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Construction and Expression of *Pet* Operon using Shuttle Vector for Mesophilic and Thermophilic Bacteria

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ABSTRAK

Konstruksi dan Ekspresi *Pet* Operon Menggunakan *Shuttle Vector* untuk Bakteri Mesofilik dan Termofilik. *Eny I. Riyanti dan Peter L. Rogers.* Keuntungan fermentasi etanol pada suhu tinggi mendorong penelitian perakitan bakteri termofilik etalogenik. Selain itu, kemampuan bakteri termofilik dalam penggunaan gula pentosa hasil degradasi biomasa memberi peluang untuk menekan biaya produksi bioetanol. Tujuan dari penelitian ini adalah untuk mengkonstruksi *pet* (*production of ethanol*) operon dengan menggunakan *shuttle vector p*MK18 dan melihat ekspresinya dalam bakteri mesofilik dan termofilik. Konstruksi dan ekspresi *pet* operon dengan menggunakan *adh*T dari bakteri termofilik dan *pdc* dari bakteri mesofilik, dan penggunaan mesofilik-termofilik *shuttle vector* sebagai *backbone*-nya baru pertama kali dilaporkan. *Pet* operon adalah suatu susunan gen penyandi produksi etanol yang terdiri dari gen *pdc* (*pyruvate decarboxylase*) dan *adh* (*alcohol dehydrogenase*). Konstruksi *pet* operon menggunakan gen *adh*T dari bakteri termofilik *Geobacillus thermoglucosidasius* M10EXG dan *pdc* (*pyruvate dehydrogenase*) dari bakteri mesofilik *Zymomonas mobilis* ZM4 telah dilakukan dengan menggunakan mesofilik-termofilik *shuttle vector p*MK18. Ekspresi *pet* operon pada bakteri mesofilik *Eschericia coli* dapat memproduksi 0,3 g/l etanol dengan aktivitas *adh*T sekitar 0,02 U/mg protein dan aktivitas *pdc* sekitar 0,004 U/mg protein. Perlu dilakukan penelitian lanjutan untuk perbaikan konstruksi *pet* operon untuk sistem termofik pada *Thermus thermophilus* HB27, karena konstruksi yang didapat belum optimum untuk sistem termofilik ini. Hasil ini diharapkan akan mengawali pengembangan teknik manipulasi genetik pada bakteri termofilik yang masih sangat terbatas, khususnya pengembangan teknik manipulasi termofilik etanologenik.

Kata kunci: Etanol, bakteri termofilik, bakteri mesofilik, *pet* operon, ekspresi gen.

INTRODUCTION

The natural ability for thermophiles to utilize a wide range of sugars, including pentose, at high temperature (Larsen *et al*. 1997) renders them as potential microorganisms for ethanol production from cheap lignocellulosic materials (Olsson and Hahn-Hagerdal 1996), which consist mainly of cellulose, hemicellulose and lignin. In addition, high temperature fermentation promises cost savings at an industrial scale, mainly through the reduction of cooling costs following enzymatic pre-treatment (Klapatch *et al*. 1994, Banat and Marchant 1995). Other advantages of fermentations carried out at evaluated temperatures include lower contamination risks (particularly by mesophilic contaminants) and reduced energy costs for ethanol recovery (Edwards 1990, Lowe *et al*. 1993, Banat *et al*. 1998).

The potential advantages associated with fermentation carried out at elevated temperatures have stimulated a significant interest in developing thermophilic ethanologens. However, naturally isolated ethanol producing thermophilic yield very low ethanol compared to mesophilic counterpart (Dien *et al*. 2003, Desai *et al*. 2004, Demain *et al.* 2005, Chinn *et al*. 2006, Keating *et al*. 2006, Stephanopoulos 2007). Current research on increasing ethanol yields in potential thermophilic ethanologens has been focused on optimization of fermentation conditions. Limited metabolic manipulations have also been reported in these thermophiles, including the development of high ethanol tolerant (Ljungdahl and Carreira 1983, Lovitt *et al*. 1988, Bryant *et al*. 1992) and LDH-negative mutant strains. So far, the metabolic/genetic manipulation of ethanol producing thermophiles was not revealed yet.

In mesophilic bacteria such as *Zymomonas mobilis*, gene *pdc* and *adh* were reported to be involved in the production of ethanol (Conway *et al*. 1987a, 1987b). A number of thermostable *adh*T, have been isolated and characterized because of possible advantages associated with the use of thermophilic microorganisms and their enzymes in biotechnological processes (Coolbear *et al*. 1992, D'Auria *et al*. 1996). Thermostable variants of ADH from the obligately fermentative *Z. mobilis* have also been investigated (Rellos *et al*. 1998). However no *adh*T expression has been reported previously in *T. thermophilus*.

There is no report of the isolation of *pdc* genes from thermophilic microorganisms or heterologous expression of thermostable *pdc* genes in thermophiles. It has been suggested that thermophilic ethanologens do not possess PDC enzymes (Payton 1984). However *pdc* genes have been isolated from a number of mesophiles with reports of at least four *pdc* genes

isolated from other bacteria being heterologously expressed in *Escherichia coli* (Raj *et al*. 2002). Interestingly, PDC enzymes from the Gram-negative mesophiles *Z. mobilis*, *Acetobacter pasteurianus* and *Zymobacter palmae* were found to be thermostable, retaining 60 to 100% activity after incubation for 30 min at 60°C, while PDC from the Gram-positive *Sarcina ventriculi* was denatured at temperatures higher than 50°C (Raj *et al*. 2002).

The homo-fermentative ethanol pathway has been metabolically engineered into autotrophic cyanobacteria via the introduction of the *pet* operon containing *pdc* and *adh* genes encoding the enzymes PDC and ADH respectively (Deng and Coleman 1999). This study was aimed to investigate the possibility of *pet* operon expression containing combination of mesophilic *pdc* from *Z. mobilis* ZM4 and thermophilic gene *adh*T (from *G. thermoglucosidasius* M10EXG) (with native promoter) using mesophilic-thermophilic shuttle vector, *p*MK18.

MATERIALS AND METHODS

Thermophilic bacteria *G. thermoglucosidasius* strain M10EXG (Fong 2004) was used as a source of *adh*T and mesophilic bacteria *Z. mobilis* ZM4 (ATCC31821) was used as a source of *pdc*.

Mesophilic bacteria *E. coli* strain DH5α (Invitrogen, USA) and JM109 (Promega, USA) were used for construction and expression study of the *pet* operon in mesophilic system, while *T. thermophilus* HB27 was used as a host for gene expression in thermophilic system.

Mesophilic plasmid *p*BBR1MCS4 (Kovach *et al*. 1995) and *p*CR®II-TOPO® (Invitrogen, USA) were used for the *pet* operon construction. pLysS (Novagen, Germany) was used as a source for *cat* (chloramphenicol acethyltransferase) gene. Thermophilic-mesophilic shuttle-cloning vector, *p*MK18 (Biotools, Spain) was used as a backbone for cloning and expression *pet* operon. The construct was then expressed in both mesophilic and thermophilic system.

T. thermophilus HB27 (Hoshino pers. com.), *G. thermoglucosidasius* M10EXG (Fong *et al*. 2006) and *E. coli* strains (DH5α, JM109, and recombinant strains) were cultured in Luria-Bertani (LB) medium (Luria and Delbruck 1943) at 70, 60, and 37°C respectively.

Z. mobilis ZM4 (ATCC31821) was cultured at 30°C in GY medium (Jeon 2004) without shaking or on GY plate with parafilm shield as *Z. mobilis* is not strict anaerobe.

Pet Operon Construction

The alkaline lysis plasmid DNA extraction method (Sambrook *et al.* 2001) was used for extraction of recombinant plasmids from *E. coli*. Genomic DNA was extracted using modifications of the method described by Pitcher *et al*. (1989). DNA purification from agarose gels (1% w/v) was carried out using a QIA quick gel extraction kit (Qiagen, California, USA), following the protocol supplied by the Manufacturer.

All PCR reactions were performed in an Eppendorf 8 MastercyclerTM. Taq polymerase and all PCR reagents are supplied by Roche (Basel, Switzerland). Sequencing reactions were performed using BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

Restriction enzyme digestions were carried out at 30°C or 37°C overnight followed by inactivation at 65°C for 20 min and were set up according to the instructtions provided by the Suppliers. Enzymes were supplied by MBI Fermentas (Massachusetts, USA). PCR products were subjected to cleanup before RE digestions. To minimize self-ligation of vectors, linearized vectors were dephosphorylated at 37°C for 15 min followed by inactivation at 65°C for 30 min using Shrimp Alkaline Phosphatase (SAP) (Roche, Germany).

DNA cleanup after RE digestion, dephosphorylation and PCR amplification was carried out using Amicon® Microcon®-PCR Centrifugal Filter Devices (Millipore, USA) following the information supplied by the Manufacturer.

Primers used for the *pet* operon construction in this study were provided in Table 1.

Touchdown PCR method was used for *pdc* and *adh*T amplifications. Touchdown PCR is a PCR method for minimizing non-specific sequence amplifycations. This method uses the melting point primers set as the upper limit of the annealing temperature. The early steps of this method were set with cycles at high annealing temperatures, and then followed by decreasing the increments for every subsequent set of the cycles. The firstly amplified sequence was the one in between regions of the greatest primer specificity; it was most likely that this was the sequence of interest. These fragments were further amplified during the subsequent rounds at lower temperatures, and will out compete the nonspecific sequences to which the primers may bind at the lower temperatures. In this study, the thermal cycling was carried out at 95°C for 5 min, followed by 20 cycles at 95°C for 30 sec, 53 to 47°C for 20 sec (-2°C per 5 cycles), 74°C for 2 min, and 10 cycles at 95°C for 30 sec, 45°C for 20 sec, 74°C for 2

Table 1. Primer used in this study.

Primers	Sequences (5'-3')	Relevance
M10*adh*T*Bam*HI F	CAG GGG ATC CGT GCG AAG TCG CC	Amplification of the whole M10EXG *adh*T with the addition of *BamH*I and *Xho*I sites (underlined) at 5' and 3' ends respectively.
M10*adh*TXhoI R	GTG TTC TCG AGG TTA ATT ATT ATT TTC	
*pdc*XhoIRBS F	GTT TCT CGA GGG AGG G AA AAG CAA TGA GT	Amplification of a 1,764-bp promoterless *pdc* from *Z. mobilis* ZM4 with the addition of a SD region (bold) and a set of inverted repeats (shaded) to the 5' and 3' ends respectively. *Xho*I RE site (underlined) was also introduced at the 5' end of the amplified product.
*pdc*T1 R	TCT GGT AAG AGA AAG GCT TT A AAC TAC	
*pdc*T2 R	AGG AGC TTG TTA TGC	
*pdc*T2*Hind*IIIR	ATG CAT AAA GCC TTT CT G GTA AGA GAA A	
CAT1 F	GGA CTA GTC CAT GGC GCC GCG GAA GAT CAC TTC GCA G	Amplification of the functional cat gene from pLysS flanked with *Spe*I RE sites (underlined), including its own promoter.
CAT2 R	GGA CTA GTC CAT GGT GGC GGC GGA ATT TCT GCC ATT CAT CC	

min, followed by a final extension step at 74°C for 7 min.

The *pet* operon constructions were done in *E. coli* hosts (JM109 and DH5α). A Heat Shock transformation (Sambrook *et al*. 2001) was used for the *E. coli* cells, while the chosen *pet* operon construct in the shuttle vector, *p*MK18-*adh*T-*pdc-Hind*III#7 was then transformed into the *T. thermophilus* HB27 cells using the electroporation method (Riyanti 2007).

Enzimatic Analysis for the *Pet* Operon

Functional expressions of the gene *adh* and *pdc* products in all the resulted recombinant strains were determined by enzymatic assays for Alcohol dehydrogenase (ADH) and PDC. Ethanol was also determined for the detection of the *pet* operon product.

Activity of *pdc* gene was measured using the Carboligase Assay as described by Breuer *et al*. (2002). The ADH enzymatic assay was performed using the method developed by Cannio *et al*. (1994).

Product of the *pet* operon, ethanol, was measured using the High Performance Liquid Chromatography (HPLC) technique with a CTO-10ASVP Column Oven (Shimadzu, Japan) fitted with an HPX-87H ion exchange column (Bio-Rad Laboratories, California, USA) as described by Riyanti (2007).

RESULTS AND DISCUSSION

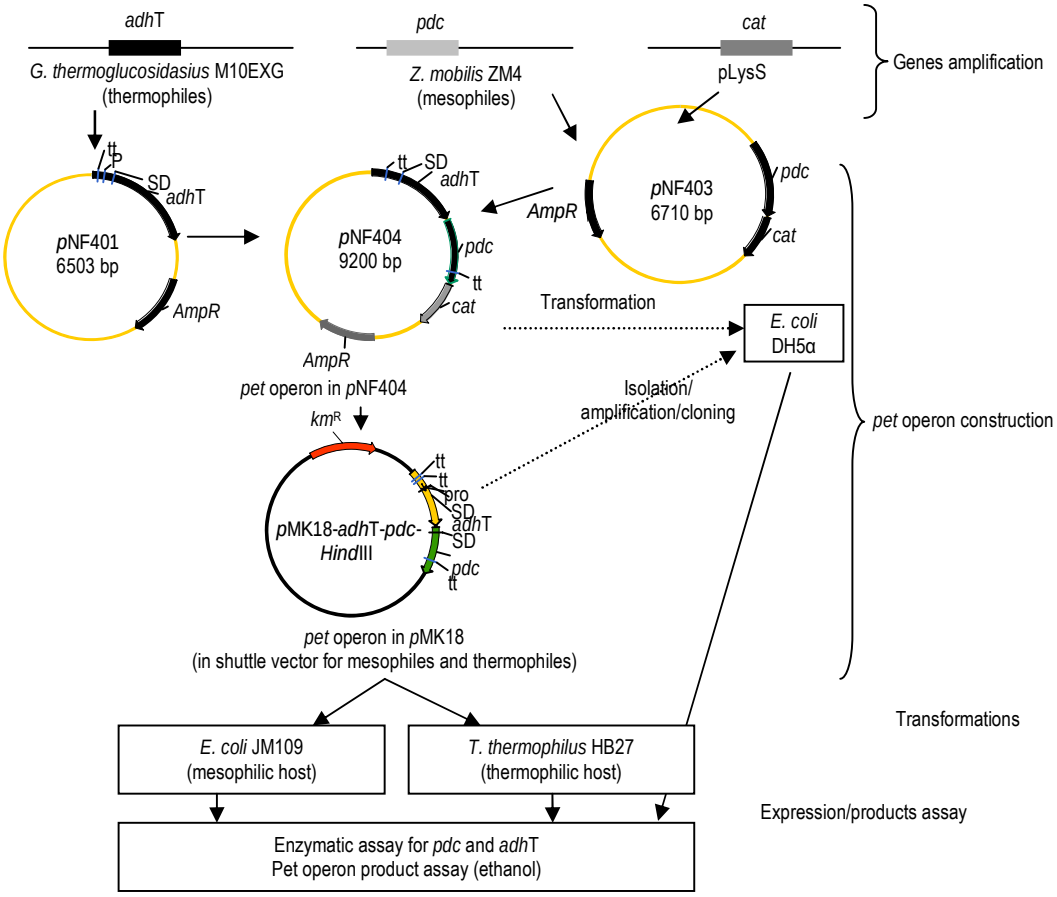
Pet Operon Construction

Strategy for *pet* operon construction was illustrated in Figure 1. The *adh*T fragment (1.553-bp) from *G. thermoglucosidasius* M10EXG chromosomal DNA (Jeon *et al*. 2008) was amplified using primers M10*adh*T*Bam*HI F and M10adhTXhoI R and cloned into *E. coli* based vector, (*p*BBR1MCS4), and was then named as *p*NF401 (Figure 1 and Figure 2B lane 1). The amplified fragment contained the native *adh*T promoter and SD regions but not the transcriptional terminators.

Promoterless *pdc* (1.747 bp) from *Z. mobilis* ZM4 chromosomal DNA was amplified using touchdown and cloned into *p*CR®II-TOPO®. Primers used were *pdc*XhoIRBS F and *pdc*T1 R, which introduced an SD region [identical to that from M10EXGT *adh*T (GGAGGG)] 7 bases from the start codon and an *Xho*I RE site at the 5' end as well as partial inverted repeats, transcriptional terminator sequence of *adhA* from *Z. mobilis* (Keshav *et al.* 1990), at the 3' end of the amplified DNA fragment. A second round of PCR amplification with primers *pdc*XhoIRBS F and *pdc*T2 R resulted in the introduction of a complete set of inverted repeats (AAAGCCTTTCT and AGAAAGGCTTT) at the 3' end of the fragment. The cloned *pdc* with terminator in *p*CR®II-TOPO® was named as *p*NF403 (Figure 1). Gel confirmation of the gene was documented in Figure 2B lane 3.

A functional 977 bp chloramphenicol acetyl transferase (*cat*) cassette that confers chloramphenicol resistance from pLysS was amplified with primers CAT1 F and CAT2R, to facilitate further sub cloning of the promoterless *pdc* fragment downstream of *adh*T. The amplified *Spe*I-flanked *cat* fragment was digested with *Spe*I and sub cloned into *p*NF403 downstream of the promoterless *pdc* fragment (Figure 1).

The *pdc-cat* fragment from *p*NF403 was isolated by gel purification of the *Xho*I-and *Nsi*I-partial digested. The approximately 2.7 kb purified fragment was cloned into *p*NF401 downstream of *adh*T via *Xho*I and *Nsi*I RE sites, generating the approximately 9.2 kb recombinant plasmid *p*NF404, which contained the *pet* operon (*adh*T and *pdc*, approx. 3.3 kb) (Figure 2B, lane 1) as well as ampicillin and chloramphenicol resistant determinants expressed via their own promoters. Figure 2 shows the construction of *pet* operon in *E. coli*-based vector *p*NF404. The map of *pet* operon in the *p*NF404 was shown in Figure 2A.



Pdc = pyruvate decarboxylase, *adh*T = alcohol dehydrogenase, *cat* = chloramphenicol acetyltransferase, *km*^{R} = kanamycine nucleotydyltrasferase, *AmpR* = ampicillin resistance gene, tt = terminator, SD = Shine delgarno region, P = promoter, *Z. mobilis* Zm4 and *E. coli* = mesophilic bacteria, *G. thermoglucosidasius* M10EXG and *T. thermophilus* HB27 = thermophilic bacteria, *p*NF401, *p*NF403, and *p*NF404 = *E. coli*-based vector, *p*MK18-*adh*T-*pdc*-*Hind*III = shuttle vector containing *pet* operon. Enzymatic assay (ADH and PDC) and product assay (ethanol) were performed for both recombinant strains *E. coli* JM109 (*p*MK18-*adh*T-*pdc*-*Hind*III) (in mesophilic system) and *T. thermophilus* HB27 (*p*MK18-*adh*T-*pdc*-*Hind*III) (in thermophilic system) and also for all controls: *E. coli* DH5α (*p*NF404) (as control of *pet* operon construction using *E. coli* based vector), and control mesophilic hosts, *E. coli* DH5α and JM109, and thermophilic hosts, *T. thermophilus* HB27.

Figure 1. Construction and cloning strategy of *pet* operon.

The sub cloning of *pet* operon in shuttle vector *p*MK18 is shown in Figure 3. The *pet* operon from *p*NF404 was double digested with *Sac*I and *Nsi*I, and a total fragment of about 3.1 kb (Figure 3B lane 4) (containing 1.553 bp *adh*T) (Figure 3B lane 1), and 1.747 bp promoterless *pdc* (Figure 3B lane 2) then gel purified. This fragment was then amplified by PCR with MT10adh*Hind*IIIF and *pdc*T2*Hind*IIIR primers. The fragment following *Hind*III digestion of *pet* operon was then ligated into *Hind*III-digested, dephosphorylated *p*MK18. Kanamycin resistance gene (~1 kb, Figure 4 lane 3) was used for selection both in *E. coli* and *T. thermophilus*.

Expression comparison in mesophilic system vs thermophilic system of *pet* operon could be investigated by using the same plasmid which can replicate in both systems. However, genetic manipulation in thermophilic organism is very limited due to the availability of convenient genetic tools. Shuttle vector *p*MK18 was used for cloning and expression study of *pet* operon in both organisms. *p*MK18 is a shuttle-cloning vector for *E. coli* and *T. thermophilus*, and the only commercially available at present. This vector has limitations such as unique cloning sites, low ligation efficiency compared to commercially available mesophilic cloning vector, and low retention in thermophilic host (Riyanti 2007).

The construction processes were done in *E. coli* JM109 (New England BioLabs, Inc, USA). JM109 was chosen instead DH5α as JM109 is an auxotrophic strain which is dam$^-$and met$^-$. These characteristics were reported to prevent methylation of foreign proteins.

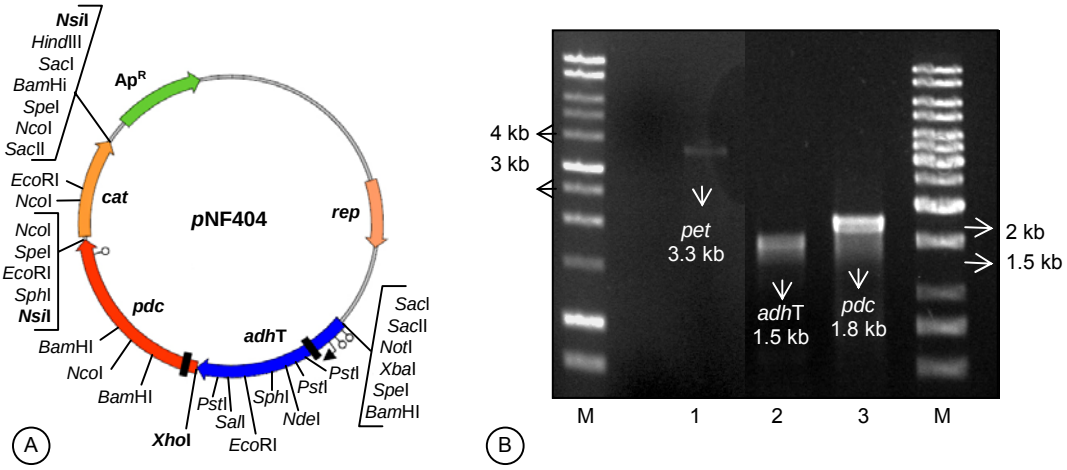


Figure 2. Map of *p*NF404 containing *pet* operon (A) and gel electrophoresis confirmation of *pet* operon construction (B). A: *adh*T = gene encoding thermostable alcohol dehydrogenase with its native promoter from *G. thermoglucosidasius* M10EXG, *pdc* = gene encoding pyruvate decarboxylase (promoterless) from *Z. mobilis* ZM4, *cat* = chloramphenicol resistant determinant (expressed via its own promoter), Ap^R = ampicillin resistant determinant, *rep* = genes required for plasmid replication. ↱ = Putative *adh*T promoter, ▌= Shine-Dalgarno region, (°) = transcriptional terminators, and commonly used RE sites are also shown; B: ~4 kb *adh*T-*pdc-cat* fragment (lane 1), ~1.5 kb *adh*T (lane 2) and ~1.8 kb *pdc* (lane 3) from *p*NF404. M = 1 kb ladder DNA marker.

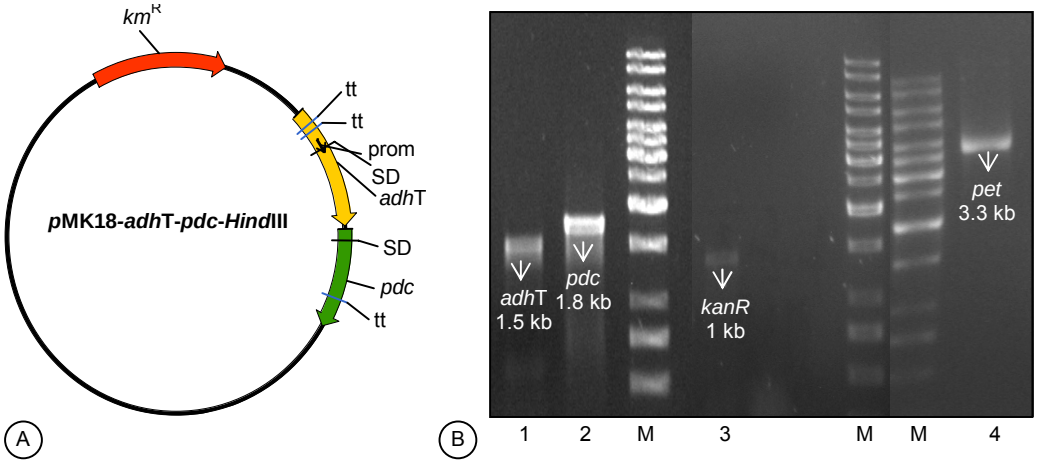


Figure 3. Construction of *pet* operon in *p*KM18 (*p*MK18-*adh*T-*pdc-Hind*III) (A) and gel confirmation of *pet* operon in the shuttle vector *p*MK18 by PCR amplification (B). A: km^R = kanamycin nucleotydiltransferase gene, *adh*T = alcohol dehydrogenase gene from *G. thermoglucosidasius* M10EXG, *pdc* = pyruvate decarboxylase gene from *Z. mobilis* ZM4, (→(promoter) = putative *adh*T promoter, tt = transcription terminators, SD = Shine-Delgarno region; B: 1kb ladder (M), ~1.5 kb *adh*T (lane 1), ~1.8 kb *pdc* (lane 2), ~1 kb kan^R (lane 3), ~3.1 kb *pet* operon (lane 4) from *p*MK18. M = 1 kb ladder DNA marker.

However it was found that the transformation efficiency of *p*MK18-*adh*T-*pdc-Hind*III into *E. coli* JM109 was low at about 1.5 x 10^2 transformants/μg DNA.

Enzymatic Analysis for *Pet* Operon Expression

The *pet* operon expression was evaluated by measuring ADH-T and PDC enzyme activities as well as ethanol production for recombinant *E. coli* JM109 carrying *p*MK18-*adh*T-*pdc-Hind*III#7 (Table 2). Control *E. coli* host strains (DH5α and JM109) and control *E. coli*-based plasmid *p*NF404 (DH5α) was included in the assay. As shown in Table 1 functional ADH-T was detected at 0.02 U/mg protein and PDC of about 0.004 U/mg protein was expressed from the *pet* operon in JM109 using shuttle vector (*p*MK18-*adh*T-*pdc-Hind*III#7). Furthermore, an increase in ethanol production (0.3 g/l) above background level (0.1 g/l) was detected in this recombinant *E. coli strain*. This activity was slightly higher compared to the expression using *E. coli*-based vector *p*NF404 using strain DH5α (0.003 U/mg protein). The control, *E. coli* (DH5α) strain JM109 (*p*MK18) (without *pet* operon) have PDC activity of

about 0.01 U/mg protein. These results show that combination thermophilic-mesophilic genes with thermophilic promoter produced functional operon in mesophilic system.

The construct of *p*MK18-*adh*T-*pdc*-*Hind*III#7 was then transferred into *T. thermophilus* HB27 by electroporation on the medium containing 40 μg/ml kanamycin (Table 3). This recombinant strain *T. thermophilus* HB27 (*p*MK18-*adh*T-*pdc*-*Hind*III#7) was then assayed for the enzymes products (PDC and ADH) and its ethanol production. Control host *T. thermophilus* HB27 was included in the assays. No additional ADH-T or PDC activity and ethanol production from any *T. thermophilus* transformants (*T. thermophilus* HB27 (*p*MK18-*adh*T-*pdc*-*Hind*III#7)) was evident compared to the wild type values (*T. thermophilus* HB27).

From the results, it is suggested that *pet* construction consisting *adh*T (from *G. thermoglucosidasius*) and *pdc* (from *Z. mobilis* ZM4) might not suitable for the expression system in *T. thermophilus* HB27 (due to different metabolic pathway, or coding region), or the concentration of enzyme produced by the *pet* operon was very low due to the low copy number and unstable plasmid in *T. thermophilus* HB27. Stronger promoter or using newly construct shuttle vector *p*POPTE which has higher stability (Riyanti 2007) is suggested for further investigation.

The comparison expression of *pet* operon on mesophilic (*E. coli* JM 109) and thermophilic organisms (*T. thermophilus* HB27) shown on Table 4. Recombinant *E. coli* JM109 (*p*MK18-*adh*T-*pdc*-*Hind*III#7) produced higher ADH activity of about 0.02 U/mg protein, compared to recombinant *T. thermophilus* HB7 (*p*MK18-*adh*T-*pdc*-*Hind*III#7) of about 0.001 U/mg protein (as a background level). No PDC activity was detected in *T. thermophilus* HB27 (*p*MK18-*adh*T-*pdc*-*Hind*III#7), while this enzyme was active of about 0.004 U/mg protein in recombinant mesophile *E. coli* JM109 (*p*MK18-*adh*T-*pdc*-*Hind*III#7). Recombinant *E. coli* JM109 (*p*MK18-*adh*T-*pdc*-*Hind*III#7) produce about 0.3 g/l ethanol, while recombinant *T. thermophilus* HB27 (*p*MK18-*adh*T-*pdc*-*Hind*III#7) produced only 0.01 g/l and it was considered as background level from the wild type (Table 3).

In comparison with other recombinant microorganisms (particularly recombinant *E. coli*) containing the *pet* operon (Ingram and Conway 1988, Ingram *et al*. 1987, Ohta *et al*. 1991), levels of ADH and PDC activity and ethanol production in JM109 (*p*MK18-*adh*T-*pdc*-*Hind*III#7) constructed in this study were low (0.02 U/mg protein, 0.04 U/mg protein and 0.3 g/l respectively). In this study, PDC activity was determined using the carboligase assay, which measures the phenylacetylcarbinol (PAC) formation from the substrates pyruvate and benzaldehyde (Rosche *et al*. 2002). This method was chosen due to interference by lactate dehydrogenase (LDH) in the ADH-coupled decarboxylation reaction used in PDC screening on the thermophilic isolates (Leong 2000). As such, it may be inappropriate to compare PDC activity determined in *p*MK18-*adh*T-*pdc*-*Hind*III#7(JM109) and other previous reported recombinant microorganisms carrying the *pet* operon in which PDC activities were measured using other methods such as the ADH-coupled decarboxylation reaction (Ingram and Conway 1988, Ingram *et al*. 1987) and the fluoride release assay (Ohta *et al*. 1991).

Although there have been reports on the construction and expression of *pet* operons in *E. coli,* these

Table 2. Specific activity of ADH-T and PDC and ethanol production of the recombinant *E. coli* strains.

Strain[a]	Specific ADH-T activity (U/mg protein)	Specific PDC activity (U/mg protein)	Ethanol (g/l)
DH5α	nd	0.001	0.1
JM109	nd	0.001	0.1
*p*NF404 (DH5 α)	0.02	0.003	0.4
*p*MK18(JM109)	nd	0.001	0.1
*p*MK18-*adh*T-*pdc*-*ind*III#7(JM109)	0.02	0.004	0.3

[a]All *E. coli* strains or clones were cultured on an LB medium, nd = non-detectable.

Table 3. Specific activities of ADH-T and PDC and ethanol production by *T. thermophilus* HB27 and its transformant.

Strain[a]	Specific ADH-T activity (U/mg protein)	Specific PDC activity (U/mg protein)	Ethanol (g/l)
T. thermophilus HB27[a]	0.001	nd	0.1
T. thermophilus HB27(*p*MK18-*adh*T-*pdc*-*Hind*III#7)[b]	0.001	nd	0.1

[a]*T. thermophilus* strains (wild type), [b]*T. thermophilus* HB27 transformants (that was obtained from transformation using *pet* construct), nd = non-detectable.

Table 4. Comparison of specific activity of ADH-T and PDC and ethanol production of recombinant *E. coli* JM109 and *T. thermophilus* HB27 strains.

Strain[a]	Specific ADH-T activity (U/mg protein)	Specific PDC activity (U/mg protein)	Ethanol (g/l)
E. coli JM109 (*p*MK18-*adh*T-*pdc-Hind*III#7)	0.02	0.004	0.3
T. thermophilus HB27 (*p*MK18-*adh*T-*pdc-Hind*III#7)	0.001	nd	0.01

nd = non-detectable.

operons were constructed with *adhB* and *pdc* from the mesophile *Z. mobilis* (Ingram and Conway 1988, Ingram *et al*. 1987, Ohta *et al*. 1991, Hespel *et at*. 1996). Variations of this *pet* operon (changes in promoter regions) have also been constructed and expressed in other microorganisms such as *Bacillus subtilis* and *B. polymyxa* (Barbosa and Ingram 1994), some lactic acid bacteria (Gold *et al*. 1996, Nichols *et al*. 2003) and the cyanobacterium *Synechococcus* sp. strain PCC 7942 (Deng and Coleman 1999). However, except in *E. coli*, expression of the *pet* operon is weak and ethanol production is low in these reported recombinant microorganisms.

This combination gene of mesophilic (*pdc*) and thermophilic genes (*adh*T) in one system of functional *pet* operon has never been reported previously. This result also shows that operon system containing combination of mesophilic gene and thermophilic gene, under thermophilic promoter could give functional expression in mesophilic bacteria. However further investigation should be performed for effective expression of *pet* operon in thermophilic system, *T. thermophilus* HB27. These results might also give evidence of suggestion that mesophiles and thermophiles do not share a common ethanol metabolism system. This report also could be used for further genetic manipulation on ethanol production using thermophilic bacteria.

CONCLUSION AND SUGGESTION

In conclusion, a *pet* operon was constructed using thermophilic *adh*T with its own promoter from *G. thermoglucosidasius* M10EXG and mesophilic *pdc* from *Z. mobilis* ZM4. The *pet* operon constructed in the present study was heterologously expressed in recombinant *E. coli* strain JM109 harboring *p*MK18-*adh*T-*pdc-Hind*III#7.

It is possible to engineer and heterologously express *adh*T from *G. thermoglucosidasius* M10EXG and *pdc* from *Z. mobilis* ZM4 as a co-transcribed *pet* operon for ethanol production in *E. coli*. Increased expression of the ethanologenic enzymes in thermophiles may be achieved with genetic improvements of the *pet* operon (such as the use of additional/stronger promoters, alternative shuttle vector, choice of gene source) as well as optimization of culture conditions for ethanol production. The *pet* operon construct should be further manipulated for better expression in thermophilic system.

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