinely used in food production and are safe for human consumption. *P. anomala* has been used extensively in alcoholic drink production such as Japanese *sake* (Yoshizawa 1979) and Indian *Hamei* (Jeyaram et al. 2008). *H. guilliermondii* has been used in large scale production of acetion, a type of food additive. Acetion is an aromatic compound in many dairy products, in particular, butter (Teixeira et al. 2002). *S. cerevisiae* has the longest history of use in food production especially bread (Dietrich et al. 2004). Due to the vast usage in food production and the promising GRAS status, *P. anomala, S. cerevisiae* and *H. guilliermondii* were subjected to further amplification and sequencing of 18S rDNA and 26S rDNA genes. The 18S rDNA and 26S rDNA data sequences further confirmed the identity of the yeast species (data not shown).

CONSTRUCTION OF p1926Zeo

Generally, integrative expression vectors are designed to integrate into the host's genome to obtain stable transformants. We have identified the rDNA sequence as a target for vector integration into the host genome (by homologous recombination). rDNA was chosen because it serves as a wide-range or universal target that can be used in a variety of fungal hosts. The rDNA also allows multiple integration as rDNA clusters are present in multiple copies

TABLE 1. Isolated yeast cultures from food sources of Malaysia

Sample	Origin	Species
1	Bangi 1-Tapai pulut (1)	*Haneispora guilliermondii*
2	Bangi 2-Tapai pulut (2)	*Candida glabrata*
3	Bangi 3 -Tapai pulut (3)	*Candida glabrata*
4	Kuala Terengganu -Tapai pulut 1 (4)	*Candida carpohila*
5	Kuala Terengganu -Tapai pulut 2 (5)	Candida tropicalis
6	Kota Baru-Tapai Pulut 1(6)	*Pichia carabicca*
7	Kota Baru-Tapai Pulut 2 (7)	*Candida tropicalis*
8	Kajang-tapai ubi 1(8)	*Isenchenkia orientalis*
9	Kajang-tapai ubi 2 (9)	*Isenchenkia orientalis*
10	Kajang-tapai nasi 1(10)	*Isenchenkia orientalis*
11	Hentian Kajang-tapai nasi 1(11)	*Isenchenkia orientalis*
12	Hentian Kajang-tapai nasi 2(12)	*Pichia anomala*
13	Melaka-tapai nasi 1(13)	*Candida tropicalis*
14	Melaka-tapai nasi 2 (14)	*Candida* sp.
15	Melaka-(ragi 1)	*Pseudozyma parantica*
16	Melaka-(ragi 6a)	*Candida metapsilosis*
17	Terengganu –(ragi 15)	*Candida sp*
18	Kedah –(ragi 17)	*Pichia anomala*
19	Sarawak –(ragi 20a)	*Pichia anomala*
20	Sabah –(ragi 21)	*Saccharomyces cerevisiae*
21	Kuala Terengganu-(ragi12)	*Saccharomycetales* sp.
22	Sarawak- (ragi 20b)	*Pichia* sp.
23	Sabah-(ragi 23)	*Saccharomycetales* sp.

in the genome (Cox et al. 2000). A ~1.5kb *S. cerevisiae* rDNA sequence was amplified and subcloned into pUC19 using *Nde*1 restriction sites (plasmid P1926S (4.1 kb)) (Figure 1(a)).

A 1.2 kb Zeocin resistance cassette was amplified from the plasmid, pPICZαC (Invitrogen, USA) and sub-cloned into pUC19 vector partially replacing the ampicilin resistance (424bp) cassette using *Sca*1 and *BsrD*1 restriction sites (Plasmid p19ZeoC (3.5 kb)) (Figure 1(b)). Zeocin resistance cassette was selected based on earlier antibiotic screening of yeasts in our collection whereby the *P. anomala, S. cerevisiae* and *H. guilliermondii* cultures were susceptible to Zeocin at 100 µg/mL (data not shown). Furthermore this selection agent is effective in multiple cell types, so these expression vectors only need to carry one drug selection marker, making the subcloning and transformation easier and more efficient (Calmels et al. 1991). Finally, the Zeocin cassette was released from p19ZeoC by restriction enzyme digestion and ligated into p1926S, by replacing the DNA fragment between the *Sca*1 and *Hin*d III restriction enzyme sites. The resulting plasmid construct was named p1926Zeo (4.9 kb) (Figure 1(c)).

TRANSFORMATION OF *H. guilliermondii* AND *P. anomala* WITH p1926SZeo

Higher transformation efficiencies will enable a higher probability of achieving transformants with high copy numbers. Yeasts transformation is usually done by electroporation; this is because electroporation is less time consuming compared to the spheroplast approach. Both approaches can yield 10^3 to 10^4 transformants/μg DNA. Much lower efficiencies are usually obtained due to use of inefficient integration sites and difficult inserts. Transformation efficiencies are even lower with chemical transformation (Invitrogen, USA).

In work described by Wu and Letchworth (2004), yeasts pretreated with 0.1 M lithium acetate (LiAc) and 10 mM dithiotreitol (DTT) before electroporation had increased transformation efficiency of approximately 150 fold compared to electroporation done without prior pre-treatment. In this work, p1926Zeo was linearised with *Bgl*II, diluted and transformed into both *H. guilliermondii* and *P. anomala* under the standard conditions described in the Materials and Methods section emulating work done by Wu and Letchworth (2004). *H. guilliermondii* highest transformation efficiency was recorded at 8.8×10^4 transformants/µg DNA when 1500 ng DNA was used and the efficiency decreased when more or less than 1500 ng DNA is used (Table 2). In *P. anomala* however, the highest transformation efficiency was observed at 4.4×10^4 transformants/μg DNA when 100 ng DNA was used and decreased with higher amounts of DNA (Table 3). On average, transformation efficiencies for both *P. anomala* and *H. guilliermondii* were in the range of 10^4 transformants/µg DNA under the conditions described in this work.

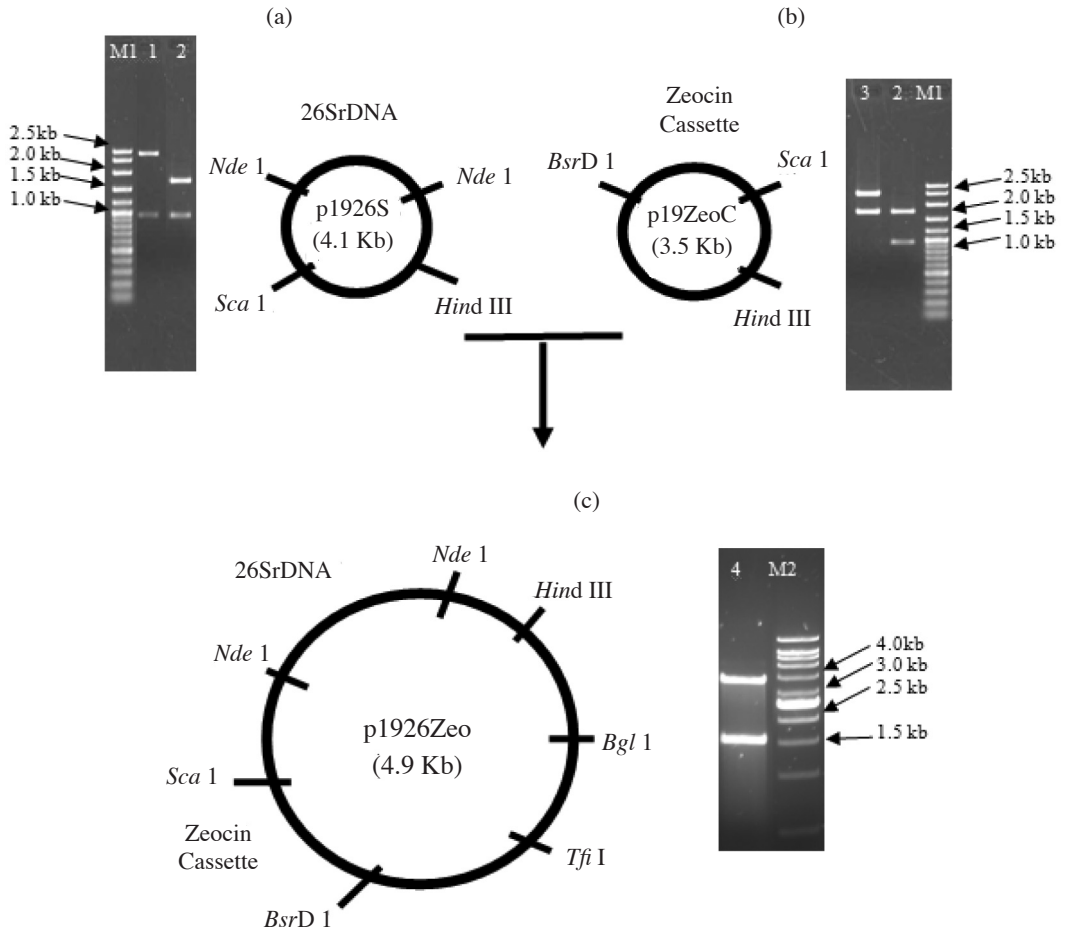

FIGURE 1. The strategy to construct the plasmid vector, p1926Zeo, involves the ligation of the Zeocin cassette from p19ZeoC (Zeocin resistance cassette inserted into pUC 19 vector using *Sca*1 and *BsrD*1 restriction sites) by replacing a DNA fragment of the p1926S vector (26S rDNA inserted into pUC 19 vector using *Nde*1 restriction sites) at the *Sca*1 and *Hind*III restriction sites. 1: p1926S digested with *Sca*1 and *Hind*III; 2:p19ZeoC digested with *Sca*1 and *Hind*III; 3: pUC19 digested with *Sca*1 and *Hind*III; 4: p1926Zeo digested with *Nde*1; M1: 100 bp marker (Vivantis) and M2: 1 kb marker (Vivantis)

TABLE 2. The effect of DNA concentration on transformation efficieny of *H. guilliermondii*

DNA (ng)	Total Transformants *(n)* ($\times 10^4$)	Transformants/µg DNA*(n)* ($\times 10^4$)
50	0.001 ± 0.002	0.05± 0.040
100	0.44 ± 0.02	4.4± 0.20
300	1.15 ± 0.03	3.5± 0.10
500	0.85 ± 0.05	1.7± 0.10

TABLE 3. The effect of DNA concentration on transformation efficieny of *P. anomala*

DNA (ng)	Total Transformants *(n)* ($\times 10^4$)	Transformants/µg DNA*(n)* ($\times 10^4$)
500	0.6 ± 0.05	1.1± 0.10
1000	4.7 ± 0.70	4.7± 0.70
1500	13.2 ± 0.60	8.8± 0.40
2000	15.8 ± 0.30	7.9± 0.30

GROWTH RATE PROFILING FOR *P. anomala* AND *H. guilliermondii* TRANSFORMANTS

Growth profiles of *P. anomala* and *H. guilliermondii* displayed typical lag, log and stationary phases under conditions carried out in this work. In this study, the growth profiles for both *P. anomala* (Figure 2(a)) and *H. guilliermondii* (Figure 2(b)) transformed with p1926Zeo resembled the wild-type growth profile. Both *H. guilliermondii* and *P. anomala* transformants took 40 hours to reach stationary phase. The near similarity between growth profiles for both *P. anomala* and *H. guilliermondii* transformed with p1926Zeo with their respective wild type growth profiles, shows that integration of p1926Zeo into the chromosomes did not interfere with the yeast metabolic pathways related to growth.

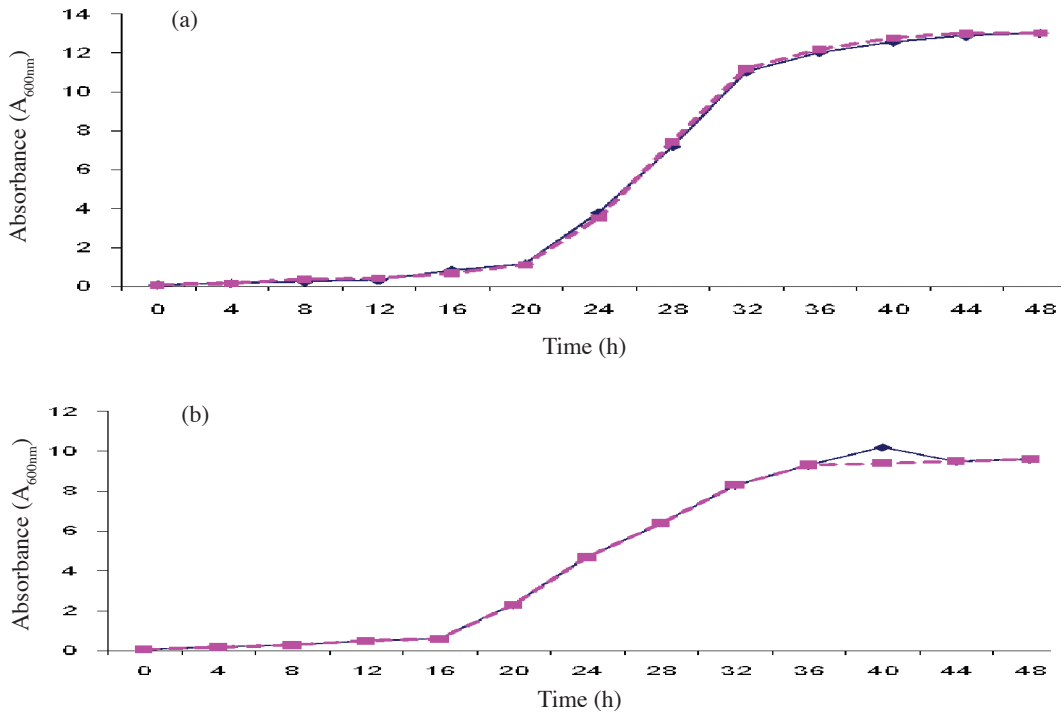

FIGURE 2. Growth curve profiles based on the spectrometry absorbance (A_{600nm}) values of cells grown over a period of 48 h for (a) wild type *P. anomala* ( ) and *P. anomala* transformed with p1926Zeo ( ), and (b) wild type *H. guilliermondii* ( ) and *H. guilliermondii* transformed with p1926Zeo ( )

STABILITY OF TRANSFORMANTS CARRYING RESISTANCE TO ZEOCIN

In this study, the transformants used for the stability test for both *P. anomala* and *H. guilliermondii* were transformants resistant up to 1500 µg/mL Zeocin. *H. guilliermondii* was cultured in YPD medium over 40 generations (80 h; doubling time of 1 h 48 min); *P. anomala* was cultured for 24 generations (48 h; doubling time of 2 h). Both *P. anomala* and *H. guilliermondii* sub-cultured on YPD medium containing 100 µg/mL of Zeocin were still able to maintain viability even after 5 cycles of sub-cultivation. This showed that after 40 generations for *H. guilliermondii* and 24 generations for *P. anomala* transformed with p1926Zeo, the transformants were able to maintain Zeocin resistance activity due to the presence of *sh ble* gene in the Zeocin resistance cassette (Cereghino & Cregg 2000).

CONCLUSION

Two highly potential GRAS yeasts, *P. anomala* and *H. guilliermondii*, were identified from Malaysian food sources. These yeasts were tested for transformation efficiency and stability with the integrative vector p1926Zeo and have shown promising results. These yeasts are currently being developed as heterologous protein expression hosts.

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