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Master's project in Bioengineering and Biotechnology

Production of a thermostable fumarase in *Saccharomyces cerevisiae* for the bioconversion of fumarate to L-malate

Carried out in the laboratory of HISAO OHTAKE
At Osaka University
Under the supervision of HISAO OHTAKE

Done by

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Under the direction of

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In the Laboratory for Environmental Biotechnology

EPFL

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OSAKA, 19 AUGUST 2011

I. Acknowledgement

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I owe special gratitude to my family, who supported me through this wonderful experience and I thank them for their patience.

Finally, I would like to thank every person who helped me through this project.

II. Summary

The gene encoding fumarase (*fum*) from *Thermus thermophilus* was expressed in yeast *Saccharomyces cerevisiae*. The recombinant cells were heated at 70°C to inactivate indigenous enzymes and used for the bioconversion of fumaric acid to L-malic acid. By heating the host cells at 70°C, substrate is able to go across the heat-damaged membrane of the microorganism and a desired product can be formed. This new concept, called Synthetic Metabolic Engineering (SME), has already been applied successfully in *Escherichia coli*. Unfortunately, *E. coli* membrane is weakened too much during the heat treatment and enzyme leakage appears. The surface structure of yeast is more rigid than that of *E. coli* and this might be taken as an advantage for application of SME. When continuous or repeated-batch reaction is carried out, enzyme leakage becomes a major drawback. It is assumed that yeast cells could overcome this problem by retaining more enzymes in the cell during and after the heat treatment.

In order to prove this hypothesis, a thermophilic fumarase (FUM) was over-expressed in two hosts, *S. cerevisiae* as well as *E. coli*. *fum* was first modified to be over-expressed in yeast cells and FUM was successfully produced in yeasts. Optimization of SME techniques was carried out for yeast cells. Then, enzyme activity and enzyme leakage was investigated for both strains. *E. coli* showed high level of FUM expression, though considerable amount of enzyme leaked to supernatant. On the other hand, even though the level of FUM expression in *S. cerevisiae* was low, yeast cells overcome leakage problem and are re-usable. This study showed the first trial of SME in yeast cells and possibility of utilization of yeast as a host strain for SME.

III. Tables

Abbreviations Table

Abbreviation	Full name
amp	Ampicillin
BSA	Bovine serum albumin
CIAP	Calf intestinal alkaline phosphatase
Cm	Chloramphenicol
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diaminetetraacetic acid
EtBr	Ethidium Bromide
FA	Fumaric Acid, fumarate
FDA	Food and Drug Administration
FUM	Fumarase, Fumarate hydratase
fum	Gene encoding fumarase
GB	Glass Beads shaking
GB-DTT	Glass beads shaking with DTT incubation
GRAS	Generally Regarded As Safe
His	Histidine
HPLC	High Pressure Liquid Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani medium
LMA	L-malic acid, L-malate
ME	Malic enzyme
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced form of NAD+
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP+
NBRP	National Bio-Resource project
OD	Optical density
ODCase	Orotidine-5'-phosphate decarboxylase
ON	Overnight
PAE	Post-alkaline extraction
PAGE	Polyacrylamide gel electrophoresis
pBS	pBlueScript vector
PBS	Phosphate buffer salines
PCR	Polymerase chain reaction
R. opacus	*Rhodococcus opacus*
rpm	Rotation per minute
RT	Room Temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SD_{min}	Synthetic dextrose minimal medium
SD+HIS	SD_{min} + histidin
SDS	Sodium dodecyl sulfate
SN	Supernatant
T. kodakaraensis	*Thermococcus kodakaraensis KOD1*
T. thermophilus	*Thermus thermophilus*
TEs	Thermophilic enzymes
TEMED	Tetramethylethylenediamine $(CH_3)_2NCH_2CH_2N(CH_3)_2$
Tris	tris(hydroxymethyl)aminomethane
URA3	Gene encoding ODCase
X-gal	Bromo-chloro-indolyl-galactopyranoside
YAC	Yeast artificial chromosome
YCp	Yeast centromere plasmid
YEPD	Yeast extract peptone dextrose medium
YEp	Yeast Episomal Plasmid
YIp	Yeast Integrative Plasmid

Origin of used chemicals, substances and instruments

Name	Details	Manufacturer
Agar	Powder	Wako
Ampicillin	anhydrous	Wako
BigDye	Terminator v3.1 sequencing	Applied Biosystems
Biorad protein assay	dye reagent concentrate	BIO-RAD
blunt end buffer	for T4 kinase treatment	Toyobo
Chloramphenicol		Wako
CIAP		Takara
Coomassie Brilliant Blue	R-250 staining solution	BIO-RAD
denaturation buffer	for T4 kinase treatment	Toyobo
dNTPs	for PCR	Takara
exTaq polymerase		Takara
exTaq polymerase buffer		Takara
Filter	Avodisc syringe filter with 0.2 µm membrane	Pall corporation
Glass Beads and tubes	BSP-11079105W	Wakenyaku
HPLC		Shimadzu
HPLC Program	LC Solution V.1.25	Shimadzu
HPLC column		Nacalai Tesque
IPTG		Nacalai tesque
kit for extraction from agarose gel	Illustra GFX PCR DNA and Gel band purification kit	GE Healthcare
kit for plasmid extraction	WizardPlus SV Minipreps DNA purification	Promega
LB broth		Nacalai tesque
M13 primers		Invitrogene
NAD+/NADH		Oriental Yeasts
NADP+/NADPH		Oriental Yeasts
PCR device	C1000 Thermal cycler	BIO-RAD
Peptone	Enzymatic digest of protein	Bacto
PrimeStar Premix	for PCR	Takara
rATP	for T4 kinase treatment	Toyobo
Restriction enzymes		Takara
Restriction enzymes buffer		Takara
Spectrophotometer	UV-2450 UV-VIS Spectrophotometer	Shimadzu
Standard buffer solutions		Nacalai tesque
T4 kinase		Toyobo
TEMED		Funakoshi
Transformation kit for yeasts	Frozen EZ yeast transformation kit	Zymo reseqrch
Tryptone	Pancreatic digest of casein	Bacto
X-gal		Nacalai tesque
Yeast Extract	extract of autolysed yeast cells	Bacto
Yeast Nitrogen without amino acids	based for classifying yeasts	Difco
10x Tris/Glycine/SDS buffer		BIO-RAD
10x loading buffer	for gel electrophoresis	Takara
2-log DNA ladder	for gel electrophoresis	New England Biolabs
2x ligation mix		Wako
Thermus HB8 genome		Harima Riken

All the other chemicals not mentioned in this list come from Wako or Nacalai tesque.

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IV. Introduction

Organisms with an optimal growth temperature above 60°C are called thermophiles. Coming from thermophiles, thermophilic enzymes (TEs) are usually resistant to inactivation at high temperature and optimally active above 60°C. TEs are nevertheless very similar to mesophilic enzymes and share with them catalytic mechanisms [Vieille *et al*., 1996]. TEs amino acid sequences are 40-85% similar to mesophilic ones and their 3-dimensional structures are alike [Vieille and Zeikus, 1996]. There is no systematic structural difference between extremely stable and normal protein. It so happens that TEs are more rigid at room temperature (RT), which gives the protein a protection from unfolding and preservation of catalytically active structure [Bruins *et al*., 2001].

TEs have many advantages compared to enzymes from mesophilic organisms [Cava *et al.* 2009] as they show increased resistance to denaturing chemical agents [Khajeh *et al*., 2001]. Thermophiles have high growth rate as well as high cell yields and their enzymes are easily crystallized. They are also the closest living representatives of the last bacterial common ancestors and, therefore, become subject matter of primitive life. They possess a large number of conserved genes. Their genome size being really small, very few functional paralogues exist and their stability is very high. Although thermophiles have such attractive features, their potential problems in cultivation have prevented application to industrial use. As they require high temperature cultivation, dehydration of the medium might happen; glass plates and thermo-resistant equipment should be used. The instability of several growth medium components as well as the combination with other extremophilic characters (such as a high or low pH, or different salt conditions), also hamper the cultivation of the organisms [Cava *et al.*, 2009].

Production of thermophilic enzymes in mesophilic hosts has many advantages to overcome above-mentioned problems in thermophiles [Honda *et al.* 2010]. Many mesophilic hosts are common organisms to use in laboratories and are then well-studied. They don't require specific growth conditions (such as a high temperature or a high pH). Moreover, the thermal properties of TEs are retained when cloned in mesophilic hosts [Vieille *et al*., 1996] and TEs are readily purified only by heating at high temperature as endogenous enzymes of mesophilic hosts are inactivated. Utilizing such features, synthetic metabolic engineering (SME) has emerged as a novel bioprocess. SME is based on expression of a series of thermophilic enzymes that catalyze objective reactions in mesophilic host and inactivation of enzymes derived from host organism at high temperature. This will eliminate unwanted side reactions [Restiawaty *et al*., 2011; Honda *et al*., 2010; Iwamoto *et al*., 2007] and only desirable product could be obtained. In addition, this treatment will partially disrupt the cell membrane and the substrate accessibility will be improved. Based on SME, construction of an artificial bio-synthetic pathway that transform fructose to 2-deoxyribose 5-phosphate has already been demonstrated [Honda *et al*., 2010]. Fructose 1,6-diphosphate from fructose and polyphosphate was also achieved based on SME [Iwamoto *et al*., 2007]. Such studies have demonstrated the power of SME using *E. coli* as a host strain.

To further expand application of SME, it is desirable to investigate other candidates as host strain, as SME employing *E. coli* has some restrictions at the present time. Although *E. coli* is

a very good host for protein expression and its gene modification tool is abundant, its thin membrane allows leakage of many proteins as well as the enzyme of interest out of the cells after heat treatment [Tsuchido *et al.*, 1985; Restiawaty *et al.*, 2011]. This therefore might make the cells not reusable. Inability of protein production requiring post-translation and production of toxic product such as endotoxin in high concentration cultivation are also disadvantages of *E. coli*.

Yeast cells provide an appealing alternative to protein expression in bacterial cells. They have many advantages compared to other kinds of hosts as their gene manipulation is easy and they are capable of secretion and modification of proteins [Barnett, 2000; Strathern, 1981]. They are also classified as Generally Regarded As Safe (GRAS) by the U.S Food and Drug Administration (FDA) [Nevoigt, 2008]. It is also believed that the yeast membrane should resist better to heat treatment, and less protein should leak out of the cell. Such attractive features of yeast promoted me to investigate possibility of yeast as a host strain for SME.

Production of TE has already been applied successfully in many kinds of yeasts [Lòpez-Lòpez *et al.* 2009; Kiiskinen *et al.* 2004; Rocha *et al.* 2010; Chen *et al.* 2007; Zamocky *et al.* 2008; Li *et al.* 2009]. All of those publications have in common the fact that the thermostable protein expressed in the specific yeast strain is secreted by the host (in an active form for some of them [Chen *et al.*, 2007]). Sometimes secreted naturally, the proteins have also been modified in order to be secreted. An example was demonstrated by Lòpez-Lòpez and co-workers [Lòpez-Lòpez *et al.* 2009], where the putative signal peptide was replaced by the *S. cerevisiae* α-factor secretion signal. Kiiskinen and co-workers [Kiiskinen *et al.* 2004], also showed that with the α-factor and modified cDNA having a stop codon after the native processing site at the C terminus, the production could be increased six folds. Although these studies successfully demonstrated secretion of TEs, intracellular production of TEs and construction of an artificial bio-synthetic pathway have not been demonstrated in yeast.

Therefore, the creation of an artificial biosynthetic pathway in the yeast cells of *Saccharomyces cerevisiae* was investigated in this project. As a model enzyme, fumarase (FUM) from *T. thermophilus* HB8 is selected for the bioconversion of fumaric acid (FA) to L-malic acid (LMA), based on SME. Fumarase can be found in two different forms. The cytosolic one is involved in the metabolism of amino acids, while the mitochondrial one is involved in Krebs's cycle. Malic acid is nowadays used mainly in the food, cosmetics and pharmaceutical industries and is mostly produced by chemical synthesis or hydratation of fumarate by either immobilized cells or fumarase. It has been identified by the U. S. Department of Energy as a substance that could be made from renewable carbohydrates in large quantities [Zhang *et al.*, 2010]. In the Krebs's cycle, fumarase is responsible for the hydratation of fumarate into L-malate, without addition of any cofactor [Koolman and Röhm, 2004]. **Another aim of this project is comparison of *S. cerevisiae* and *E. coli* as host strains for SME**. Therefore FUM was also expressed in *E. coli* and enzyme production and activity were compared with yeast strain. Enzyme leakage and reusability of cells expressing FUM were also compared. This is the first report of SME on yeast and comparison of SME on *E.coli*.

V. Materials and Methods

V.1 Organisms

V.1.1 Escherichia coli

The bacterial strains used in this study are *E. coli* Rosetta (DE3) pLysS, for protein expression, and DH5α and JM109 for plasmid amplification. The three of them were available in our laboratory, in the glycerol stock.

JM109 is a derivative of K strain bacteria and has *recA* and *endA* genotype. This strain also has *lacZ* mutation (allowing for blue/white selection). Sensitive to all common antibiotics, JM109 transformants can be obtained using antibiotic marker gene on plasmid, and transformants were obtained by heat-shock method.

DH5α strain is a versatile *E. coli* strain that carries the *end*A1, *rec*A1 and *lacZ*ΔM15 mutations. The *end*A1 mutation increases plasmid yield, while the *rec*A1 mutation ensures insert stability. Blue/white screening is also possible due to the *lac*Z mutation.

DH5α and JM109 were used for plasmid amplification and transformation with pBlueScript (pBS) plasmid for sequencing.

Carrying the T7 RNA polymerase gene on DE3 (a λ prophage), Rosetta (DE3) pLysS are used for protein expression. Protein expression is induced by addition of Isopropyl β-D-1-thiogalactoèyranoside (IPTG) in liquid medium. Expression is almost eliminated without induction by IPTG. Rosetta (DE3) pLysS are resistant to Chloramphenicol (Cm).

V.1.2 Yeast Strain

The yeast strain used is BY5208 MATα *ura3-52 his3-Δ200*. This strain was provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan.

Encoding for Orotidine 5-phosphate decarboxylase (ODCase), *URA3* was mutated in the strain used in this experiment. This allows for positive as well as negative selection. ODCase is responsible for catalyzation of the transformation of orotidine 5-phosphate to uridylic acid.

Table 1 Summary of main differences between *E. coli* and *S. cerevisiae*

Escherichia coli	**Saccharomyces cerevisiae**
Gram-negative; rod-Shape	Eukaryotic; round
2µm-long, 0.5µm diameter → 1.57µm^3 of volume	5µm diameter → 523µm^3 of volume
Easily manipulated genetically, easily grown	Easily manipulated genetically, easily grown, not weakened by antibiotics
Incapable of secretion	Capable of secretion
Pathogenic	Considered as GRAS by the FDA
Thin cell wall, low pH tolerance	Robust, tough cell walls, wide physiochemical tolerance

V.1.3 Extremophiles

T. thermophilus is a thermophilic gram-negative bacterium that grows under aerobical conditions (oxygen being the only electron acceptor) from 62°C to 75°C. It has a high growth rate, good cell yield, does not require any specific amino acids or vitamins to grow and is tolerant to a number of stress conditions. Genome's information on two different strains is available (HB27 and HB8) and both of them are composed of 1.9 Mbp and a mega-plasmid of 0.23 Mbp. Their GC content is equal to 69%, their coding density to 95%, coding for approximately 2,200 proteins (more than 600 having been crystallized). *T. thermophilus* also exhibits the highest transformation frequencies among *Thermus*, with a velocity of 40 kb/s/Cell [Averhoff *et al.*, 2010]. Facultative strains of *Thermophilus* can exhibit denitrification under anaerobic conditions [Cava *et al.* 2009; Jenney *et al.* 2008].

Thermus thermophilus (*T. thermophilus*) is a great source of TEs. Since its discovery in 1974 [Oshima *et al.* 1974], interests have been driven by the fact that its macromolecules were very well adapted to function at very high temperatures. One good example of such a fact is the use of the DNA polymerase, Taq polymerase, of *Thermus aquaticus* in the Polymerase Chain Reaction (PCR). Thermophiles' natural adaptation was achieved thanks to gene mutation, intramolecular recombination, differential gene deletion and horizontal gene transfer.

Thermophiles are a good source of novel catalysts, proteases, starch-converting enzymes (such as cyclodextrin glycosyl transferases or glucose isomerase), DNA processing enzymes or lipases [Bruins *et al.*, 2001].

The stabilization of proteins is due to a small number of extra salt bridges, hydrophobic interactions, hydrogen bonds [Bruins *et al.*, 2001]. Basic mechanisms of stability are summarized in Table 2.

It is possible to engineer mesophilic enzymes to make them more stable, but the gain is usually small. The strategy used is to improve the core packing of the protein. If the stability of a protein can be improved, the sensitivity will be lower, as well as the viscosity. The heat and mass transfer rate will be improved.

Table 2 Basic mechanisms of TEs stability [modified from Bruins *et al.*, 2001]

1	High packing density
2	Optimum charge patterns
3	Minimization of accessible hydrophobic surface area
4	Helix stabilization
5	Subunit assembly
6	Oligomer formation
7	Other environmental factors

In this study, *T. thermophilus* genome was purchased from Harima Riken Institute. *T. thermophilus* HB8 expression plasmids were constructed by "Whole-Cell Project of a Model Organism, *Thermus thermophilus* HB8", and deposited by Dr. Seiki Kuramitsu, SR

System Biology Research Group, RIKEN Harima Institute. Fumarase enzyme gene (*fum*) comes from *T. thermophilus* HB8 genome.

Thermococcus kodakaraensis (*T. kodakaraensis*) is a hyperthermophilic archaeon isolated from a solfatara in Japan, whose genome was sequenced [Atomi *et al.*, 2004]. This strain is obligate heterotrophic and grows in the presence of sulfur. In this study, it is used for obtaining the gene encoding malic enzyme (ME).

V.2 Plasmids

V.2.1 Bacterial plasmid

The plasmid used for FUM expression is pET-11a-FHA and purchased from Harima Riken Institute.

The plasmid used for ME expression is pET21a-ME, already available in our laboratory. This plasmid is constructed by ligated ME gene from KOD1 strain with pET21a.

Both plasmids were introduced into *E. coli* Rosetta (DE3) pLys strain.

V.2.2 Yeast Plasmids

The two different plasmids used in this study are p803 (pNV11) and p804 (derivative of p803 that does not contain stuffer cDNA). The plasmids were provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan. 10 µL of each plasmid were received, with a concentration of 0.12 mg/mL for p803 and 0.11 mg/mL for p804.

Four main types of vectors are available for the transformation of yeasts. Yeast Artificial Chromosomes (YACs) are used to clone genes up to 3000 kb. Yeast Episomal Plasmids vectors (YEps) are autonomously-replicating high-copy-number vectors. Yeast Integrative plasmids vectors (YIps) are non-autonomous but are able to perform a homologous recombination, as a single copy. Yeast Centromere plasmid vectors (YCps) are autonomously replicating, low copy-number vectors [Hadfield in Johnston, 1994; Mishra and Baranwal in Satyanarayana and Kunze, 2009]. In this study, a YEp was chosen, as it has a high copy-number and, even if it is unstable, should be efficient enough to see if the production of fumarase is possible. This vector is composed of a GAP promoter, a DNA stuffer, Ap^r, 2 µm ori, URA3 as well as some restriction enzyme sites.

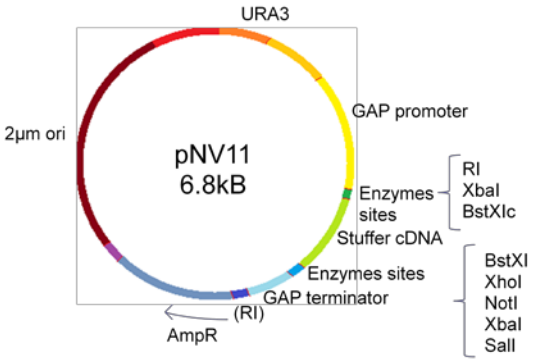

Figure 1 YEp plasmid map used in this study. This plasmid carries amp resistance to be amplified in *E. coli*. It also carries GAP promoter, for protein production in *S. cerevisiae*, without induction. P803 contains a stuffer cDNA, while p804 does not.

V.3 Molecular Biology

V.3.1 Competent cells

Theory

Transformation, the process in which a foreign DNA is imported to a bacteria, was first demonstrated by Stanley Cohen Laboratory, in 1973 [Thiemann and Palladino, 2004]. It was discovered that plasmids could be incorporated into pre-treated bacterial cells. Once the plasmid DNA incorporates inside the bacteria, it can replicate and be expressed. Naturally, bacteria do not take up DNA easily. Therefore, bacterial cells need to be treated with, for example, an ice-cold solution of CaCl2 as such treatment gives to bacterial cells competence. The treated cells are called competent cells and used for bacterial transformation.

Protocol (for 80 x 200 µL E. coli)

i. *Pre-culture cells from glycerol stock on LB plate (with appropriate selection)*
ii. *Inoculation of 1 colony in 5 mL LB, incubation overnight at 37°C*
iii. *Add 1% cell (v/v) to 200 mL SOB medium*
iv. *Incubation at 37°C until OD_{660} = 0.5*
v. *On ice 30 min*
vi. *Centrifugation 5,000 rpm in 50 ml tubes 4°C 5 min, keep cells*
vii. *Resuspend cells in 17 mL TB buffer, on ice 10 min*
viii. *Centrifugation 5,000 rpm 4°C 5 min, keep cells*
ix. *Resuspend cells in 6 mL TB buffer, on ice 10 min*
x. *Centrifugation 5,000 rpm 4°C 5 min*
xi. *Resuspend cells in 4 mL TB buffer and 0.6 mL DMSO*
xii. *Dispense 200 µL in micro tubes*
xiii. *Freeze -80°C*

V.3.2 Transformation

Theory

After cells have been made competent, they can undergo transformation and are therefore able to take up foreign DNA. In this study, bacterial cells were transformed by heat shock. Yeast transformation was done thanks to the ZYMO RESEARCH Frozen EZ yeast transformation kit. Yeast transformation usual process is usually more tedious than bacterial transformation [Hinnen *et al.*, 1978; Ito *et al.*, 1983; Gietz *et al.*, 2001, Gellissen *et al.*, 1992]

Protocol

a. Bacterial cells

i. *1 to 10% (v/v) of vector solution to competent cells*
ii. *On ice 30 min*
iii. *45 sec at 42°C*
iv. *On ice 2 min*
v. *Mix with 1 mL SOC medium*
vi. *1 hr at 37°C*
vii. *Plate 200 µL on appropriate plate*

b. Yeast cells

Yeasts transformation with p803-FUM was performed using the ZYMO RESEARCH Frozen-EZ yeast transformation kit. Cells were first grown to an OD_{600} of 1 in a 5mL tube. 1mL was taken from this culture and pelleted. Competent cells were produced every time needed, and not kept at -80°C. Despite the fact that the kit composition is not know, it can be guessed that it is based on the LiAC/SS carrier DNA/PEG method demonstrated first by Gietz [Gietz and Woods, 2001; Gietz and Schiestl, 2008; Gietz and Schiestl 2008].

V.3.3 Polymerase Chain Reaction

Theory

The polymerase chain reaction (PCR) allows the amplification of specific DNA fragment without using any living organism. It is composed of three steps that can be repeated to form a cycle.

The first step is denaturation, done by heating the DNA template. This allows breakage of hydrogen bonds between the complementary two DNA strands, yielding single-stranded DNA molecules. The second step is annealing. During this step, the oligonucleotide primers anneal to the complementary sequence on the single strands. This temperature depends on the primer sequence and is therefore crucial for reaction efficiency. It should be around 5°C lower than melting point of primer. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence closely matches the template sequence. The third step is the elongation, done by a heat-stable DNA polymerase (e.g. the Taq polymerase, whose optimal temperature is around 72°C) and polymerase synthesizes a new DNA strand complementary to the template by condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the extending DNA strand. Repeated cycles of those three steps lead to an exponential amplification of the sequence of interest [Thiemann and Palladino, 2004; Vosberg *et al*., 1989, Lodisch, 2008].

Agarose gel electrophoresis, which separates DNA by size, can then be done to check the PCR results.

Protocol

Primers used in this study were designed from OligoPerfect Designer and provided by Gene Design Inc.

PCR was performed in order to amplify *fum* from *T. thermophilus*. Restriction enzymes sites were added on both end of the gene, and start codon was modified. Primer sequences are listed in Table 3. PCR can also be performed in order to check whether transformants possess the target gene or not. Transformants are firstly boiled, in order to break the cell membrane. Then, knowing the gene sequence on the plasmid used, PCR can be performed. This is called colony PCR.

Table 3 Primers sequences. Modified from information received from GeneDesign.

Name	%GC	Size [bases]	Tm [°C]	OD	Molecular Weight	Nmol	Sequence
Forward	37.5	32	59.7	3.1	9939	8.0	gaattcaaaaaaatggaataccggattgagcg
Reverse	72.7	22	67.0	3.2	6648	15.0	gtcgacctacgccccctcgtgg

i. Mix MilliQ, primers, DNA of interest gene, PrimeStar Premix.

PCR of fumarase gene Master Mix

MilliQ	19 µL
Diluted forward primer	2 µL
Diluted reverse primer	2 µL
FUM cDNA	2 µL
PrimeSTAR Premix	25 µL
Total	50 µL

ii. Run PCR

	98°C	30 sec
30 cycles	98°C	10 sec
	60°C	15 sec
	72°C	1.5 min
	72°C	5 min
	15°C	Hold

Colony PCR of E. coli cells

i. Pick colony and put in PCR tube with 20 µL of MilliQ
ii. Heat 2 min at 98°C
iii. Spin 2 min
iv. Take 4 µL of heated sample, add 2 µL of dNTP, 2 µL of exTaq buffer, 1 µL of M13 forward primer, 1 µL of M13 reverse primer, 11 µL of MilliQ and 0.1 µL of exTaq polymerase. Mix
v. PCR conditions: •98°C for 30 sec • 98°C for 10sec • 60°C for 30 sec • 72°C for 1 min 30 sec • 72°C for 5 min • 15°C hold. Repeat 30 times.

Colony PCR of Yeast cells (adapted from Breeden Laboratory, Hutchinson Cancer Research Center, Seattle)

i. Prepare Master Mix: 2.5 µL 10x buffer; 2.5 µL 2.5 mM dNTPs; 0.5 µL forward primer (100 pmol/µL); 0.5 µL reverse primer (100pmol/µL); 20 µL MilliQ.
ii. Pick Colony and resuspend in 26 µL of Master Mix.
iii. Add 0.1 µL of exTaq polymerase
iv. Perform PCR
v. PCR conditions: 95°C for 10 min, 98°C for 30 sec, 60°C for 30 sec, 72°C for 1 min 30 sec, 72°C for 3 min, 15°C hold; repeat cycle 35 times

V.3.4 Agarose gel electrophoresis

Theory

Electrophoresis is used to separate molecules by size, conformation or charge. A matrix is cast and immersed in a buffer, providing ions to carry a current. The matrices used in this project are agarose gels that have a low resolving power, but a large range of separation. As DNA is negatively charged, it will migrate through agarose gels with a velocity inversely proportional to the $\log_{10}$ of its molecular weight. Circular forms of DNA will migrate in the gel differently from the linearized DNA, even if they have the same mass. Undigested supercoiled plasmids are meant to migrate quicker than digested plasmids while undigested nicked circle plasmids are meant to migrate later than digested plasmids. Examples of such a fact can be seen in figure 2. Some factors can affect the mobility of DNA inside the gel, such as the agarose concentration, the buffer used, the voltage applied, and the use of ethidium bromide (EtBr), which is used as a stain, to be able to visualize DNA under UV light.

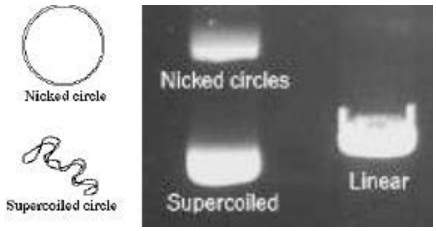

Figure 2 Examples of migration of fragment of DNA of the same size. Supercoiled plasmids, which are undigested, will migrate faster than linear plasmids that are digested. Nicked circle will migrate more slowly.

Electrophoresis was performed in this project to be able to visualize the result of the enzymes digestion and PCR. Pictures were taken after 30 to 45 min running time and directly after the electrophoresis.

Protocol

i. Prepare TAE buffer
ii. Prepare agarose gels 1%
iii. Mix sample with 10x loading buffer
iv. Load 10 µL of sample per well
v. Load Marker DNA in at least one well
vi. Run for 40 min
vii. Take picture

V.3.5 Restriction enzymes

Theory

Restriction enzymes (or restriction endonucleases) are found primarily in bacteria and archea and are able to digest double-stranded DNA at precisely-located sites, called restriction sites. Digesting DNA with a specific enzyme will produce fragments, that all have a precise length and a specific nucleotide sequence. There are three different types of restriction enzymes. Type I enzymes require ATP as well as S-adenosyl-l-methionine and cleave at sites away from recognition site. Type II enzymes often require magnesium and cleave close to or within the recognition site. Type III enzymes digest DNA after their recognition site and require ATP. Enzymes that digest straight symmetrically across the DNA produce the so-called “blunt ends”. Digesting DNA in an offset fashion, producing ends with overhanging pieces of single-stranded DNA result in the so-called “sticky ends”. Every enzymes used in this project create sticky ends and are Type II restriction enzymes.

Protocol

i. Mix at least 20 µL of plasmid, 3 µL of corresponding buffer, 1 µL of specific enzyme, 6 µL of water.
ii. Incubate at least 2 hrs at 37°C.
iii. Perform electrophoresis

V.3.6 Extraction from gel

Theory

DNA product can be extracted from agarose gels after electrophoresis. This is especially useful, in order to confirm DNA length of plasmid or fragment before use, without wasting part of the sample. Plasmids were retrieved using Illustra GFX PCR DNA purification kit.

Protocol (from Illustra GFX kit)

a. Production of cleared lysate

i. Centrifugation 1 min, 15,000 rpm up to 2 mL of culture, keep cells
ii. Add 250 µL resuspension buffer, vortex
iii. 250 µL of cell lysis solution
iv. Invert 4 times
v. 10 µL of alkaline protease solution
vi. Invert 4 times
vii. Incubate 5 min RT
viii. 350 µL of neutralization solution
ix. Invert 4 times
x. Centrifugation 20 min RT 15,000 rpm

b. Binding of plasmid

xi. Put mixture into binding column
xii. Centrifugation 1 min RT 15,000 rpm
xiii. Discard flowthrough

c. Washing

xiv. Add 750 µL of wash solution in column
xv. Centrifugation 1min RT 15,000 rpm
xvi. Discard flowthrough
xvii. Add 250 µL of wash solution in column
xviii. Centrifugation 5 min RT 15,000 rpm

d. Elution

xix. Put column in a sterile tube
xx. 100 µL of nuclease-free water
xxi. Centrifugation 1min RT 15,000 rpm
xxii. Keep flowthrough

V.3.7 Plasmid extraction of large preparation

In order to retrieve a large amount of plasmid, a large preparation was done. Plasmids were retrieved without any kit.

Protocol

i. Centrifuge overnight culture (100 mL) of transformed bacteria
ii. Resuspend in 5 mL Solution I
iii. Add 10 mL Solution II
iv. Incubate at RT 5min
v. Add 5 mL Solution III, centrifugation 10 min, keep SN
vi. Phenol-chloroform purification
vii. Ethanol precipitation
viii. Add 500 µL MilliQ
ix. Add 5 µL RNase solution
x. Incubation 1 hr at 37°C
xi. Phenol-chloroform purification
xii. PEG precipitation

V.3.8 Sequencing

Theory

pBlueScript (pBS) is a phagemid that is commercially available. It includes a polylinker sequence located into the *LacZ* gene and an amp resistance. This allows the selection of transformed bacteria thanks to Ampicillin as well as blue/white screening. IPTG and X-gal are added on the LB plate. If the DNA of interest was correctly integrated into pBS, the *LacZ* gene will be disrupted and the colony will appear white. On the other hand, the colony will appear blue if the *LacZ* gene is still intact [Alting-Mees *et al*., 1989; Short *et al*., 1988].

pBlueScript phagemid needs first to be digested with a blunt end restriction enzyme. Here, *Eco*RV was used. The digested vector needs to be purified after, either by gel electrophoresis or by phenol-chloroform purification followed by ethanol precipitation. To prevent self-ligation, the plasmid is treated with calf intestinal alkaline phosphatase (CIAP), to be dephosphorylated. In order for CIAP not to strip the 5' phosphates off of the DNA insert, it needs to be inactivated. This is done by phenol-chloroform purification, followed by ethanol precipitation. The prepared pBS can then be ligated with the prepared insert. This insert, after having been extracted from gel, needs to be purified by ethanol precipitation, followed by a T4 polynucleotide kinase treatment. This enzyme will phosphorylate the insert, and a

phosphate group is added to the 5' end. After incubation, phenol-chloroform purification and ethanol precipitation can be done in order to separate the DNA of interest from the mixture of kinase, rATP and buffer.

Ligation of the modified fumarase gene with pBS was performed in order to sequence the gene with the Sanger method. Ligation was performed using the Ligation Mix 2x and incubation at 16°C for at least 2 hours. Ligated vectors were then used for the transformation of *E.coli* cells, by heat shock. Cells were then incubated overnight at 37°C. Growth and color were checked after at least 20 hours of incubation. pBS was prepared using *Eco*RV and therefore digested in a blunt end manner. X-gal and IPTG were added on agar plates before plating bacteria as indicator of insertion of fumarase gene. IPTG is an inducer for β-galactosidase and X-gal is substrate of this enzyme. Only when fumarase gene is not inserted into pBS, β-galactosidase is expressed. As a result, X-gal was degraded and blue color appeared.

protocols

a. pBS preparation protocol

i. Mix 40 µL pBS extracted , 10 µL 10x H buffer, 2 µL EcoRV, 48 µL MilliQ
ii. Incubation ON at 37°C
iii. Phenol-chloroform purification
iv. Ethanol precipitation
v. Add 43 µL of MilliQ
vi. Mix with 2 µL of ClAP and 5 µL of 10x alkaline phosphate buffer
vii. Incubation ClAP 15 min at 50°C
viii. Phenol-chloroform purification
ix. Ethanol precipitation
x. Add 30 µL of MilliQ

b. T4 kinase treatment protocol

i. Gel electrophoresis and extraction of PCR
ii. Ethanol precipitation
iii. Add 10 µL of MilliQ water
iv. Add 75 µL of denaturation buffer for kinase treatment
v. Heat 90°C for 2 min
vi. Put on ice for 3 min
vii. Add: -10 µL 10x blunt end buffer, -10 µL 10 µM rATP, -2 µL T4 kinase, -3 µL MilliQ
viii. Incubation 1 hr at 37°C
ix. Heat at 90°C for 3 min
x. Phenol/chloroform purification
xi. Ethanol purification
xii. Add 20 µL of MilliQ water

c. blue and white screening

pBS ligated with the gene of interest can be amplified by *E. coli* transformants. The cells having correctly integrated the gene of interest on the plasmid will appear white. The cells with an empty plasmid will appear blue. Cells that have not been correctly transformed should not grow, due to amp in the medium. White colonies can then be used for colony PCR. Colonies carrying the gene of interest can then be grown into a bigger flask, and plasmid can be extracted in order to sequence the gene of interest.

d. BigDye sequencing

The BigDye Terminator v3.1 Cycle Sequencing Kit was used for FUM gene sequencing.

i. Extract plasmid from bacteria
ii. Prepare PCR reaction mix
iii. Thermal Cycling 96°C 1 min, 96°C 10 sec, 50°C 5 sec, 60°C 4 min, 4°C hold, 25 cycles
iv. Add 5 µL 125 mM EDTA (pH8.0) to each PCR tube
v. Add 60 µL of 99.5% Ethanol
vi. Mix, centrifuge 10min 15,000 rpm

vii. *Remove SN, rinse with 30 µL of 70% Ethanol*
viii. *Centrifuge 5 min 15,000 rpm*
ix. *Remove SN, dry under vacuum, wrap tubes with aluminium foil*
x. *Suspend in 15µL Hi-Dye formamide*
xi. *Boil 2 min, cool on ice*
xii. *Transfer to sequencer plate*

PCR reaction Mix

BigDye Terminator Ready Reaction Mix	3µL
Template	*
Primer	3.2pmol
Distilled Water	Up to 15µL
	15µL

Template DNA	
Single-stranded DNA	25-50ng
Double stranded DNA	150-300ng
PCR products (100-200bp)	1-3ng
(200-500bp)	3-10ng
(500-1000bp)	5-20ng
(1000-2000bp)	10-40ng
(>2000bp)	20-50ng

V.3.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Theory

SDS-PAGE allows the separation of protein in function of their size. As proteins have tertiary and quaternary structure, the first step is linearization by using SDS as a denaturing agent. SDS breaks up hydrophilic areas and coats proteins with negative charges. The second step is electrophoresis in a gel-like matrix. Small protein will migrate faster than big protein through gel. Denaturated proteins are negatively charged and will therefore move toward the plus size. The last part is to stain the gel, in order to visualize the proteins.

The gel used in this study is composed of two parts: separating gel and stacking gel. Stacking gels is made of large pores, while separating gel is made of small pores, high salt concentration and high pH.

Protocol

Table 4 Gels recipe for SDS-PAGE (for 2 gels)

	Separating gel (15%)	**Stacking gel**
Solution A	9 mL	900 µL
Solution B	4.5 mL	0 mL
Solution C	0 mL	1.5 mL
MilliQ water	4.5 mL	3.6 mL
Ammonium peroxodisulfate	½ small spoon	½ small spoon
10%SDS	180 µL	60 µL
TEMED	10 µL	10 µL

Sample preparation

i. *Mix protein extract with same amount of SDS sample buffer*
ii. *Boil 3 min*
iii. *Load on gel, up to 20 µL.*
iv. *Run gel at 180 V for approximately 1 hr.*
v. *Stain with Coomassie Brilliant Blue 20 min at RT.*
vi. *Unstain with unstaining solution ON*
vii. *Take picture*

V.3.10 Protein extraction

Proteins can be extracted from cells in order to perform SDS-PAGE or enzyme assay. Depending on the microorganism, the extraction process will be different. In this study, proteins were extracted from bacteria cells as well as yeasts cells. Sonication was used to extract proteins from bacterial cells. Post-alkaline extraction, glass beads as well as DTT treatment were performed to extract proteins from yeast cells.

a. Sonication

i. Harvest culture by centrifugation at maximum speed, 4°C, 10 min
ii. Resuspend in buffer (Phosphate buffer salines (PBS) or 0.2%Phosphate buffer)
iii. Divide sample to get 10 mL per tube at most
iv. Sonication 3 min (10 sec sonication and 20 sec wait, on ice)
v. Centrifugation 10 min 4°C

b. Post-alkaline extraction (PAE) (modified from Kushnirov et al., 2000)

i. Overnight culture or until OD_{600} = 2.5 (~2.3mg of wet weight)
ii. Harvest by centrifugation
iii. Resuspension un 100 µL MilliQ
iv. Add 100 µL 0.2M NaOH
v. Incubation 5min at RT
vi. Harvest by centrifugation
vii. Resuspend in 50 µL SDS Sample buffer
viii. Boil 3 min
ix. Pellet
x. Load 6 µL SN¨

c. Glass beads shaking (GB)

Glass beads protocol was modified from Wakenyaku Co. LTD. Minibeads beater 3110BX Handling tips. In principle, 2 to 5 min of glass beads shaking should be enough to recover 90% of proteins from cells.

Resuspended cells at a concentration maximum of 400 mg/mL were shaken for 1 minute with 0.5 mm glass beads. This sample was then kept on ice 1 min. This was repeated up to 4 times.

d. Glass beads – DTT (GB-DTT) (modified from LeBel et al., in Yeast Protocols, p. 300)

This protein extraction protocol is designed to retrieve low-abundance enzymes, such as telomerases.

Protocol

i. Pick a colony and inoculate in appropriate medium ON at 30°C.
ii. Centrifuge 8,000 rpm 4°C 5 min.
iii. Rinse with ice-cold MilliQ 1 mL.
iv. Centrifuge 4°C 8,000 rpm 5 min.
v. Rinse with ice-cold TMG/NaCl buffer 2.5 mL.
vi. Centrifuge 4°C 8,000 rpm 5 min
vii. Resuspend in 750µL TMG/NaCl/DTT buffer, transfer in micro tube for glass beads.
viii. Add beads.
ix. Vortex 30 sec. Let stand 30 sec on ice. Repeat 25 times.
x. Remove liquid part, transfer in new micro tube.
xi. Centrifugation 15 min, 15,000 rpm 4°C
xii. Transfer SN in new micro tube
xiii. Centrifuge 30 min 4°C 15,000 rpm

V.4 Enzyme experiments

V.4.1 Spectrophotometry

Light absorbed by a sample can be measured by a spectrophotometer. Intensity of light through a liquid sample at a specific wavelength is measured. This is then converted to an absorbance value, expressed as $A = log \frac{I_0}{I}$, where I is the intensity of light through the sample and I_0 is the intensity of light through the blank sample (or without any sample).

V.4.2 Enzyme assay

Theory

Enzyme activity was first tested in vitro, by spectrophotometer. As all the enzymes produced in this study are TEs, protein extract from cells were first heated at 70°C for 20 min, in order to inactivate hosts proteins. Samples were then centrifuged and SN kept to be used at 70°C. Two different enzyme assays were done, to check FUM activity. The first one, fumarase assay, was done by monitoring fumarate consumption or production. The second one was done by coupling FUM with ME, in order to be able to monitor $NADP^+$ reduction to NADPH. ME converts LMA to pyruvate by using $NADP^+$ as a cofactor.

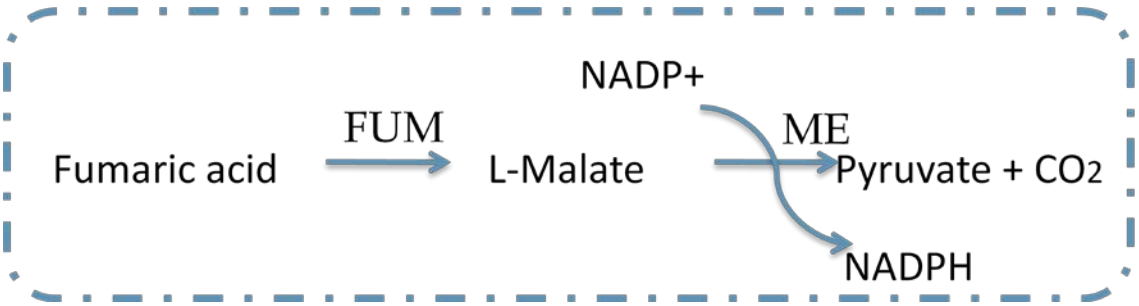

Figure 3 Coupled enzymes assay equation used in this study. $NADP^+$ conversion to NADPH can be monitored at 340 nm.

Protocol

a. Fumarase assay (modified from sigma Aldrich)

i. Resuspend ON culture of cells over-expressing FUM in potassium phosphate buffer to get 100 mg/mL of cells
ii. Extract proteins
iii. Heat protein extract at 70°C for 20 min
iv. Centrifugation 10 min 8,000 rpm
v. Stabilize spectrophotometer for 240 nm and heat it at 70°C
vi. In spectrophotometer glass cuvette, mix 50 µL of FUM protein extract, 100 µL of HEPES-NaOH pH 8.0 0.5M; 825 µL of MilliQ
vii. Autozero, wait 3 min (or until stable)
viii. Add 25 µL of substrate (fumaric acid 50mM or L-malic acid 50mM)
ix. Record absorbance over 3 min

b. Coupled enzymes assay

i. Resuspend ON culture of cells over-expressing FUM in potassium phosphate buffer to get 100 mg/mL of cells
ii. Resuspend ON culture of cells over-expressing ME in potassium phosphate buffer to get 100 mg/mL of cells
iii. Extract proteins from both samples

iv. *Heat protein extracts at 70°C for 20 min*
v. *Centrifugation 10min 8,000 rpm*
vi. *Heat Spectrophotometer at 70°C and stabilize it at 340 nm*
vii. *In spectrophotometer glass cuvette, mix 800µL of ME extract; 50 µL of FUM extract; 100 µL of HEPES-NaOH pH8.0 0.5M; 1 µL MnCL2 50 mM; 10 µL NADP+ 10 mM; 14 µL MilliQ*
viii. *Autozero, wait 3min (or until stable)*
ix. *Add 25 µL of substrate (Fumaric acid 50 mM)*
x. *Record absorbance over 3min*

Before recording FUM activity, ME activity should be checked. Therefore, the mix composition will be changed. No FUM extract and fumaric acid should be added, but 64 µL of MilliQ and 2 5µL of 50 mM LMA should be used instead

V.4.3 Bradford protein assay

Theory

The Bradford protein assay is used to quantify the concentration of a protein in a solution. It involves the binding of Coomassie Brilliant Blue to protein. The red form of Coomassie Brilliant Blue is converted to the blue form after binding with the protein. This binding can be monitored at 595nm, as it increases the absorption maximum. This rapid process has a good color stability (approximately 1 hour) [Bradford, 1976].In order to quantify correctly the amount of protein in the sample, the assay can be done with a known concentration from another protein. In this study, bovine serum albumin (BSA) was used as a standard. After calculating the standard curve, the unknown concentration can be found. Standard values used can be found in Table 5.

Protocol

a. Protein standard

Table 5 Protein standard for Bradford assay

Sample number	BSA concentration [mg]	2mg/mL BSA solution [µL]	MilliQ [µL]
1	0	0	20
2	0.5	5	15
3	1	10	10
4	1.5	15	5
5	2	20	0

b. Sample assay

i. *Take 20 µL of protein sample to analyze as sample 6*
ii. *Add 800 µL MilliQ to every sample*
iii. *Add 200 µL BioRad Protein Assay*
iv. *Vortex*
v. *Wait 10 min*
vi. *Check absorption at 595 nm; sample 1 is blank.*
vii. *Record absorbance for sample 2 to 5 as well as unknown concentration of protein*

V.4.4 L-Malic acid detection assay

Before performing High Performance Liquid Chromatography (HPLC) experiment, it appeared important to check the detection limit of HPLC instrument with the product expected in this study. This was done in order to know if a negative result comes from the fact that the product was not formed or not detected.

Protocol

i. Prepare solution of 300 mM of LMA in water.
ii. Make dilution of this solution to get 150 mM, 100 mM, 50 mM, 30 mM and 25mM
iii. Make serial dilutions of those solutions (4 dilutions by 10).
iv. Analyze by HPLC.
Solution range: from 300 mM to 0.0025 mM. The detection limit is reached when the sample cannot be detected by HPLC anymore.

V.4.5 Fumaric acid solubility assay

In order to know more precisely the behavior of FA at 70°C, its solubility was checked. It appeared that at RT, it was not possible to dissolve 100 mM of FA. Therefore, the highest concentration of solution used in this study was 50 mM. FA solubility was then calculated for 8 different temperatures.

Protocol

i. In PCR tube, put at least 2 mg of fumaric acid.
ii. Add 200µL of water.
iii. Incubate for 24 hrs at different temperatures. Here: 50°C, 51.5°C, 54.5°C, 58.9°C, 64.4°C, 69.0°C, 73.0°C
iv. Repeat with at least 5mg and 10 mg of FA.
v. Analyze concentration by HPLC.
Only the highest concentration over the three experiment is shown in the result.

V.4.5 Bioinformatics tools

a. BLAST

Blast analysis of the fumarase gene from *T. thermophilus* HB8 was performed. Analysis of similarity was performed using T-coffee program. T-coffee is a multiple alignment sequence alignment program that uses ClustalW [Notredame *et al*., 2008].

b. ExPASy calculation

The protein of interest, FUM, was analyzed by ExPASy [Gasteiger 2003].

V.4.6 Whole cell experiment

Theory

Synthetic Metabolic Engineering methods were performed by the so-called whole cell experiment. As explained earlier, SME is based on the expression of TEs in mesophilic hosts [Restiawaty *et al*., 2011]. Indigenous enzymes are inactivated by heating hosts cells at 70°C. By doing so, it is believed that no side reactions should occur and that the cell membrane of the host is partially disrupted. The first step of SME is the transformation of the host by a plasmid carrying the enzyme of interest. After expression of the target enzyme in step 2, cells are heated at 70°C. Due to the heat treatment, substrate can go through the damaged cell membrane, and conversion can happen.

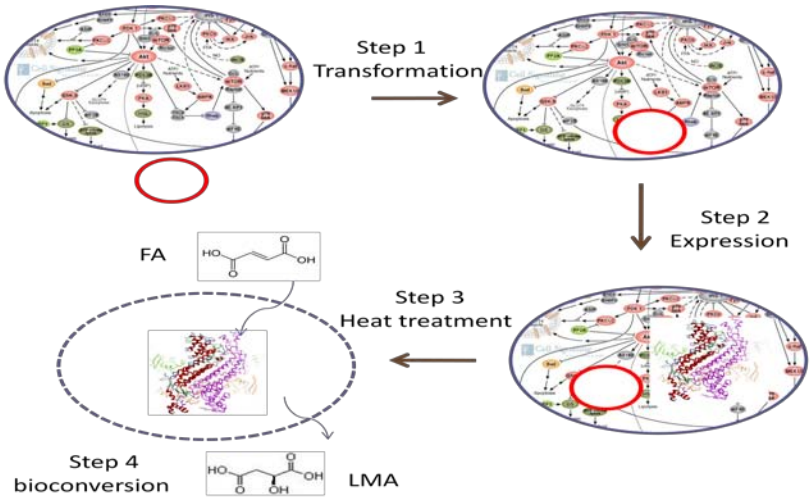

Figure 4 Summary of Synthetic Metabolic Engineering. During the first step, cells are transformed with a plasmid carrying the target thermo-stable gene. After transformation, the protein is overexpressed in the cell. Cells are then heated at 70°C, in order to inactivate the mesophilic enzymes and get no undesired side reactions.

Protocol

i. From plate, inoculate 5 mL tube (with appropriate selection). Incubate 8 hours.
ii. Inoculate flask 100 mL of medium, incubate 2 hours at 37°C
iii. Add inducer (if needed)
iv. Incubate overnight
v. Divide in 50 mL centrifuge tube, centrifuge 8,000 rpm, 10min
vi. Keep cells, resuspend to get 400 mg/mL in appropriate buffer
vii. Divide in microtubes 15 µL of cells, 105 µL of MilliQ
viii. Incubate at 70°C 20 min
ix. Add cofactor and substrate up to 30 µL
x. Incubate at 70°C.
xi. stop the reaction by adding 150 µL of ice-cold phosphate buffer and keeping samples on ice
xii. Centrifuge 5 min, 5,000 rpm 4°C, keep SN
xiii. Load 100 µL of SN into HPLC tube

V.4.7 High Performance Liquid Chromatography (HPLC)

Theory

HPLC is used to identify, quantify and/or purify particles. An adsorbent, called stationary phase, is packed in a column and eluted. The sample which contains the particle to be analyzed is washed through the column thanks to the eluting liquid (called mobile phase). Components adsorbed weakly on the surface of the stationary phase will travel faster than the ones strongly adsorbed [Lindsay and Barnes, 1992]. There are two variants in use in HPLC, known as the normal phase HPLC and reversed phase HPLC. Normal phase is used for non-polar solvent, while reversed phase is used for polar solvent. In this study, only reversed phase HPLC was used. The column silica surface has long hydrocarbon chains attached to it. Non-polar compounds will therefore pass more slowly through the column than polar ones.

Protocol

Mobile phase preparation

Solvent used in this study is 0.1% phosphate solution. 0.1% Phosphoric acid is mixed with deionized water, and filtered through a hydrophilic filter. This solution is kept at RT.

Sample preparation

Samples to be analyzed were first centrifuged for 5 min at 8,000 rpm. 100 µL of SN were loaded to the HPLC tubes to be analyzed.

Instruments preparation

The column used in this study is Cosmosil packed column, from Nacalai Tesque (Code number: 38145-21, size: 4.6x250 mm; $5C_{18}$-AR-II; type: water; manufacturer number: K54659). After 25 min of purge, and 1 hr of washing with water, the column was inserted in the instrument and filled with water. The column was then washed for another hour with water. Mobile phase was then changed to 0.1% phosphate buffer, and it was pumped through the column until stabilization of detector (or at least 30 min). Analyze was then started. After analyze of every sample, column and instrument were washed for 1 hr at least.

HPLC Instruments parameters [Detector parameters modified from Prezecki et al., 2005]

Data Acquisition
LC Stop time: 12 min
Sampling: 500 msec
Start time: 0.00 min
End time: 12.00 min

Pump
Mode: Isocratic Flow
Pump A Flow: 0.7000 mL/min
Pump B Flow: 0.000 mL/min
Configured pumps: Pump a and B LC20-AD
Pressure limits (Pump A and B): Maximum 20.0 MPa, Minimum 0.0 MPa

Detector UV/VIS
Model: SPD-20A
Lamp: D2
Polarity: +
Response: 1.0 sec
Cell temperature: 48°C
Wavelength: 210 nm
Output intensity unit: Volt
Auxiliary Range: 1.0 AU/V
Recorder range: 0.0100

Column oven
Model: CTO-20A
Oven temperature: 50°C
Maximum temperature: 85°C

Controller
Model: CBM-20A

Autosampler
Model: SIL-20A
Sample Rack: Rack 1.5 mL 105 vials
Rinsing volume: 200 µL
Needle Stroke: 52 mm
Control Vial Needle Stroke: 52mm
Rinsing speed: 35 µL/sec
Sampling speed: 15 µL/sec
Purge time: 25.0 min
Rinse mode: Before and after aspiration

V.5 Medium

All media were autoclaved before use

V.5.1 Bacteria culture

LB Broth

2% (w/v)	LB broth
2% (w/v)	Agar (if solid medium)
	Antibiotics (if needed)
	Water

Bacteria cells were replated every month on a new plate. After 12 hours growth, plates were kept at +4°C.

V.5.2 Yeasts culture

YP Broth (from Romanos et al., 1995)

Yeast Extract	1% (w/v)
Bacto peptone	2% (w/v)
Water	1L

YEPD (from Curran et al. in Xiao et al.)

Yeast Extract	1%
Tryptone	2%
Glucose	2%
Agar (if needed)	2%
Water	

Selection Medium SD+HIS

Yeast Nitrogen without amino acids	6.7 gr
Glucose	20 gr
Agar (if needed)	20 gr
Histidine	0.02 gr
Water	1L

Table 6 Summary of yeasts media and their composition

Medium name	*Ingredients*	*Per liter of water*	*Modified from*
YEPD	***Yeast Extract*** ***Tryptone*** ***Glucose*** ***Agar*** ***Water (to 1L)***	***10g*** ***20g*** ***20g*** ***20g***	***Curran et al. in Xiao et al.***
YP broth	***Yeast extract*** ***Bacto Tryptone*** ***Water (1L)***	***10g*** ***20g***	***Romanos et al., 1997***

Yeasts cells stored at +4°C and received from NBRP were taken and put (approximately 40µL) into one tube of YP broth and on one YEPD plate, without adding any antibiotics. After 3 days of incubation at 30°C, growth could be seen in the plate as well as in the tube. One colony from the plate were picked and replated in another YEPD plate. This ensures that the cells that will be picked for future work will come from one colony and therefore one clone. After three days incubation, the plate was put at +4°C for future uses.

After transformation, yeasts were grown on selection medium SD+HIS. This ensures that only the cells that still carry the plasmid of interest can grow.

Yeast cells were replated every month on a new plate. After 60 hours incubation at +30°C, cells were kept at +4°C.

V.6 Basic Protocols

V.6.1 Ethanol Precipitation

i. *Add 3 M Sodium Acetate 1/10*
ii. *Add 100% ethanol 2.5x*
iii. *Store on ice 30min*
iv. *Centrifugation 15 min, discard SN*
v. *Add 70% ethanol 2.5x*
vi. *Centrifugation 10 min, discard SN*
vii. *Dry at 37°C*

V.6.2 Phenol/Chloroform purification

i. *Add phenol ½Vol_{tot}*
ii. *Add chlofororm (IAA) ½ Vol_{tot}*
iii. *shake 1 hr at RT*
iv. *Centrifugation 10 min*
v. *Transfer supernatant to a new tube*

V.6.3 Polymerase Chain Reaction for modification of fumarase

Mix : 19 µL of MilliQ water, 2 µL of diluted forward primer, 2 µL of diluted reverse primer, 2 µL of fumarase gene, 25 µL of PrimeSTAR Premix.

Run : ·98°C for 30 sec · 98°C for 10 sec · 60°C for 15 sec · 72°C for 1 min 30 sec · 72°C for 5 min · 15°C hold. Repeat 30 times.

V.6.4 PEG Precipitation

i. *Add 500 µL of 13% PEG*
ii. *Incubation on ice 1 hour*
iii. *Centrifugation 15 min*
iv. *Keep pellet*
v. *Rinse with 70% EtOH 1 mL*
vi. *Centrifugation 15,000 rpm 5 min, remove SN*
vii. *Dry in vacuum 1 hr*
viii. *Resuspend in 100 µL MillQ*

V.7 Solutions

V.7.1 SOC medium

Tryptone	2%(w/v)
Yeast Extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM
Water	

V.7.2 TB buffer 1Liter

PIPES	3 g
$CaCL_2 \cdot 2H_2O$	2.2 g
KCl	16.6 g
$MnCl_2 \cdot 4H_2O$	10.9 g
Water	1 L

Adjust pH to 6.7 with KOH, filter sterilize

V.7.3 TAE buffer

Tris	40 mM
Acetic acid	20 mM
EDTA	1 mM
In water	

V.7.4 Agarose Gel (1%) (for 150mL)

Agarose S	1% (w/v)
EtBr added after cooling	10 µL
In TAE buffer	

V.7 5 Solution I (for DNA extraction)

Glucose	50 mM
Tris-HCl pH 8.0	25 mM
EDTA pH 8.0	10 mM
Water	

V.7.6 Solution II (20mL)

NaOH	0.16 g
10%SDS	2 mL
MilliQ	Up to 20 mL

V.7.7 Solution III

Potassium Acetate	3 M
Acetic acid (fold)	5 M
Water	

V.7.8 SDS-PAGE Sample buffer

0.5M Tris-CL (pH 6.9)	125 µL
10% SDS	200 µL
2-mercaptoethanol	50 µL
BPB solution	100 µL
	475 µL

V.7. 9 Bromophenol blue (BPB solution)

0.5M Tris-CL (pH6.8)	1.25 mL
Bromophenol Blue	5 mg
Glycerol	7 mL
Deionized water	1.75 mL
	10 mL

V.7.10 Solution A (30% acrylamide)

acrylamide	29.2 g
N,N'-methylen bis-acrylamide	0.8 g
Water	Up to 100mL

V.7.11 Solution B (1.5 M Tris-HCl buffer ; adjust pH to 8.8)

Tris	18.2 g
Water	100 mL

V.7.12 Solution C (0.5M Tris-HCl buffer. Adjust pH to 6.8)

Tris	6.1 g
Water	100 mL

V.7.13 TMG/NaCl buffer

Tric-HCl pH 8.0	10 mM
$MgCl_2$	0.1 mM
NaCl	200 mM
water	

V.7.14 TMG/NaCl/DTT buffer

DTT	0.1 mM
TMG/NaCL Buffer	

V.7.15 10x Phosphate-buffered saline (PBS)

NaCl	8 g
KCl	0.2 g
$Na_2HPO_4°12H_2O$	2.9 g
KH_2PO_4	0.2 g
Water	100mL

V.7.16 Ampicillin

100 mg/mL of ampicillin in MilliQ, filter sterilize, freeze at -20°C. Final concentration in medium: 200 mg/L

V.7.17 IPTG

0.2M IPTG in MilliQ, filter sterilize, freeze at -20°C. Final concentration in medium: 0.2mM

V.7.18 Cm

34 mg/mL of Cm in EtOH, freeze at -20°C. Final concentration in medium 34 mg/L

VI. Results

Two plasmids were given by the NBRP, p803 and p804. As it can be seen on the map in figure 1, p803 and p804 plasmids are really similar except that p803 contains stuffer DNA on the downstream of GAP promoter. The both carry two selections markers, an amp resistance as well as URA3. The constitutive GAP promoter and terminator are present on the plasmid and, therefore, there is no need of induction to express the protein of interest. It first appeared important to check whether restriction sites representing on the given plasmid maps are available or not, in order to remove the stuffer DNA and insert FUM DNA.

Part 1: Enzyme digestion

Table 7 List of enzymes on pnv11 and NEBcutter digestor results

Enzyme name	Number of digestion in FUM cDNA
***Bst*XI**	4
***Eco*RI**	0
***Not*I**	1
***Sal*1**	0
***Xba*I**	0
***Xho*I**	1

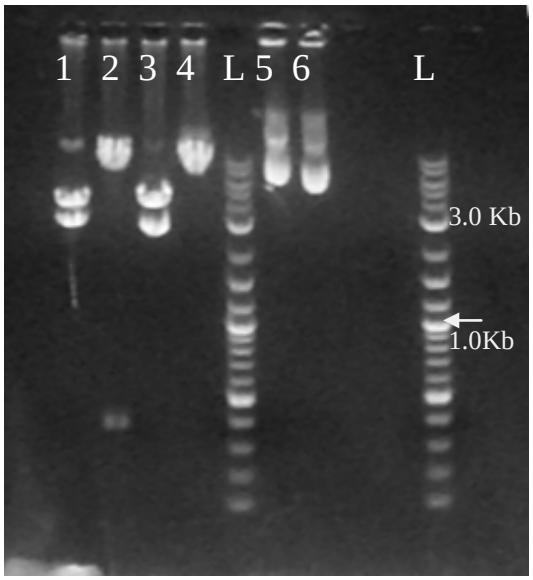

Figure 5 Electrophoresis of large preparation of plasmids p803 and p804, with *Xba*I or *Xho*I. Lanes: 1: p803 digested with *XbaI*; 2: p803 digested with *Xho*I; 3: p804 digested with *Xba*I; 4: p804 digested with *Xho*I; 5: p803 undigested; 6: p804 undigested; L: 2-log DNA ladder 10 µL

As it can be seen in Table 6, only 6 enzymes sites are present on p803 and p804. Among those enzymes, only 3 do not digest *fum* [results found with Nebcutter, Vincze *et al.*, 2003]. It was therefore decided to focus only on *Eco*RI, *Sal*I, *Xba*I, and *Xho*I enzymes. A larger preparation of the plasmids was done, in order to make digestions with the targeted restriction

enzymes. This step was performed using *E. coli* JM109 cells transformed with the plasmids and selected thanks to amp resistance marker. After preparation of the plasmids, digestion with the targeted restriction enzymes was carried out, and digestion was confirmed by electrophoresis. The control undigested plasmids were also put on the gel.

Figure 5 shows the result of digestion with *Xba*I or *Xho*I. Different from given information, digestion with *Xba*I brings two size of bands (lanes 1 and 3). On the other hand, digestion with *Xho*I brings expected size of bands (lanes 2 and 4). As expected, the undigested control plasmids (lanes 5 and 6) migrate further than the digested plasmids (lanes 2 and 4) as undigested plasmids are supercoiled, while digested plasmids are linearized.

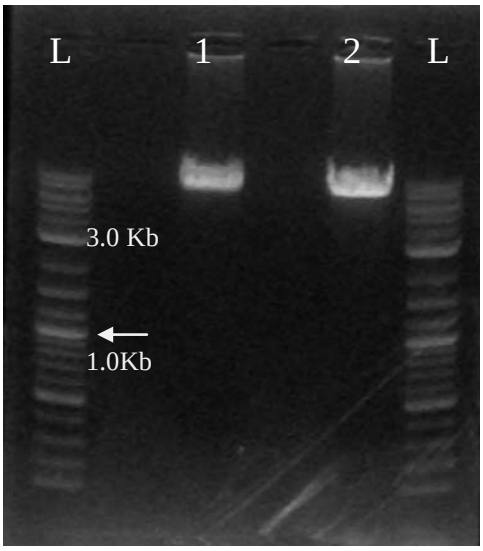

Figure 6 p803 and p804 digested with *Eco*RI. Lanes: L: 2-log DNA ladder, 1: p803 digested with *Eco*RI, 2: p804 digested with *Eco*RI The waiting time for the digestion was around 5 hours. 1: L: 2log DNA ladder 10µL, 1: p803 digested with *EcoRI*; 2: p804 digested with *EcoRI*; 3: p803 digested with *EcoRI* and *SalI*; 4: p804 digested with *EcoRI* and *SalI*

Subsequently, digestion was performed with *Eco*RI. Two *Eco*RI sites were present on the given plasmids map (figure 1), with one between brackets. It so appeared important to first test if the plasmids were digested once or twice with this enzyme. The results in figure 6 show that *Eco*RI digests the plasmids only once. This was also different from given information.

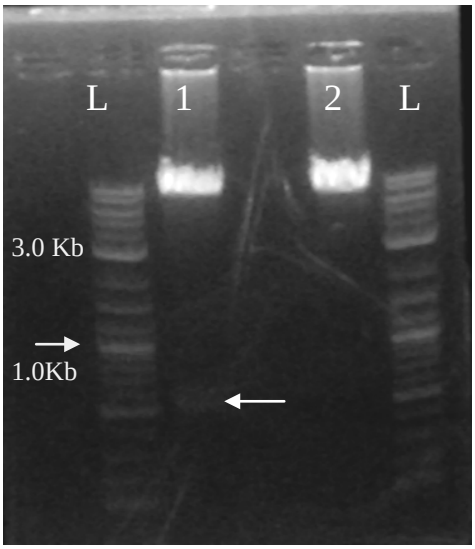

Figure 7 p803 and p804 digested with *Eco*RI and *Sal*I. Lanes: L : 2log DNA ladder 10 µL; 1: p803 digested with *Eco*RI and *Sal*I, 2: p804 digested with *Eco*RI and *Sal*I. A light band can be seen in well 1, representing the cDNA stuffer. The waiting time for the digestion was around 5 hrs.

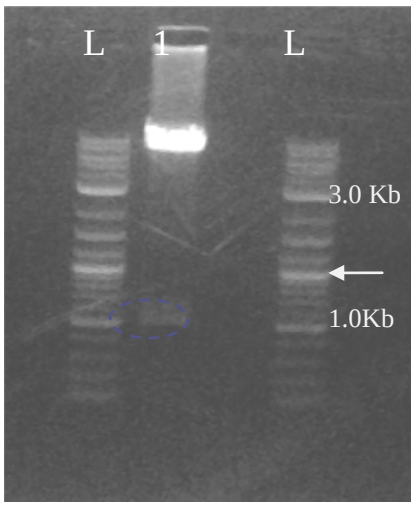

Figure 8 p803 digestion with *Eco*RI and *Sal*I. A light band can be seen in well 1, representing the stuffer DNA. Plasmid p803 was then extracted from this gel and kept at -20°C; Lane : L: 2-log ladder 10µL; 1: Plasmid digested with *EcoRI* and *SalI* 30 µL and 3 µL of dye

Digestion with *Eco*RI and *Sal*I was also carried out and result showed that this combination of enzymes enables to remove the stuffer DNA from p803. Two digestion were performed and can be seen in figure 7 and 8. Therefore, *Eco*RI and *Sal*I were chosen as enzymes used for following plasmid construction.

Gene modification

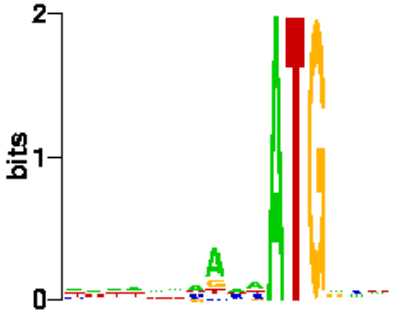

Figure 9 *S. cerevisiae* RBS consensus sequence, created with seqLogo. The sequences used were retrieved from UCSC Table browser, with the genome of *S. cerevisiae*, assembled in June 2008, track: SGC genes strand + and entire genome

The gene available commercially from *T. thermophilus* was already engineered to be produced in bacterial cells more effectively. This sequence, available in the laboratory, can be seen in appendix. It starts with a "GTG" start codon and ends with a TAG stop codon. As codon bias is known to play a role in gene expression [Sharp *et al*,. 1986], it was decided to focus on the upstream part of the gene sequence as well as the start codon itself. Figure 9 was therefore created. This figure shows the ribosome binding site (RBS of *S. cerevisiae*) (the initiation codon as well as a few bases upstream).

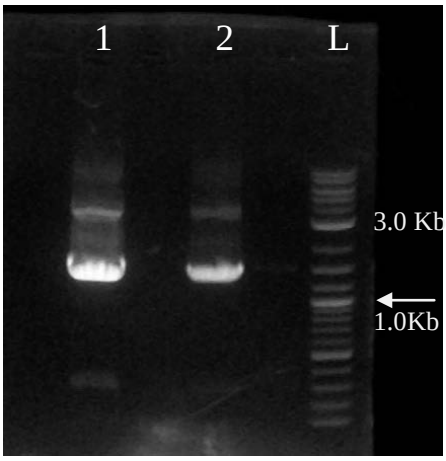

Figure 10 Fumarase gene after PCR with PrimeStar premix. 20 µL loaded in each well, 10 µL of 2-log ladder was used.

According to this consensus sequence, FUM gene of *T. thermophilus* HB8 was amplified with upstream sequence modification using the primers received from GeneDesign Inc. The primers were also designed to add *Eco*RI site to 5' terminal and *Sal*I site to 3' terminal of FUM gene, respectively. Primers were designed thanks to OligoPerfect Designer webtool. Dilutions of the primers received were completed in order to obtain 0.2-0.3 µM of primers (final concentration). PCR was performed using the PrimeSTAR HS (Premix) to amplify the gene and make the correct change in the sequence. The amplification of modified *fum* gene was confirmed by electrophoresis. As shown in figure 10, the band corresponding to expected

size of modified *fum* (1,400 bp) was observed. Amplified fragment was then inserted into pBS plasmid by following procedure. pBS plasmid was digested with *Eco*RV and treated with CIAP. The DNA insert was treated with T4 kinase. Ligation of the treated DNA insert and pBS was carried out with Ligation Mix. The ligated plasmid (pBS-FUM) was transformed to *E. coli* cells by heat shock treatment.

Several trials of pBS preparation and ligation were carried out. As the PCR product of FUM gene was too concentrated and it was hard to find the good proportion between FUM and pBS vector no colonies were obtained at first after the ligation of *fum* with pBS. A good ration between pBS and the gene of interest is important. Generally, 0.15 pmol of insert and 0.03 pmol of pBS vector are needed for the ligation to happen. By measuring the absorbance of DNA solution using spectrophotometer, the amount of µL needed can be calculated. The length of the gene being known, the pg of DNA needed can be calculated. After several trials, I succeeded to obtain transformants. 9 colonies were obtained, out of them 3 were blue.

Sequencing of gene modifications

Ligation of the modified gene with pBS is checked by blue/white screening after transformation of competent bacteria.

8 colonies were picked and checked by colony PCR with the usual protocol. As shown in figure 11, colonies 1 to 9 have integrated *fum* that is approximately 1,500 bp length. This length is slightly longer than the expected length from sequence of *fum* (ca. 1,400 bp). As it appeared that a large quantity was loaded on the gels, a shift in the gene length could be occurred. However, the shift seen here seems somehow too large. It could be interesting to make a further analysis on this result. The insert found here could be purified from the gel and then digested with *Eco*RI and *Sal*I. The length of the digested DNA could then be checked, in order to see if the insert found is really fumarase.

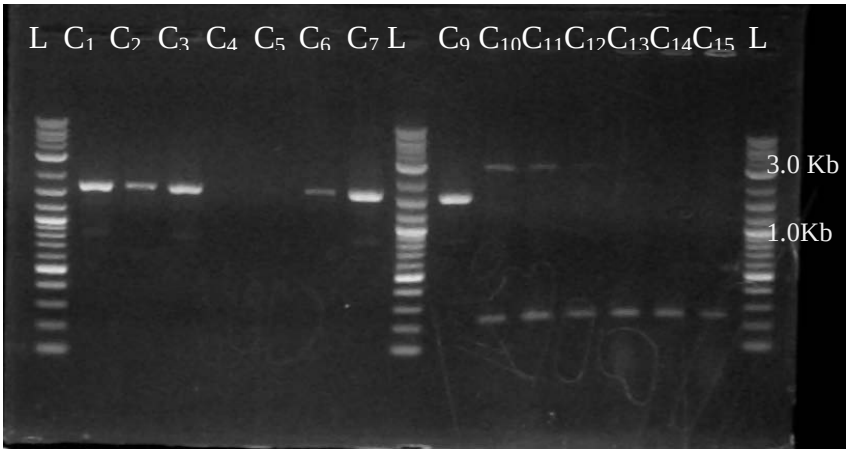

Figure 11 Colony PCR of pBS ligation with *fum*. Lanes: L: 2-log ladder 6µL; C1-15: Colony PCR 20 µL and 2 µL of dye. Colonies 1-9 come from one preparation of pBS, colonies 10-15 come from another preparation of pBS

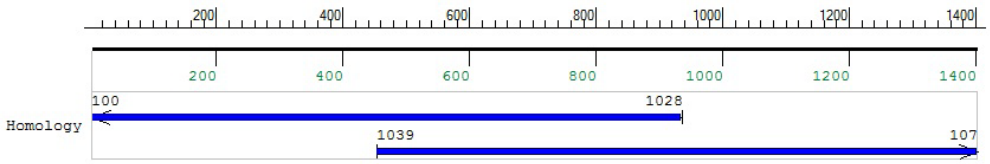

Figure 12 Overlapping region of sequenced gene. Two primers were used, separately, to sequence the gene of interest.

As ligation with pBS was done, the gene was sequenced as follows. From colony 1 and 7, pBS-FUM was extracted by the Miniprep Kit. PCR was then carried out using the BigDye terminator reaction mix. DNA in PCR samples were precipitated by EtOH containing EDTA and wash by 70% EtOH. Samples were resuspended in HiDye formamide, boiled, and cooled on ice and then sequenced. Primers used for sequencing are M13 forward and reverse primers. The sequencing overlap can be seen in figure 12. M13 primers will start sequencing a few base upstream or downstream of start and stop codon. As during sequencing, the first few bases sequenced is without credibility, it seems important to choose primers that will start amplification far enough from the start and stop codon.

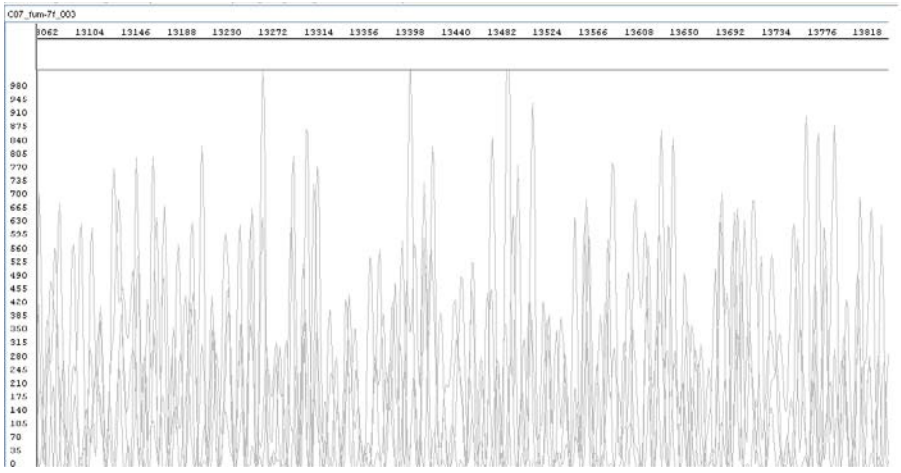

Figure 13 Sequencing of colony 7. As it can be seen, no clear results were obtained

As shown in figure 13, sequencing of the gene from colony 7 failed. On the other hand, successful sequencing of the gene from colony 1 was achieved using forward primer (data not shown) and sequence homology reached 100% compared to predicted sequence. The sequencing using reverse primer also showed 100% homology after analysis of sequence wave profile (figure 14). These results demonstrated successful modification of FUM genes for expression in *S. cerevisiae*.

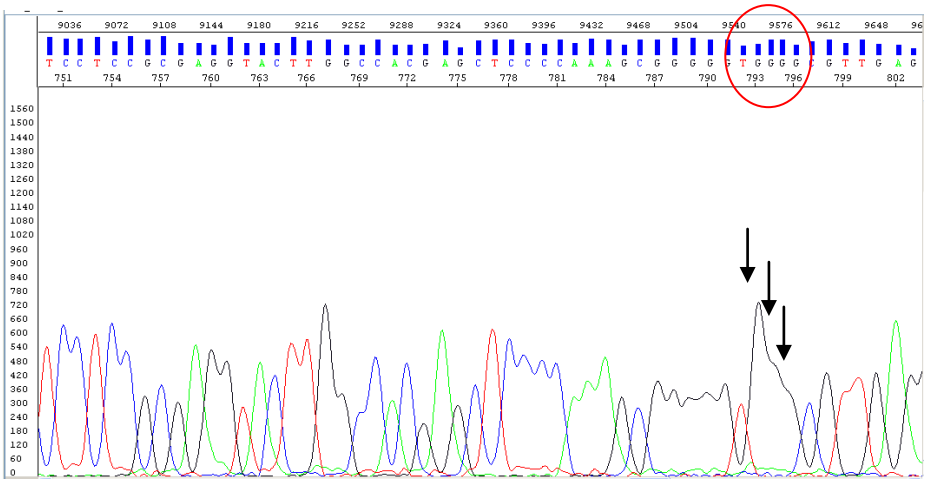

Figure 14 Details of sequencing of colony 1. It can be seen that 4 G were found from nucleotide 793 to 796, while the graph shows only 3 peaks and therefore 3 G only.

BLAST analysis/T-coffee

Since the sequence of fumarate hydratase from *T. thermophilus* is available only in strain HB27, a blast analysis was performed on fumarase from *T. thermophilus* HB8, the strain used in this study. The blast analysis demonstrated that it shares a 100% similarity with fumarate Hydratase from *T. thermophilus* HB8. It so appears that fumarase of *S. cerevisiae* and the one of *T. thermophilus* share approximately 60% of affinity (results found by comparing the two sequences with T-coffee and ClustalW2). The Blast analysis with the search set modified for *S. cerevisiae* (taxid:4932) results show a 98% coverage with fumarase [*Saccharomyces cerevisiae*] (accession number AAA66909.1), with a E-value equal to 3e-150.

Part 2: Yeast transformation

To construct expression plasmid of FUM gene in yeast, the correctly modified FUM gene was transferred from pBS-FUM to yeast expression plasmid p803 by digestion with *Eco*RI and *Sal*I and following ligation using ligation mix. Resulting plasmid was designated as p803-FUM. DH5α was transformed with p803-FUM plasmid and grown on LB-Amp medium. Colonies obtained were then checked by colony PCR.

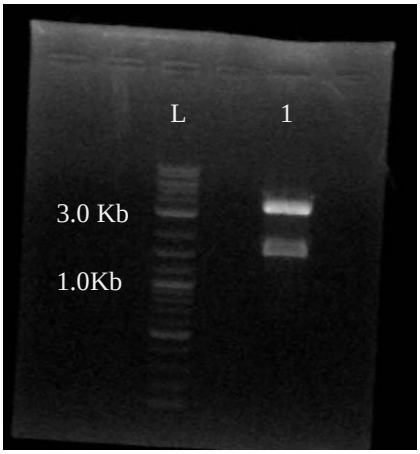

Figure 15 pBS digested with *EcoRI* and *SalI*. Lanes; L: 2-log ladder 6 µL; 1: 30 µL of digestion of 20 µL of purified pBS with *EcoRI* and *SalI* and 3 µL of dye.

As shown in figure 16, colony 6 has no plasmid, while colony 4 and 5 didn't give any clear results. Colony 10 was then chosen for amplification of plasmid, and inoculated in a 5mL LB-amp tube. p803-FUM plasmid was then extracted using the miniprep protocol.

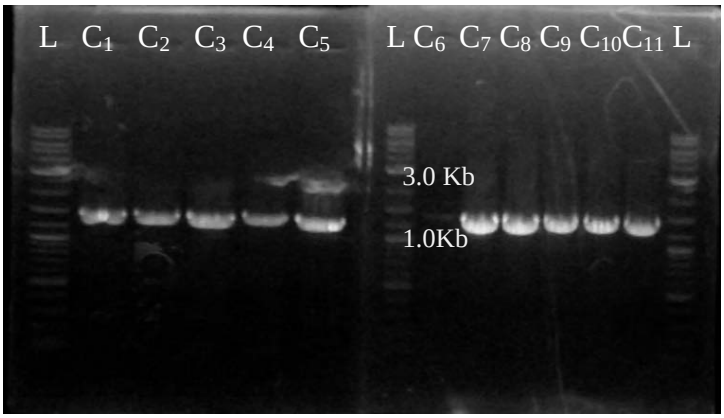

Figure 16 colony PCR of DH5α bacteria cells transformed with p803-FUM plasmid for plasmid amplification. ; L: 2-log ladder 6 µL; C1-C11, colony PCR of colony 1 to 11

Using p803-FUM, yeast cells (strain BY5208) were transformed by the ZYMO kit. Competent yeasts were created thanks to the kit first step. Cells were then transformed with the kit second step, based on the LiAC/SS carrier method. The newly transformed yeasts were then plated on a SD+HIS plate. Rosetta cells were also transformed by pet11a-FHA in order to produce FUM in both microorganisms.

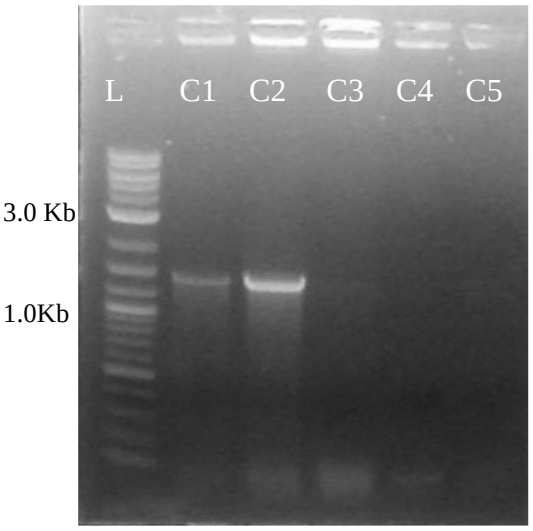

Figure 17 Yeast colony PCR. Lanes: L: 2-log DNA ladder. C1 to C5 : 5 different colonies. Amplification done by M13 primers

Colony PCR protocol was first optimized for yeasts and successful results were obtained. The results of colony PCR of BY5208 containing p803-FUM can be seen in figure 17. The bands corresponding to *fum* were confirmed in colonies 1 and 2. This result indicated that successful introduction of p803-FUM into BY5308 strain. The growth of BY5208-FUM was monitored (figure 18). By monitoring the OD at 600 nm, it can be found that around 20 hrs of incubation is enough to harvest cells being still in the exponential phase.

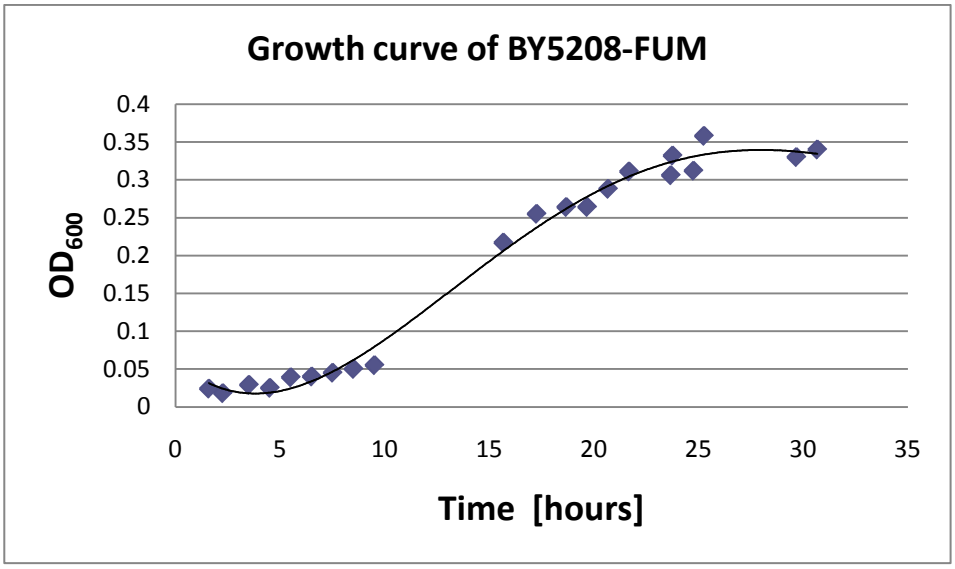

Figure 18 Growth curve of BY5208-FUM in SD+HIS. Three different cultures were used. The trend line is an order 4 polynomial curve

Part 3: Protein production and extraction

To confirm whether introduced FUM gene was correctly expressed in *E. coli* and yeast strains or not, protein production was investigated by SDS-PAGE. Protein extract for SDS-PAGE from Rosetta cells harboring pET11-a-FUM plasmid that contains FUM gene in pET11-a plasmid were obtained after induction of IPTG, resuspension in PBS, sonication and mix with SDS sample buffer. In the case of yeast, three different protocols were followed in order to obtain protein extract for SDS-PAGE. The first protocol followed is the same of the Rosetta protocol, without induction by IPTG. Samples were resuspended in PBS and sonicated. The second protocol is the so-called post-alkaline extraction (PAE). Cells were treated by 0.2M NaOH, incubated 5 min at room temperature, pelleted and resuspended in SDS sample buffer. The last protocol is the glass beads with DTT incubation (GB-DTT) protocol, from LeBel *et al*. Cells were harvested, washed with water, rinsed twice with TMG/NaCl buffer, resuspended in TMG/NaCL/DTT buffer. Glass beads were added and samples were vortexed 30 sec, and then kept on ice 30 sec. This was repeated 25 times. After the transfer in new microtubes, samples were centrifuged and mix with sample buffer for SDS.

First trial was carried out using sonication and PAE method. As shown in figure 19, no band corresponding to FUM gene product (ca. 50 kDa) was observed for samples from yeast strain (lanes 1 to 6). On the other hand, thin bands were observed for samples from E. coli strains (lanes 7 to 10). Second trial was carried out using PAE and GB methods for yeast and sonication method for *E. coli*. Samples from *E. coli* shows clear band corresponding to size of FUM was observed in any conditions (lanes 7 to 12), while samples from yeast showed band only in the case of using PAE method (lanes 13, 17, and 18).

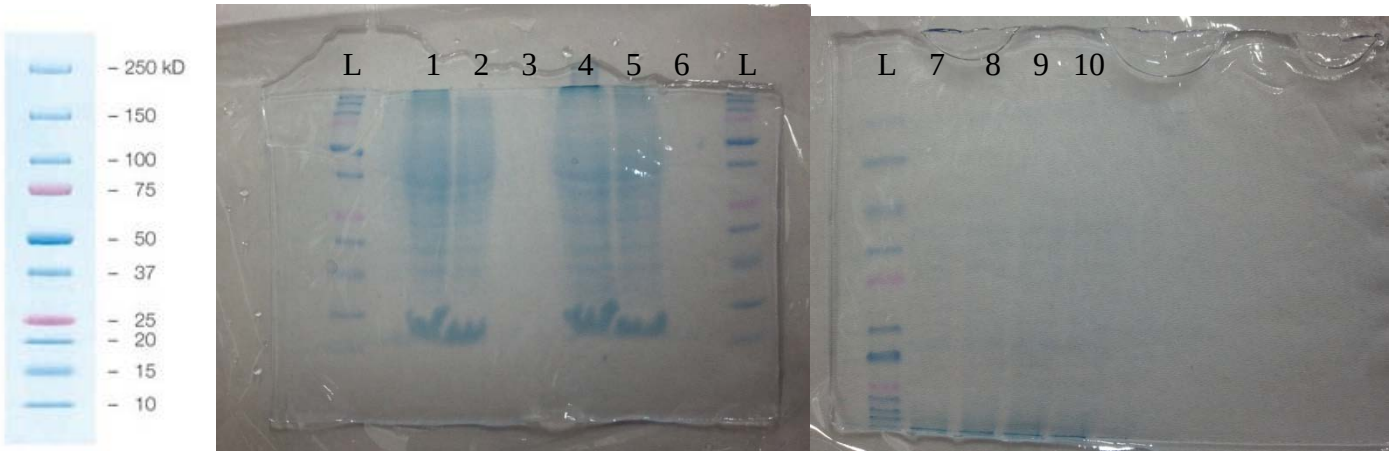

Figure 19 SDS-PAGE first attempt. L = protein ladder 3µL; 1: yeasts transformed with p803-FUM, post-alkaline extraction; 2: yeasts transformed with p803-FUM, post-alkaline extraction from frozen cells; 3: yeasts transformed with p803-FUM, sonication; 4: yeast cells not transformed, post-alkaline extraction; 5: yeast cells not transformed, post-alkaline extraction, from frozen cells; 6: yeasts not transformed, sonication; 7-10: rosetta-pet11-a-FUM, sonication, from 4 different colonies

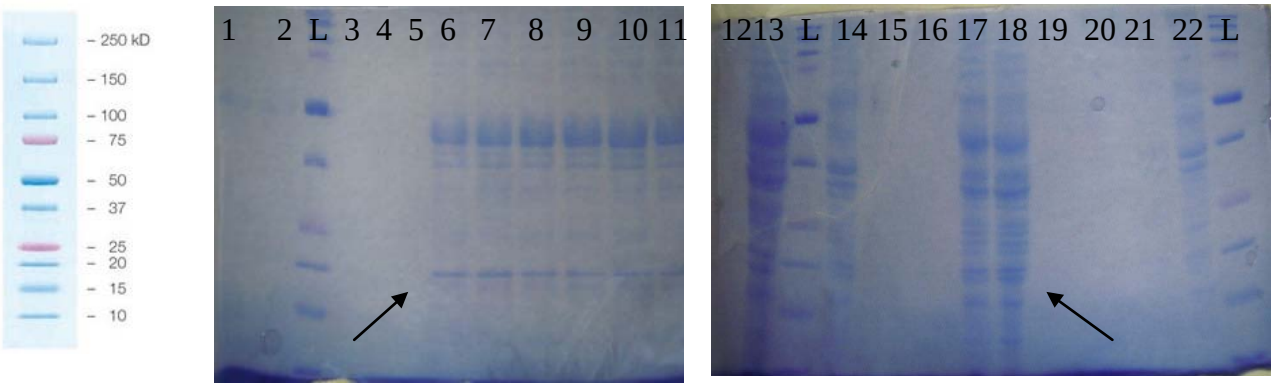

Figure 20 SDS-PAGE Second attempt. L : Ladder 3µL ; 1 : Yeast-FUM glass cell lysis; 2: Yeast-FUM glass cell lysis, 70°C 20min; 3: yeasts not transformed, glass cell lysis; 4: yeasts not transformed, glass cell lysis, 70°C 20min; 5: Rosetta-FUM, colony 6; 6: Rosetta-FUM colony 6, 70°C 20min; 7: Rosetta-FUM, colony 5; 8: Rosetta-FUM colony 5, 70°C 20min; 9: Rosetta-FUM, colony 4; 10: Rosetta-FUM colony 4, 70°C 20min; 11: Rosetta-FUM, colony 3; 12: Rosetta-FUM colony 3, 70°C 20min; 13: Yeast-FUM post-alkaline extraction, 10µL; 14: Yeast not transformed, post-alkaline extraction, 10µL; 15: Yeast SD+HIS medium,70°C, 20min 30 µL; 16: Yeast SD+HIS medium 30 µL; 17: Yeast-FUM post-alkaline extraction, 70°C, 20min 6µL; 18: Yeast-FUM post-alkaline extraction, 6µL; 19: Yeast YPD medium from untransformed cells, 70°C, 20min, 30 µL; 20: Yeast YPD medium from untransformed cells 30µL; 21: yeasts not transformed, post-alkaline extraction, 70°C, 20min, 6µL; 22: yeasts not transformed, post-alkaline extraction, 6µL. Arrows show the band that is meant to be fumarase

Trial for protein extraction from yeast cells was again carried out by GB method. This extraction was checked by spectrophotometry as well as SDS-PAGE. Due mainly to the presence of tyrosine and tryptophan, proteins exhibit a distinct UV light absorption maximum at 280 nm [Layne, 1957]. Nucleic acid, which absorbs strongly light at 260 nm, also slightly absorbs at 280 nm. Possible contamination by nucleic acids can unfortunately happen during glass beads extraction. Grove equation can be used, in order to reduce this contamination as follows. $Protein\ concentration\ \left[\frac{mg}{mL}\right] = 1.55 \times Abs_{280} - 0.76 \times Abs_{260}$.

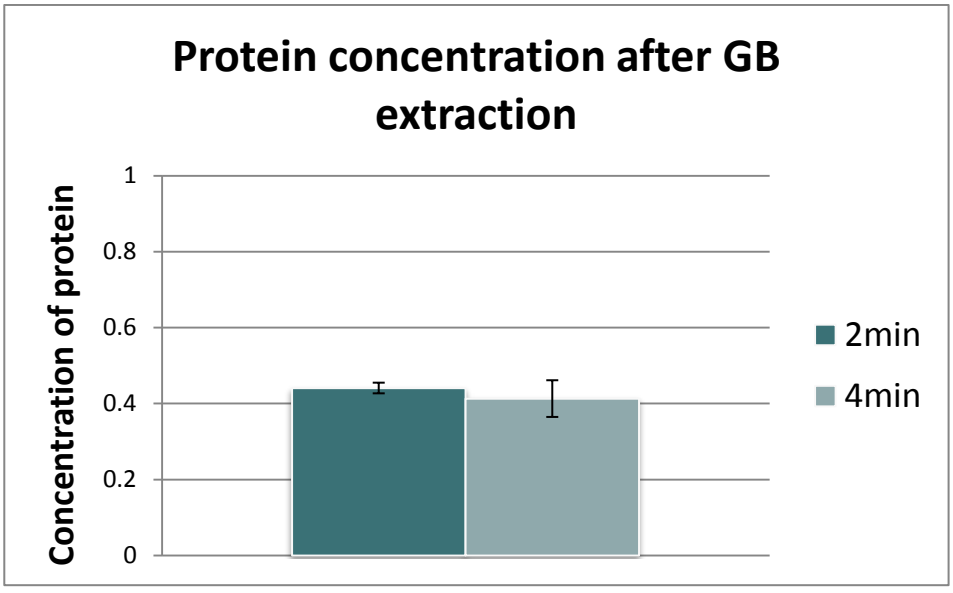

Figure 21 Protein concentration after glass beads extraction. 2 min samples was shaken for 1 min then kept on ice 1 min. This was repeated twice. 4 min sample was shaken for 1 min then kept one ice 1 min. This was repeated 4 times. Final OD value found were transformed by the Grove equation. This result was done in triplicate

As shown in figure 21, protein production was confirmed in yeast strain using GB method as the extraction samples showed absorption at 280 nm. SDS-PAGE results also showed the bands corresponding to FUM gene product by both PAE and GB method (figure 20). These results showed successful expression of FUM in yeast and *E. coli* and that PAE and GB methods are suitable methods for extraction of proteins from yeast.

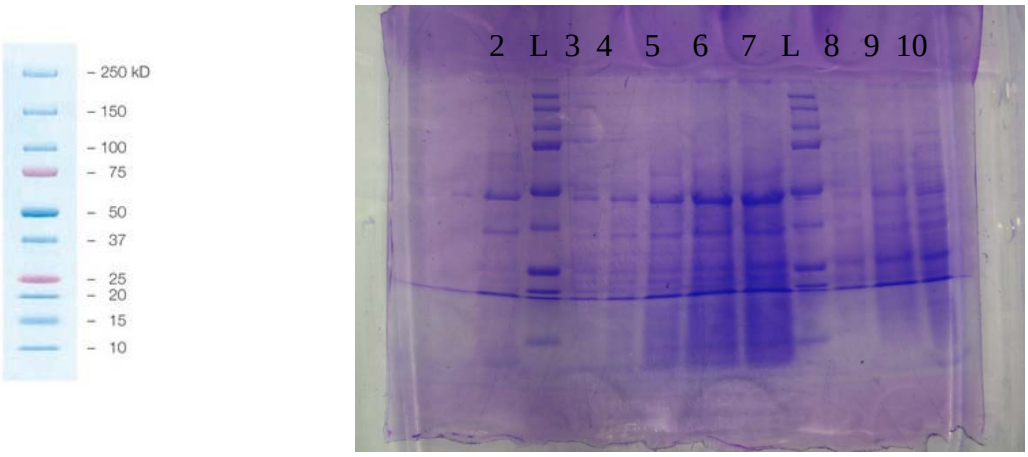

Figure 22 SDS-PAGE of Yeasts samples by PAE and GB. Lanes: L: Ladder; 2: 30 µL of Y-PAE; 3: 2 µL of Y- GB 2minutes; 4: 5 µL of Y-GB 2 min; 5: 10 µL of Y- GB; 6: 20µL of Y- GB; 7: 40 µL of Y- GB; 8: 0.5 µL of Y- PAE; 9: 4 µL of y- PAE; 10: 0.1 µL of Y PAE

Part 4: In vitro Enzyme Analysis

Enzyme assay

Table 8 Units/mL of enzyme of FUM. Three different extraction techniques were used (result done in triplicate).

	Units/mg of cells	Standard deviation
Rosetta-FUM	0.51	0.15
Yeast-FUM Post Alkaline Extraction	0.017	0.0001
Yeast-FUM Glass Beads	0.033	0.01

Encouraged to the result that both strain successfully produced FUM, enzyme activity was measured in vitro by spectrophotometer. By mixing protein extract containing FUM, buffer, substrate, co-factor and water, and heating this mixture at 70°C,

Fumarate production or consumption were monitored at 240 nm. Enzyme activity was first checked at 240 nm. Unfortunately, it appeared that the spectrophotometer results are really unstable at such a low wavelength. To overcome this problem, FUM was used coupled with ME. FUM converts FA to LMA and ME can convert LMA to pyruvate in the presence of $NADP^+$. Therefore FUM activity can be measured by monitoring NADPH production. As shown in Table 8, both strains successfully produced FUM with retaining its activity.

It was first proven that the absorbance measured is really due to FUM activity and that ME concentration is high enough for not having any impact on the reaction rate. This was done by

measuring the absorbance with different concentrations of FUM. If the concentration of FUM is increased by two, absorbance should also increase by two. This was proven and those results were triplicated (data now shown)

In vitro leakage assay

To evaluate enzyme leakage from cells, SN as well as cell extract samples were prepared after different incubation time at 70°C. Protein concentration per mL of enzyme was then calculated by the Bradford assay. As shown in figure 23, half of proteins leaked in early phase of incubation using *E. coli*, while proteins derived from yeast strain gradually leaked. After 3 hrs of incubation percentage of leakage of yeast reached that of *E. coli*. These results indicated that protein was more stably retained in yeast cells than *E. coli* cells.

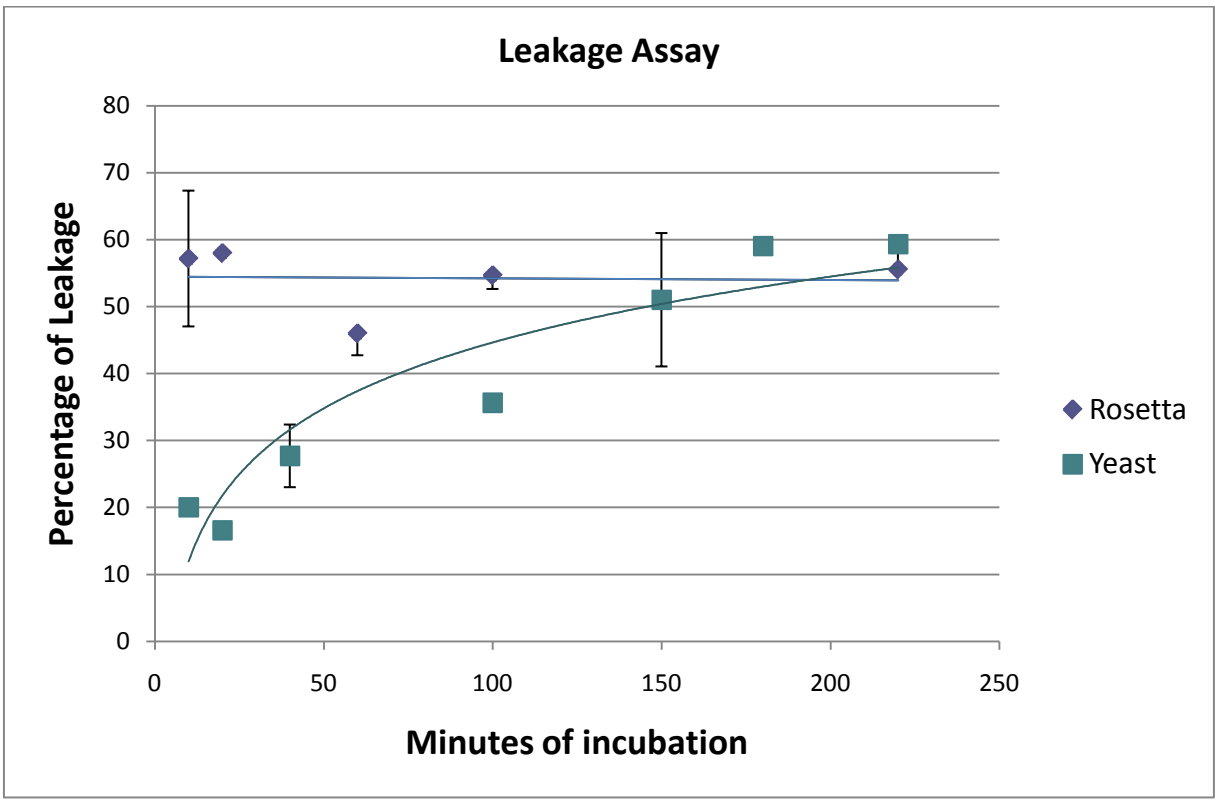

Figure 23 Percentage of leakage in bacteria and yeast cells, over time. In vitro (result done in triplicate).

In vitro leakage assay in the presence of cations

To the purpose of preventing enzyme leakage, whole cells of *S. cerevisiae* as well as *E. coli* were pre-incubated 4 hrs with different divalent cations. Manganese (Mn) and Calcium (Ca) were tested. Results of incubation with different concentration of $MnCl_2$ and $CaCl_2$ were shown in figures 24 and 25. The final percentage of leakage for every sample was compared with the leakage obtained for the control sample (same incubation time, without ion). As shown in these figures, addition of divalent cation brought in decrease of enzyme leakage in yeast while enzyme leakage increases in *E. coli*. Since high concentration of $CaCl_2$ (> 10

mM) brought in inactivation of FUM (Table 9), addition of 5 mM of is preferable for preventing enzyme leakage in yeast.

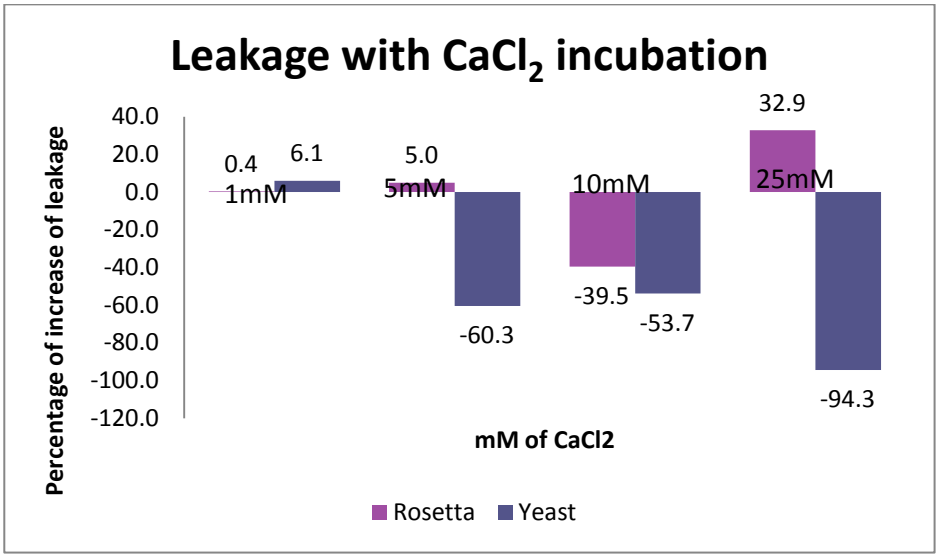

Figure 24 Leakage percentage with presence of $CaCl_2$. It can be seen really clearly that leakage increases in Rosetta cells with presence of $CaCl_2$, while SN activity was reduced by 94% in yeast cells. Enzyme unit with no ion was set to be 100%. This result shows the percentage of decreases of unit.

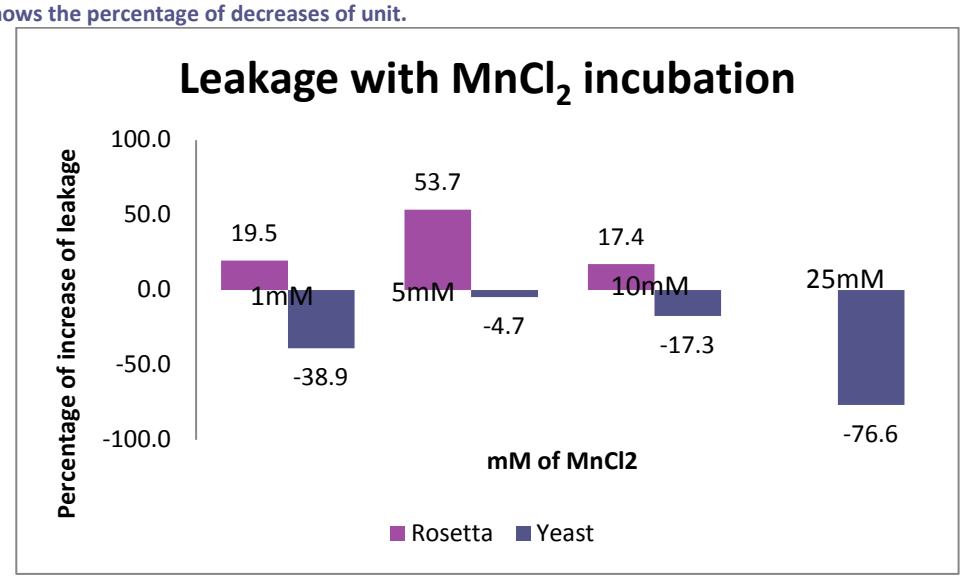

Figure 25 Leakage percentage in presence of $MnCl_2$.25mM $MnCl_2$ for Rosetta sample was not tested. Enzyme unit with no ion was set to be 100%. This result shows the percentage of decreases of unit.

Table 9 Percentage of enzyme activity in presence of $CaCl_2$. Enzyme unit with no ion was set as being 100%.

$CaCl_2$ concentration	25 mM	10 mM	5 mM	1 mM
Rosetta	inactivation	Inactivation	160%	142%
Yeasts	inactivation	Inactivation	231%	204%

Part 5: L-malic acid production

L-malic acid detection

Since FUM activity was confirmed in both yeast and *E. coli* strains, LMA production from FA was carried out. Before carrying out the production, some preparation was carried out. It first of all appeared important to check the detection limit of LMA by HPLC. Different concentrations of LMA in solution were used. As shown in Table 10, 0.15 mM seems to be the detection limit of LMY by HPLC.

Table 10 L-malic acid detection limit by HPLC

L-Malic acid detection limit	0.15mM

Fumaric acid solubilization

Subsequently, solubility of FA was investigated. As the enzymes used in this study are TEs, they have an ideal working temperature around 70°C that is far from conventional condition. The substrate used in this study, FA cannot be dissolved in water at RT with a low concentration equal to 100 mM. Its solubility was tested at different temperatures, up to 73°C, as shown in figure 26. At 50°C, around 100 mM could be dissolved in water. At 60°C, the highest concentration was observed, being equal to nearly 240 mM. This experiment was triplicated, and only the highest concentration dissolved in shown in figure 26.

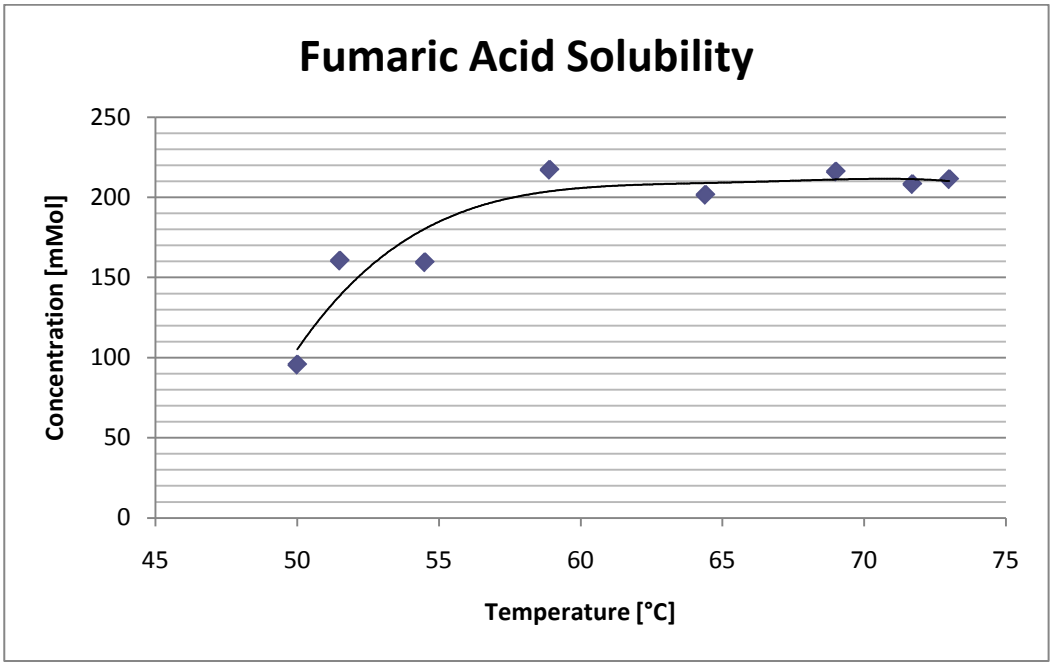

Figure 26 Fumaric acid solubility. This result was triplicated and only the highest concentration found from the triplicate experiment is shown on this figure.

L-malic acid production

After preliminary experiments mentioned above, LMA production was carried out. Principles of SME were applied during the whole cell experiments. Untreated cells were resuspended in phosphate buffer, and incubated at 70°C for 20 min. After having cooled them down on ice, FA was added, up to a concentration of 10 mM. Cells were then incubated at 70°C for different period of time. Reaction was stopped by adding the same volume than the sample volume of phosphate buffer, and samples were cooled down on ice. LMA production was then evaluated by HPLC.

LMA production was first checked up to 2 hours (figure 27). Yeasts cells were used with a concentration of 80 mg/mL. Rosetta cells were used with a concentration of 40 mg/mL.

Long-termed LMA production was also carried out to check the bioconversion after 2 hours. As shown in figure 28, conversion using Rosetta reached approximately 90% really fast, while conversion using yeast cells reached approximately 80% even after 5 hours.

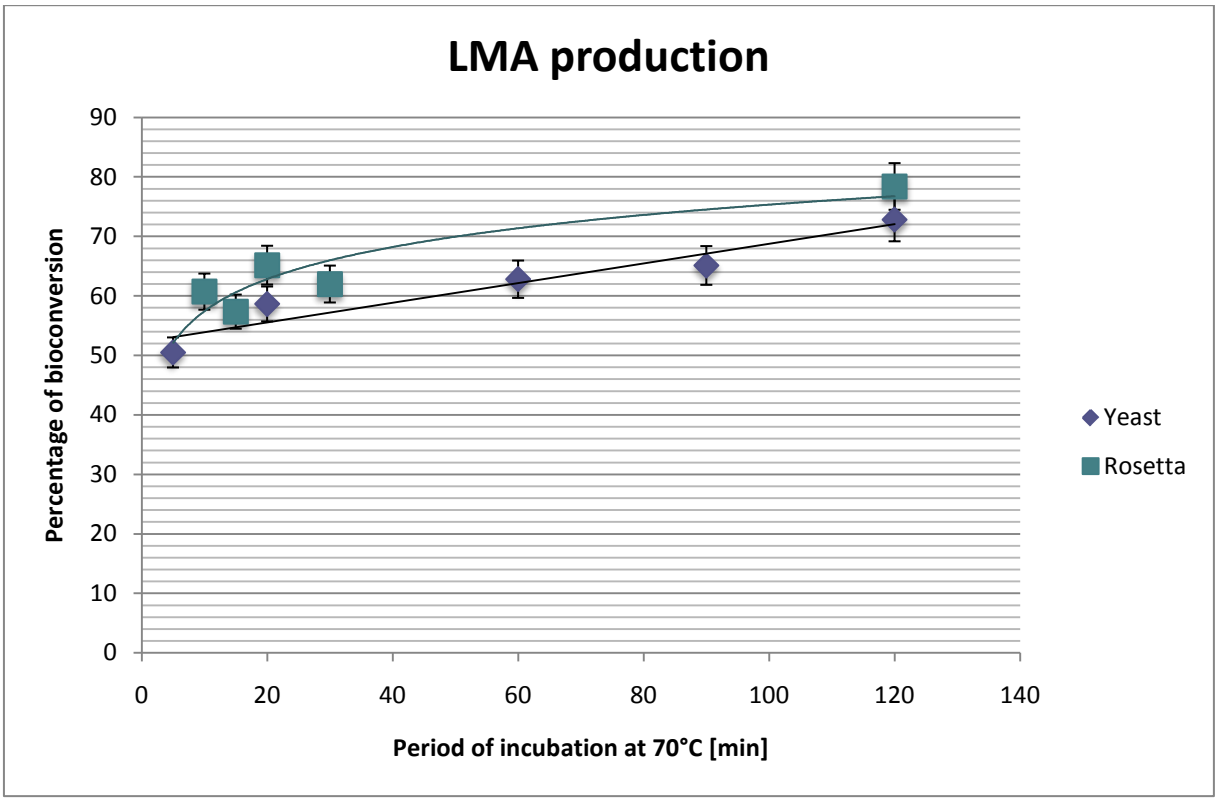

Figure 27 LMA production from 0 to 120 minutes. The percentage of bioconversion is shown. Results were done in triplicate.

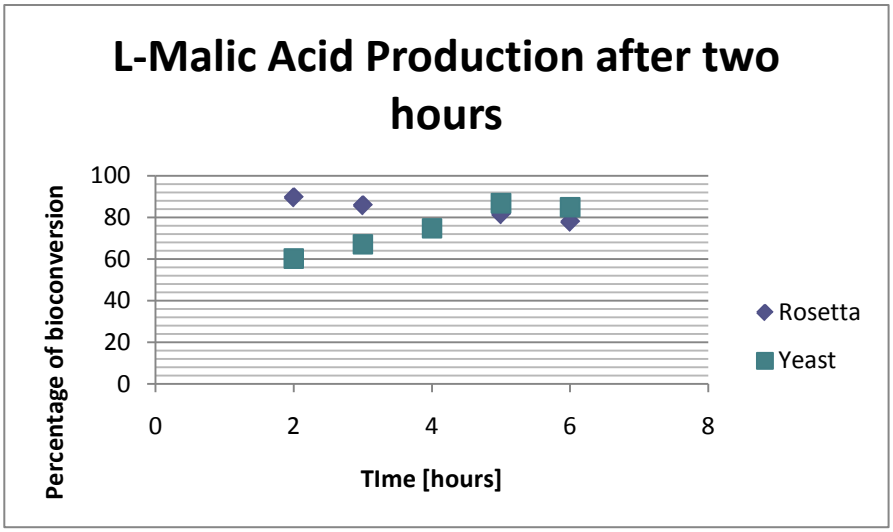

Figure 28 L-malic acid production for different periods of time over two hours

Leakage assay

Enzyme leakage from cells was also tested in whole cell experiment. Yeast cells over-expressing FUM were incubated at 70°C for 1, 2 and 3 hours. After centrifugation, SN and cells were kept separately and cells were resupsended in phosphate buffer. FA was then added to both cells and SN sample to final concentration of 10 mM. All samples were then incubated 3 hours at 70°C. Reaction was then stopped by adding phosphate buffer, and samples were cooled down on ice. After centrifugation, 100 µL of sample was subjected to HPLC analysis. As shown in Table11, no LMA was produced using SN samples. This result indicated that most of enzyme was kept in yeast cells after 2 and 3 hours incubation at 70°C.

Table 11 Bioconversion of FA to LMA, after 2 and 3 hours of incubation at 70°C. SN was then collected, and incubated with FA (10 mM) for 3 hours. Result was done in triplicate

Time of incubation [hour]	2	3
Bioconversion	0	0

Part 6: Reusability assay

To confirm whether FUM-expressing cells can be used for several batch reactions, reusability assay was performed over four hours. Whole cells of Rosetta and yeast that over-expressing FUM were incubated 20 min at 70°C. FA was then added up to a concentration of 10 mM. Samples were then incubated up to two hours at 70°C. After two hours, samples were centrifuged and cells were kept and washed with 0.2% phosphate buffer. Phosphate buffer and water and 10 mM FA were then added. Samples were incubated at 70°C for up to two hours again. LMA production and FA consumption were measured by HPLC. As shown in figure 29, conversion rate of FA to LMA dramatically decreases when second batch reaction was carried out using *E. coli* cells. On the other hand, conversion rate of FA to LMA did not decrease when yeast cells were used (figure 30). The same experiment was also carried out, in presence of $MnCl_2$ (figures 29 and 30). 10 mM of $MnCl_2$ final concentration was used. $MnCl_2$

was added directly when the cells were mixed with water. Cations were therefore present for the pre-incubation of 20 min. These results strongly indicated that yeast cells expressing FUM have reusability and can be used for repeated reaction.

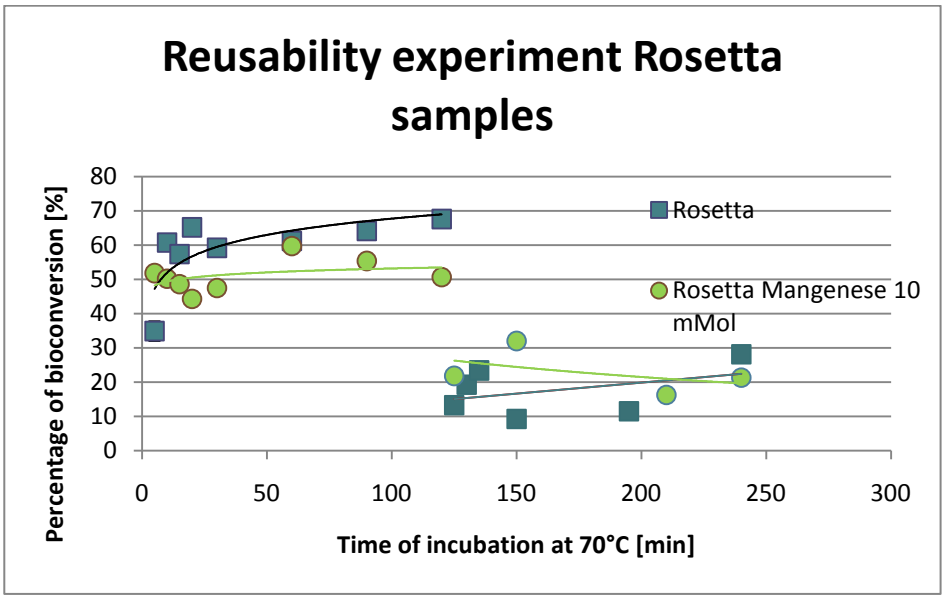

Figure 29 Reusability assay over 4 hrs for Rosetta over expressing FUM. Result was done in duplicate.

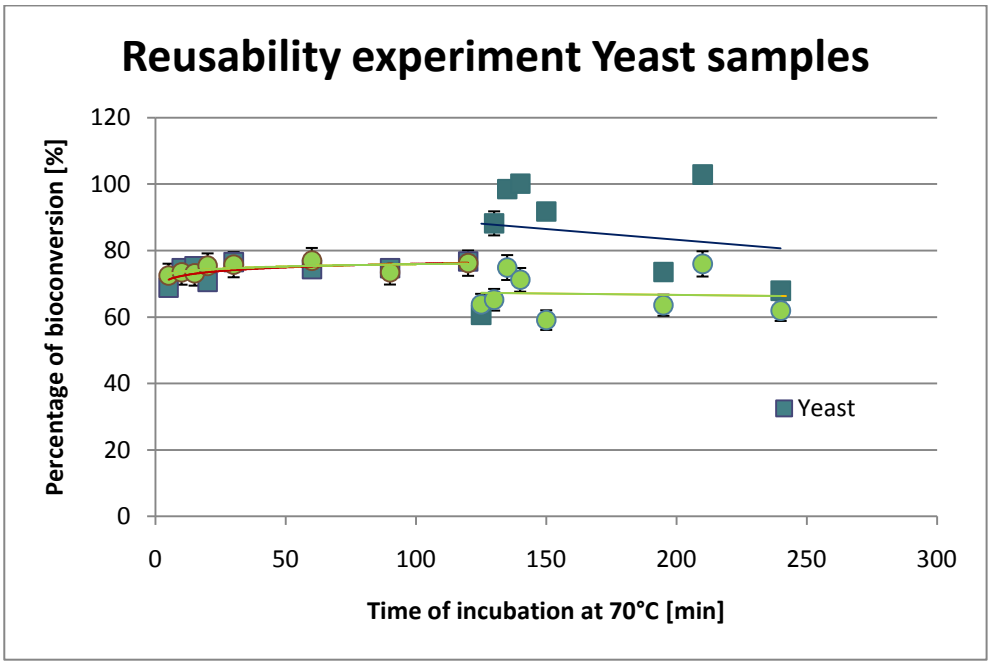

Figure 30 Reusability assay for Yeast samples over expressing FUM over 4 hrs. Result was done in duplicate

Part 7: Yeast cells characteristics

Pictures of cells

By simply looking at the heated samples, it seemed that *E. coli* cells lyses more easily than *S. cerevisiae* cells. In order to confirm this point of view, pictures of cells were taken under microscope, 40 x 0.60, Plan Fluor, Ph2 DM ELWD. Rosetta and yeasts cells before heat treatment can be seen in figure 31. As it can be seen in this, bacterial cells are too small to be correctly seen under this magnification. Nevertheless, yeast cells were heated 30 min at 70°C and a picture was taken after heat treatment. This can be seen in figure 31. No difference in either size or shape can be seen. It would nevertheless be interesting to take pictures of the cells with a higher magnification, in order to be able to see more clearly the membrane structure.

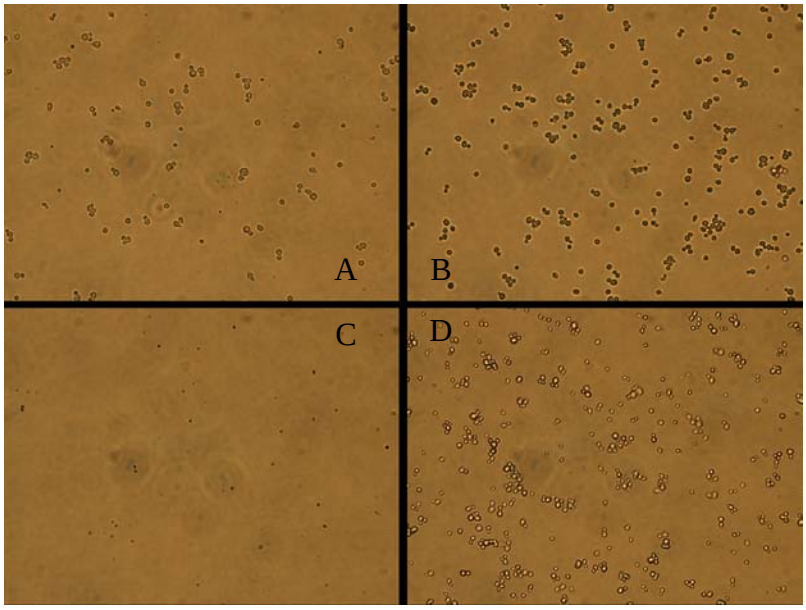

Figure 31 Yeast and Rosetta sample A Yeast cells B Yeast cells after 30 min heat treatment C Rosetta cells D Yeasts and Rosetta cells after heat treatment

Sedimentation assay

In order to prove the advantage of yeasts cells compared to *E. coli* cells, sedimentation of cells was tested. This was carried out by monitoring the OD_{600} or OD_{660}, for yeasts and bacteria respectively, over approximately 3 hours, in a cuvette where the bottom and the top was hidden. Only an area of approximately $1cm^2$ was still not hidden. As shown in figure 32, yeast cells showed better sedimentation property than *E. coli* cells.

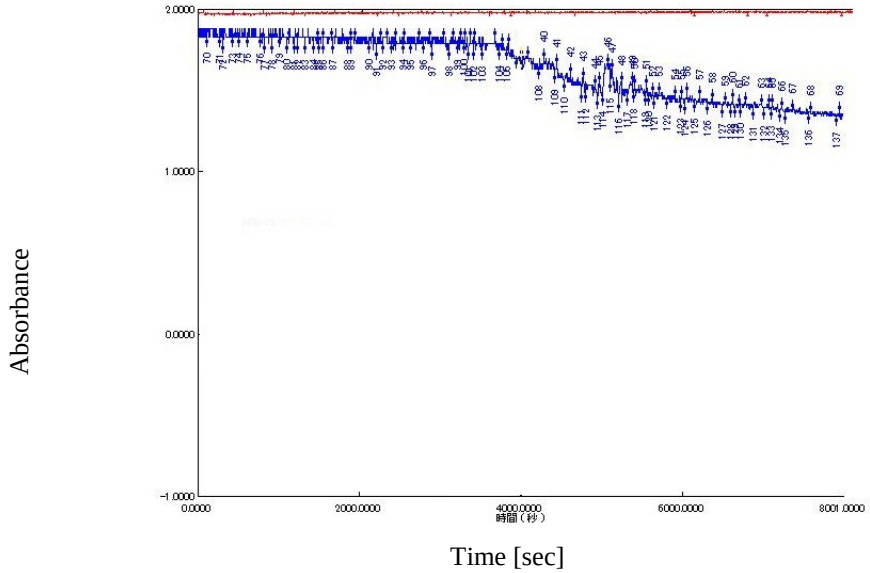

Figure 32 Sedimentation assay. In red: *E. coli* cells, measured at OD_{660}. In blue: Yeast cells, measured at OD_{600}, for 2 hours and 20 min, approximately

Table 12 Sedimentation Ratio after 8,000 seconds (2.22 hours)

Sedimentation Ratio After 2.22hours	
E. coli	~100%
Yeast	28.88%

A better separation of biomass by yeast cells can also be seen by looking at micro tubes used for the application of SME. In figure 33, Rosetta and yeast cells at a concentration equal to 100mg/mL were incubated at 70°C. After the heat treatment, bacteria cells seem to have lysed, as the sample become really sticky. After centrifugation of 5,000 rpm (5 min, 4°C), it is not possible to separate bacterial cells from SN (figure 31.B). In the case of yeasts, 5 min centrifugation is enough to separate cells from SN.

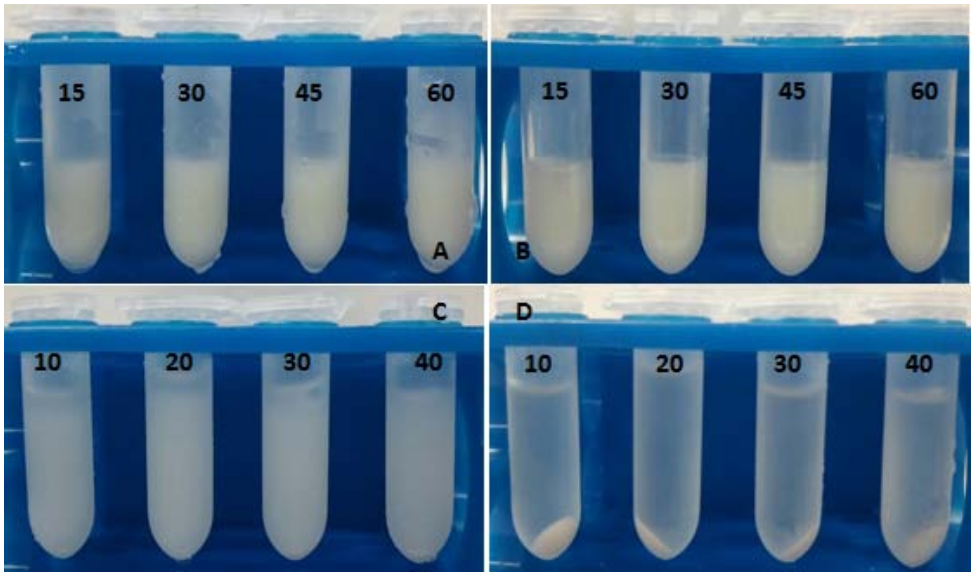

Figure 33 A: Rosetta cells after incubation at 70°C; B: Rosetta cells after incubation at 70°C and centrifugation at 5,000 rpm; C: Yeast cells after incubation at 70°C; D: Yeast cells after incubation and centrifugation at 5,000 rpm

VII. Discussion

The first part of this project was the construction of the correct plasmid, in order to produce FUM in *S. cerevisiae*. This was carried out by ligating *fum*, the gene encoding fumarase, on p803 plasmid, given by the NBRP. The restriction sites on the plasmid were first checked and *fum* already available in our laboratory was modified. Those modifications were checked by sequencing, and *fum* was then ligated on p803 plasmid. Finally, the newly ligated plasmid was amplified in *E. coli*.

Part 1 Plasmid creation

Enzymes restriction

Results in figure 5 showed that *Xba*I digests the plasmids at two different sites as two size of band was observed. Unfortunately, this was not mentioned on the plasmid map given (this can be seen in figure 1 as well as appendix). The restriction site seems to be unique. This result was duplicated and confirmed that *Xba*I digests both plasmids (p803 and p804) at two different sites, giving an approximately 3,000 bp-length and a 4,000 bp-length fragments. It can also be seen that *Xba*I does not remove the stuffer DNA.

As the sequence of stuffer cDNA is unknown, there is the possibility that *Xba*I digested in the middle of stuffer DNA. However, this digestion does not give 4,000 bp and 3,000 bp-length bands. Another possibility could have been a contamination during the digestion. This seems nevertheless unlikely, as all digestions have been at least duplicated. The reason is unclear, but there seems to be something wrong in given sequence of p803 and p804.

Figure 5 also showed that *Xho*I removes the stuffer DNA only in plasmid p803, and not in plasmid p804. Unfortunately, *Xho*I also digests *fum*. Therefore, *Xho*I is not available for cloning of *fum*.

Digestion of the plasmids with *Eco*RI and *Sal*I was performed and the stuffer was correctly removed from p803, but not p804 (figure 7 and 8). In addition, *Eco*RI and *Sal*I sites are not contained in *fum*. Therefore, p803 was chosen as the vector plasmid for plasmid construction. The digested plasmid was retrieved from the gel, thanks to the GE Kit in approximately 40 µL of MilliQ water.

In all the results obtained, it can be seen that the plasmids are around 7,000 bp, which was corresponding to the size from given information. The stuffer removed in figures 7 and 8 seems to be approximately 400 bp-length. Since the length of stuffer DNA is 300 bp, it is considered that enzyme sites exist near the stuffer DNA. It is still not sure if plasmid p804 contains a stuffer DNA or not. With all the digestions done, no stuffer was removed.

Gene modifications

On figure 9, a consensus sequence composed of AAAATG seems to be the most appropriate one for *S. cerevisiae*. This figure was created with seqLogo [Schneider *et al*., 1990], by retrieving sequences from UCSC Table browser, with the genome of *S. cerevisiae* assembled in 2008 (track SGC gene strand + and entire genome). The perl code used to create the sequences database can be found in appendix.

Studies about the translation initiation regions of *S. cerevisiae* have already been done [Hamilton *et al*., 1987]. It was proven that the start codon context is different from animal mRNAs [Kozak, 1987], with a consensus sequence shown in figure 34. In *S. cerevisiae*, no exception from AUG start codon was found. In highly expressed genes, the consensus sequence of RBS would be AAAAAA ATG TCT, with a C avoided in the +4 position, G avoided in the leader position and an A in positions -1 to -6 at more than 50% [Hamilton *et al.* 1987].

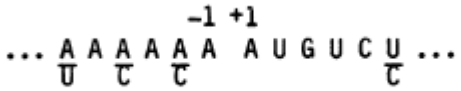

Figure 34 Consensus sequence for the AUG context in highly expressed genes [Hamilton *et al*., 1987]

In lower eukaryotes, such as *S. cerevisiae*, AUG is the only codon recognized as the translational initiator [Chang and Wang, 2004, Hamilton *et al*., 1987]. Therefore, if the first AUG codon is mutated, initiation of translation begins at the next available AUG on the mRNA. Yeasts cannot efficiently use non-AUG codon as translation start sites. Examples of native non-AUG initiation have not been reported in *S. cerevisiae*.

Table 13 Codon Usage Table (modified from http://crumb.stanford.edu/community/codon_usage.shtml), from 435 genes found in GenBank 63

Amino Acid	*Codon*	*Number*	*/1000*	*Fraction*	*Percentage*
End	***TGA***	***151.00***	***0.70***	***0.34***	***34%***
End	***TAG***	***78.00***	***0.36***	***0.17***	***17%***
End	***TAA***	***219.00***	***1.10***	***0.49***	***49%***
Met	***ATG***	***4610.00***	***21.31***	***1.00***	***100%***

As shown in Table 13, ATG is the most frequent start codon and TAA is the most frequent stop codon in the yeast strain used in this project. For proper protein production in *S. cerevisiae*, it seemed therefore important to modify at least the start codon and add a poly-A before the initiation codon as well as restriction enzymes sites on both ends. Six A were then added upstream of the start codon after the restriction enzyme site. *EcoR*I was added on the forward primer, while *Sal*I was added on the reverse primer to be able to ligate the gene of interest into p803 plasmid. Primers sequence is shown in Table 3.

The electrophoresis result after PCR of *fum* is *shown* in figure 10. The size of amplified gene was 1,400 bp that is corresponding to expected size, and this result indicates that the PCR have worked correctly. PCR conditions did not need to be optimized. This was one of the issues that could have been raised, as extremophiles are known to have a high GC content. It also seems that the concentration obtained is high. The modified gene was purified from the gel with the GE Kit and dissolved in approximately 40 µL of MilliQ water.

Sequencing of gene modifications

Before transformation of *S. cerevisiae*, it is important to sequence the modified gene. Ligation of the gene with pBS allows an easier sequencing. pBS was digested in a blunt end manner, which reduces its efficiency. The ratio of the gene to be ligated and pBS is really important and colonies can be obtained only if this ratio is optimal. Thanks to the blue/white screening, colonies that have integrated gene will appear white. As a control, bacteria were also transformed with circular pBS. All of them appeared blue, as expected.

Only white colonies were picked and insertion of *fum* was confirmed by colony PCR as shown in figure 11. Blue colonies were also picked as controls. None of them had the insert (data not shown).

As shown in figure 13, colony 7 did not give any results for sequencing. However, homology reached 100% for colony 1 for the forward sequencing. Only one nucleotide on the reverse sequencing seemed to be out of place. By looking at figure 14, which is a detail of the whole sequenced gene, it can be seen that the graph and the sequence found have small differences. From nucleotide 793 to 796, the sequence found was GGGG while the graph shows only 3 distinct black peaks. Therefore, the graph can be trusted more than the sequence given by the sequencer program. The modified gene reached 100% homology for the forward and reverse sequencing and every expected change were introduced into *fum*. These results showed successful modification of *fum*.

The correctly modified gene from colony 1 was then digested from pBS plasmid by *Eco*RI and *Sal*I. The digested gene can be found in figure 15. The two bands were obtained and one had approximately 1400 bp-length, which is corresponding to the length of fumarase gene, and the other had approximately 3,000 bp-long, which is corresponding to the length of pBS plasmid digested. The gene was retrieved from the gel using the GE kit.

FUM was then ligated into p803 pre-digested and amplified in *E. coli*. After the retrieval of the plasmid from *E. coli*, *S. cerevisiae* cells were transformed.

Part 2: Yeast transformation

After transformation of yeast cells, and three days of incubation at 30°C, a dozen of colonies could be seen. 5 were picked to check the insertion of p803-FUM. As shown in figure 17, only colony 1 and colony 2 carry the plasmid. Colony 2 was selected and plated again on an SD + HIS plate. This colony is the only one used for all the future experiments. The plasmid used in this study carries a dual host capacity. It can therefore be amplified in bacteria and expressed in yeasts [Hadfield in Johnston *et al.*, 1994]. It also carries the 2 µm ORI. This implies that, depending on the transformants, expression level can be different.

This study shows the first trial of SME in yeast cells. SME was proven to be really successful in E. coli [Honda *et al.*, 2010; Iwamoto *et al.*, 2007; Restiawaty *et al.*, 2011]. SME was also applied on *Rhodococcus opacus* [Klinger J., Master thesis, 2010]. In that trial, it appeared that a maximum of 60% bioconversion could be achieved.

Part 3: Protein production and extraction

The production of FUM in yeast can then be checked by SDS-PAGE. It is also important to produce FUM in Rosetta, in order to compare both organisms. Rosetta were therefore transformed with pET11-a-FUM and grown in LB Cm Amp medium plates.

The first attempt at SDS-PAGE is shown in figure 19. As it can be seen, a too low amount of protein extract was loaded for the Rosetta samples, while a too high amount of protein extract was loaded for the yeast samples. Yeast not transformed were also grown and used as a control and to compare the difference in protein production. As a result, from the first attempt, fumarase production could not be confirmed. It can nevertheless be found that sonication protocol does not work on yeasts. This was actually assumed as yeasts membrane is different from *E. coli* membrane.

The second attempt at SDS-PAGE is shown in figure 20. Protein extract from yeasts cells transformed and not transformed was prepared with post-alkaline extraction as well as glass beads - DTT lysis. Every sample was separated in two portions and one of them was heated at 70°C for 20 min. The samples derived from four different colonies of Rosetta were also prepared by sonication, and half of the samples were also heated at 70°C for 20 min. Yeasts medium from the transformed and the not transformed cells was also loaded in order to see if the protein is secreted or not. As it can be seen, the Rosetta samples gave satisfactory results as 50 kDa size of the bands corresponding to FUM were observed. No evident change between the heated samples and not heated samples can be seen. For yeast samples, it can be seen that GB - DTT lysis seems to have failed. Only PAE gave protein extract concentrated enough to detect on SDS-PAGE, even if a too high concentration was loaded on the gel. As same as for Rosetta samples, no evident difference with the heated sample compared to the non-heated samples can be seen. It can also be seen that no protein were stained in the medium samples. Therefore, it seems that no protein was secreted into YPD or SD+HIS medium.

GB extraction was tried as shown in figures 21 and 22. Two different numbers of repetitions of shaking were carried out. 2 min of GB extraction showed slightly higher protein concentration than 4 min of GB extraction. Therefore, the samples after 2 min GB extraction were analyzed by SDS-PAGE as shown in figure 22. As a result, expression of *fum* was confirmed in both yeast and *E. coli* strains. Due to the 2 μm ORI, some transformants can have a lower expression level. Protein extract was checked by SDS-PAGE (date not shown) from different transformants by GB and no difference between intensity of FUM band were seen.

Part 4: In Vitro Enzyme Analysis

Enzymes are proteins that catalyze chemical reactions [Lodish *et al*., 2008; Claessen *et al.*, 1983]. They increase the rate of a reaction, without affecting its extent and without permanently changing their conformation. Michaelis and Menten showed that the rate of an enzymatic reaction is proportional to the substrate concentration, only if this one is low. At high concentration, the rate reached a maximal velocity V_{max}, V_{max} being proportional to the

enzyme amount. This saturation is due to the binding of the substrate to a limited number of enzyme sites. The rate of formation of a specific product at a specific substrate concentration [s] is given by the so-called Michaelis-Menten equation

$$V_0 = V_{max} \frac{[s]}{[s] + K_m}$$

where K_m is the substrate concentration that yields a half-maximal reaction rate and is called Michaelis constant. The smaller the K_m value, the faster the reaction, and therefore the more effective the enzyme is. The rates of reactions vary among enzyme. This rate was found in this study by spectrophotometer.

The units/mL of enzyme is found by the following equation:

$$\frac{Units}{mL} enzyme = \frac{\Delta(A_{340}/\min test - \Delta A_{340}/\min blank) \cdot V \cdot df}{6.022 \cdot V_{enz}}$$

Where

V = Volume of assay [mL]

V_{enz} = Volume of enzyme used [mL]

Df = Dilution factor

ΔA_{340} = Difference in absorbance at 340 nm per minute¨

6.022 = extinction coefficient of $NADP^+$

This result was then converted per unit/mg of protein by dividing it by the mg of protein of enzyme (found by the Bradford Assay). It can also be converted to unit/mg of solid, by dividing it by the mg of cells / mL of enzyme.

One unit of enzyme will convert 1.0 mmol of product to substrate per min.

Enzyme assay

The result of enzyme assay is shown in Table 8. As it can be seen, enzyme activity of cells is approximately 50 times higher for Rosetta samples than yeast samples extracted with PAE and approximately 15 times higher than the one for yeasts extracted with GB. This result clearly showed that expression level of FUM by Rosetta is higher than yeast one. It is therefore assumed that the bioconversion of FA to LMA will be more slowly for yeasts cells than bacteria cells.

As PAE involves incubation with NaOH, it was anticipated that the enzyme activity would change. As it can be seen in Table 8, GB seems to be a better extraction method than PAE. It was therefore decided to use only GB for further extraction of proteins. In Table 8, the units of the enzyme are shown as per mg of cells. It is therefore difficult to know if FUM produced in yeasts is really less active than the one produced in Rosetta, or if this difference is due to the extraction method. It is believed that GB can extract 90% of proteins. This result was

triplicate and the average is also shown in Table 8. While enzyme activity is different between both strains, these results showed that FUM was expressed as active form in both strains.

In vitro leakage assay

One of the biggest problems of SME is the leakage of the enzyme out of the cell [Restiawaty *et al*., 2011; Tsuchido *et al*., 1985]. Therefore, after incubation of cells at 70°C for different period of time, enzyme leakage was estimated by measuring enzyme activity of SN samples in vitro. The unit/mg of protein was calculated from SN as well as remaining cells after centrifugation (proteins were extracted from those cells). The percentage of leakage shows the result of the following calculus

$\%\ leakage = \frac{U_{SN}}{U_{SN} + U_C}$, where U_{SN} represents the unit/mg of protein of the SN and U_C represents the unit/mg of protein of remaining cells.

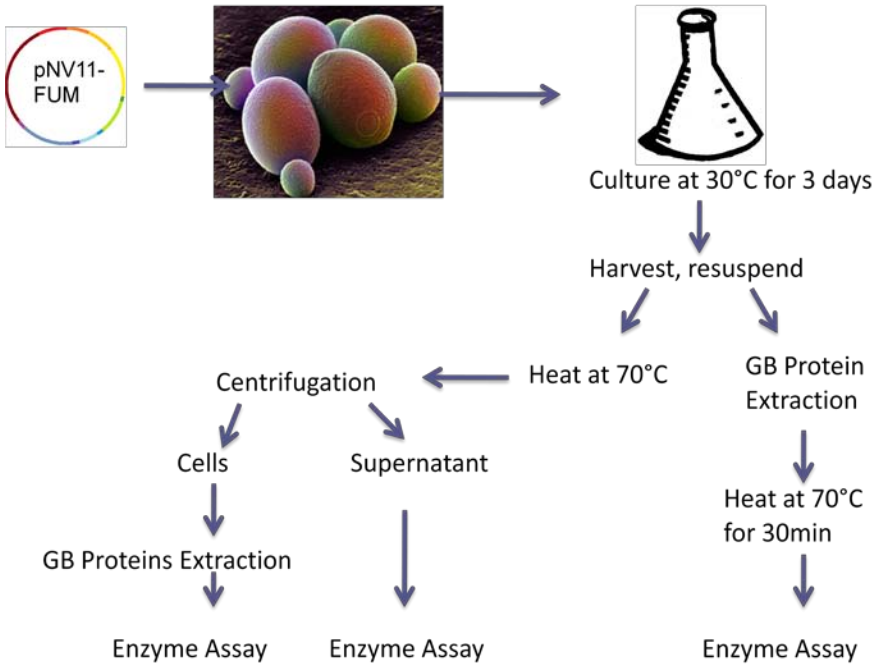

Figure 35 In vitro leakage assay methodology. Yeast cells were first transformed by p803-FUM, and then culture for at most 3 days. Enzyme assay was carried out for remaining cells, SN as well as protein extracted before heat treatment. Cells culture was done up to three days for untransformed yeasts, while transformed yeasts and Rosetta were cultured up to 12 hours.

The results of leakage estimation over time are shown in figure 23 (average of triplicate results). This figure shows the percentage of enzyme activity outside the cell compared to the overall enzyme activity. It can be seen that even after only 5 min, nearly 60% of enzymes

have leaked from bacterial cells. On the other hand, only around 35% of enzymes have leaked from yeast cells, even after 100 min of incubation. These results strongly insist that yeast becomes a useful host for SME as it can stably retain enzymes even at high temperature.

In vitro leakage assay in presence of cations

To prevent enzyme leakage, addition of divalent cation to reaction mixture was examined. As shown in figures 24 and 25, both cations inhibit enzyme leakage for yeast cells. On the other hand, addition of cations has a different impact on *E. coli* samples. A too high concentration of ion increases the leakage, while a low concentration of ion seems to not change the leakage value. Coupled enzyme assay was then performed in presence of different concentration of $CaCl_2$.As shown in Table 9, high concentration of $CaCl_2$, such as 25 mM and 10 mM inactivated FUM, while 5 mM and 1 mM activated FUM. FUM from yeast was even more activated than FUM from bacteria.

Part 5: L-malic acid production

L-malic acid production

As FUM was expressed in active form in both strains, LMA production from FA was carried out. As shown in figure 27, Rosetta cell bioconversion followed an exponential curve and reached 80%. Yeast cells bioconversion followed a linear curve and reaches 70% after two hours. Higher production rate of LMA in Rosetta cells was expected due to their higher FUM activity. LMA production from FA by *S. cerevisiae* has already been done. Wang and co-workers [Wang et al., 1996] proved that the conversion of FA to LMA was possible with *Brevibacterium flavum* immobilized cells. Unfortunately, they found that succinic-acid was formed as a side-product. In the current study, no high concentration of side-product was found.

Neufeld and coworkers [Neufeld et al., 1991] used immobilized cells of *S. cerevisiae* for conversion of FA to LMA. They could show that in free cells experiment, FA was converted to LMA as a rate equal to 65 mM g^{-1} hr^{-1}. 87% of FA was converted to LMA in 45 min. This conversion rate is higher than the one found in this study, but the main advantage of SME is its easy application. Compared to SME, the protocol used by Neufeld and coworkers is more difficult.

LMA is currently produced by chemical synthesis via hydratation of FA. This leads to a racemic mixture of malic acid, from which LMA should be purified. Another way currently followed to produce LMA is by enzymatic process [Wang *et al.*, 1998, Wang *et al.*, 1998].

Presecki and coworkers [Presecki and Vasic-Racki, 2005] used permeabilized *S. cerevisiae* for the production of LMA. A study of different strain was done and a proposed model for batch production of LMA was also performed by Presecki and coworkers. They could prove that the permeabilization process removes the barrier for free diffusion across cell membrane and empties the cell of most of the small molecular weight cofactor. Overall the yeast strains tested, permeabilized *Saccharomyces bayanus* showed the highest activity.

Leakage assay

During production of LMA using yeast, samples were collected and LMA production was also carried out using SN samples to estimate whether FUM leaks from yeast cells or not. As shown in Table 11, no LMA could be detected by HPLC after 3 hours of incubation. This result indicates that FUM did not leak to SN in detectable level from yeast cells. In vitro enzyme experiment uses a really small amount of substrate and a small enzyme concentration. In vitro experiment is more sensitive than HPLC. The fact that no product was detected by HPLC is therefore compatible with the fact that activity was found in the SN.

Part 6: reusability assay

Reusability assay

Encouraged to the result, yeast cells stably retained FUM intracellular, repeated batch reaction was carried out to compare reusability of cells between *E. coli* and yeast. As shown in figure 29, Rosetta lost 40% of activity after first batch reaction.

On the other hand, as it can be seen in figure 30, yeast cells did not show decrease of enzyme activity after first batch reaction. This successful result showed that nearly no enzyme was removed during the washing of the yeast cells. The leakage of the enzyme is considered as being one of the biggest problems of SME in *E. coli*. Yeasts cells seem to overcome this problem.

Reusability assay in the presence of $MnCl_2$

In figures 29 and 30, it seems that $MnCl_2$ presence does not really have an impact on the leakage from bacterial cells. It also seems that, at first, $MnCl_2$ will have a negative impact on the enzyme activity. This seems to be the case also for yeast cells. This impact is nevertheless really small.

Leakage during second batch experiment was tested in *E. coli* [Restiawaty *et al.*, unpublished]. To overcome the leakage problem, it was proven that linking the protein of interest to a membrane protein of *E. coli* is suitable. This is nevertheless a really tedious protocol and shows clearly one of the major problems of *E. coli* for the application of SME.

Bressler and coworkers [Bressler *et al.*, 2002] attempted to use a bioreactor based on supported liquid membranes for production of LMA from FA. This complicated design ensures mass flow and feasibility of continuous production of LMA from FA. A conversion rate of 84% (above the calculated equilibrium value) was demonstrated. Continuous production of LMA was demonstrated and it is believed that yeasts can become cheap and accessible biocatalyst [Sikorski *et al.*, 1989; Presecki and Vasik-Racki, 2005; Wang *et al.*, 1996]

Part 7: Yeast cells characteristics

Sedimentation assay

As shown in figures 31, yeast cells are bigger than bacteria cells. It is considered that yeast cells weight more than bacteria and they will show good sedimentation. After 130 min, approximately 15% decrease of the OD of yeasts cells was observed, while bacteria samples OD did not vary. Sedimentation ratio after 2.22 hours is shown in Table 12.

The advantage of using yeast over E. coli for use of a bioreactor was analyzed by Domingues and co-workers [Domingues *et al.*, 2000]. It was proven in their study that yeasts are more resistant to contamination at a high-cell density than bacteria. As heat treatment is part of SME process, contamination should also be reduced. Those two promising facts shows that yeasts could be considered as a really good host for SME application

Different yeast characteristics were also monitored. The results in this study show clearly that a better biomass separation can then be achieved with yeasts compared to bacteria. Yeasts are very well-known organisms, used in industry for production of proteins or ethanol. It can be added that the production of recombinant proteins for therapeutic uses in *S. cerevisiae* is nowadays done. One example would be rh Insulins, by Novo Nordisk, produced by secretion. Vaccines for Hepatitis B virus or Human Papilloma Virus are also produced in *S. cerevisiae* (Recombivax-HB, by Merck and Co, Engerix-B by GlaxoSmithKline, Gardasil, by Merck and Co.), as well as semi-synthetical molecules, such as Vitamin C and Artemisin [Walsch, 2010]. Yeasts are mainly used for their capacity to secrete proteins. Secretion of protein of interest is not wanted for application of SME. Plasmid used should therefore be cautiously chosen.

VIII. Conclusion

In this study, SME was applied to *S. cerevisiae* cells, in order to produce LMA from FA. *Fum* was first modified in order to produce FUM in yeast cells. The modifications were confirmed by sequencing the gene. The correctly modified gene was then ligated to a YEp plasmid, p803, carrying GAP promoter. p803-FUM was amplified in *E. coli* cells and *S. cerevisiae* cells were transformed with the extracted plasmids.

Enzyme production was confirmed using different protein extraction protocols by SDS-PAGE. The most suitable protocol for this study was the GB protocol. *E. coli* cells were also transformed to express FUM. FUM activity and LMA production were then compared between both microorganisms.

FUM activity was first confirmed in vitro by coupling it with ME. In this study, the gene of interest was obtained from *T. thermophilus*, a thermophilic bacterium. The main advantages of performing processes at higher temperature are the reduction of microbial contamination, reduction of viscosity, improvement of mass transfer rate and substrates solubility [Bruins *et al*., 2001]. The main drawbacks are the instability of cofactors, substrates and products. In this study, the solubility of the substrate, FA, is increased with higher temperature.

It appeared that expression level of FUM in yeast cells is less than that in *E. coli*. Nevertheless, a high percentage of bioconversion from FA to LMA was achieved with yeasts.

Enzyme leakage during incubation at high temperature was tested for both microorganisms. It had already been reported that, by heating cells at such high temperature, leakage can happen [Restiawaty *et al.*, 2011]. This is especially inconvenient for SME application as cells could not be re-used for bioconversion. Leakage of enzyme was therefore investigated in both microorganisms. Enzyme leakage was first estimated by in vitro enzyme assay. While enzyme leakage was observed to some extent in yeast cells, the level of leakage was less than that in *E. coli*. Enzyme leakage during LMA production was also carried out in yeast cells. After different period of heat treatment, reaction mixture was collected and SN was obtained. Using SN, conversion of FA to LMA was examined for up to 3 hours. No fumaric acid was produced even after 3 hours of incubation.

Enzyme activity was also estimated in presence of cations in vitro. Two cations used in this study, $MnCl_2$ and $CaCl_2$ showed an impact on enzyme activity in SN. It was proven that $CaCl_2$ has a negative impact on the enzyme activity.

The reusability of cells was then checked, in presence and in absence of cations. $MnCl_2$ was shown to have an impact of leakage by reducing it. It appeared that reusability of yeast cells is indeed better than bacterial cells. These results strongly demonstrated superiority of yeast as a host for SME as expressing enzyme stably retains in the cell and whole cell biocatalyst can be repeatedly used, while expression level of enzyme is less than in *E. coli*.

Table 14 Comparison between the two microorganisms used in this study: *E. coli* and *S. cerevisiae*

	Escherichia coli		*Saccharomyces cerevisiae*	
	Disadvantages	**Advantages**	**Disadvantages**	**Advantages**
Generalities		Well known host		Well known host
		Easy cultivation		Easy cultivation
		Fast growth	Slow growth	
		Easy manipulation		Easy manipulation
		Growth on varieties of medium		Growth on varieties of medium
	Small size of cells			Big size of cells
Expression techniques		Easy transformation		Easy transformation
		Used for production of many chemicals, pharmaceuticals, proteins, vitamins	Mainly used for production of ethanol and protein	
		No secretion	Secretion of protein	
Type	Prokaryote		Eukaryote	
Membrane Tolerance	Thin cell wall			Tough cell wall
	Low pH tolerance			High pH tolerance from 3 to 9
Protein production	Cannot make post-translational modification			Post-translational modification partially possible
Biomass	Thin cell membrane			Thicker cell membrane
	Bad sedimentation			Good sedimentation
	No separation at low speed centrifugation			Good separation at low speed centrifugation
Application of SME		Already proven to be successful	First trial	
		Fast bioconversion	Slow bioconversion	
		High expression level	Low expression level	
	Cell lysis at high temperature			Nearly no cell lysis at high temperature
	Enzyme leakage			Enzyme leakage partially reduced
Safety	Production of endotoxins			Considered as GRAS by the FDA

Characteristics of cells were also analyzed. Yeast cells are known to be bigger than bacterial cells. This is an advantage for a better separation of cells. It was proven that a better sedimentation is achieved with yeasts in this study. This means that a lower centrifugation speed is needed to separate yeast cells compared to *E. coli* cells. Moreover, their size being bigger, membranes or filter to remove cells could have larger pores. Despite such differences bring only a small advantage in academic research, sedimentation properties and size of cells are linked to cost of instruments and processes on industrial production.

S. cerevisiae and *E. coli* are both well-known organisms and have already used for industrial production of pharmaceuticals, chemicals and proteins. Yeast cells are eukaryotes and have a thicker cell membrane. They are capable of post-translational modification, and glycosilation. They are also considered as GRAS by the FDA, while *E. coli* cells produce endotoxins. The membrane composition of both microorganisms is also different.

In summary, the first trial of SME in *S. cerevisiae* was performed. Advantages and disadvantages of *S. cerevisiae* and *E. coli* are summarized in Table 14. It was shown that a good percentage of bioconversion could be achieved by yeast cells and enzyme stably retained in the cell, despite the fact that the enzyme expression level in yeast is lower. On the other hand, *E. coli* has advantage as the reaction rate is higher, while *E.coli* has the major drawback of enzyme leakage. Other features of both strains were also discussed in this thesis.

The choice of microorganisms used for SME application ultimately depends on target product. Fulfillment of host strain for SME would bring expanded use of SME. This is the first report of using yeast as a host strain for SME.

IX. References

VII.1 Articles

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X. Appendix

1. Fumarate hydratase from *T. thermophilus* HB8 Nucleotides Sequence

gtggaataccggattgagcgggacaccatgggcgaggtgcgggtgccggcggacaagtactggggcgcgcagac
ccagcgctccctggagaacttcaggatcgggaccgaccgcttccgcatgcccctggagatcatccgggcctacgg
gatgctgaagaaggcggccgcgagagccaacctggagctcggggagctccccgaggagatcgccaaggccatcat
ccaggcggccgaggaggtggtccaggggaagtgggacgaccacttccccctggtggtcttccagacgggcagcgg
cacccagaccaacatgaacgtcaacgaggtcatcgccaaccgggcctcggagatcctggggaagcctctggggag
caagtacgtccaccccaacgaccacgtgaaccgggggcagagctccaacgacaccttccccaccgccatgtacgt
ggccgtggccctggcgctccaccagaggctctatcccgcggtggaaggcctgatccggaccttcacggccaaggc
ccaggcctttgaccagatcgtcaaggtggggcggacccacctgatggacgccgtgcccatcaccctgggacagga
gatcggcagctgggccgcccagctcaagaccaccctcgccgccgtcaaggaaatggaaaagggcctctacaacct
cgccatcggcgggacggcggtgggcacgggcctcaacgcccacccccgctttggggagctcgtggccaagtacct
cgcggaggagacggggctccccttccgggtggcggagaaccgcttcgccgccctggccgcccacgacgagctggt
caacgtcatgggggccatccgcaccctggccggggccctgatgaagatcggcaacgacgtccgctggctcgcctc
cggcccctacgcgggcattggggagatcaccatccccgccaacgagcccgggtcctccatcatgccggggaaggt
caaccccacccaggtggaggccctcaccatggtggtggtccgggtctacggcaacgaccacaccgtggccttcgc
gggaagccaggggaacttccagctcaacgtctacaagccggtgatggcctacagcaccctggagagcatcaacct
cctcgcggacgccgtggcctcctttgacgcccatctggcccaggggattgagcccaacctggagcgcattgagga
gcacctgcagaagaaccccatgctggccaccgccctcaacaaggccatcggctacgacaaggcggcggagatcgt
taagaaggccctcaaggagaagaagaccctgaagcaggcggccctcgagctcggctacctcacggaggaggagtt
tgaccgcatcgtggtccccatgaggctcgccaagccccacgagggggcgtag

2. Fumarate hydratase from *T. thermophilus* HB8, amino acid sequence

MEYRIERDTMGEVRVPADKYWGAQTQRSLENFRIGTDRFRMPLEIIRAYGMLKKAAARANLE
LGELPEEIAKAIIQAAEEVVQGKWDDHFPLVVFQTGSGTQTNMNVNEVIANRASEILGKPLG
SKYVHPNDHVNRGQSSNDTFPTAMYVAVALALHQRLYPAVEGLIRTFTAKAQAFDQIVKVGR
THLMDAVPITLGQEIGSWAAQLKTTLAAVKEMEKGLYNLAIGGTAVGTGLNAHPRFGELVAK
YLAEETGLPFRVAENRFAALAAHDELVNVMGAIRTLAGALMKIGNDVRWLASGPYAGIGEIT
IPANEPGSSIMPGKVNPTQVEALTMVVVRVYGNDHTVAFAGSQGNFQLNVYKPVMAYSTLES
INLLADAVASFDAHLAQGIEPNLERIEEHLQKNPMLATALNKAIGYDKAAEIVKKALKEKKT
LKQAALELGYLTEEEFDRIVVPMRLAKPHEGA

3. Fumarate hydratase information from Harima Riken Institute

Thermus thermophilus HB8 TTHA0558
Fumarate hydratase class II

Category: Energy metabolism
Locus: 1:complement(519024…520424)
Synonym: TT0543
Stop codon: TAG
DNA Length: 466
Molecular weight: 50889.05
Absorption coefficient: 43680
Number of methionine 15
Expression Plasmid ID: PC010558-41
Vector: pET11a
PDB code: 1vdk

4. Enzymes that don't digest fumarase gene [modified from nebcutter]

Acc65I (G^GTAC_C)
AclI (AA^CG_TT)
AflII (C^TTAA_G)
AflIII (A^CRYG_T)
AgeI (A^CCGG_T)
AhdI (GACNN_N^NNGTC)
AlwNI (CAG_NNN^CTG)
ApaLI (G^TGCA_C)
ApoI (R^AATT_Y)
AscI (GG^CGCG_CC)
AseI (AT^TA_AT)
AsiSI (GCG_AT^CGC)
AvrII (C^CTAG_G)
BaeI (_NNNNN^NNNNNNNNNNAC NNNNGTAYCNNNNNNN_NNNNN^)
BamHI (G^GATC_C)
BbvCI (CC^TCA_GC)
BcgI (_NN^NNNNNNNNNNCGANNNNN NTGCNNNNNNNNNN_NN^)
BciVI (GTATCCNNNNN_N^)
BclI (T^GATC_A)
BfaI (C^TA_G)
BglII (A^GATC_T)
BlpI (GC^TNA_GC)
BmgBI (CAC|GTC)
BmtI (G_CTAG^C)
Bpu10I (CC^TNA_GC)
BsaI (GGTCTCN^NNNN_)
BsgI (GTGCAGNNNNNNNNNN NNNN_NN^)
BsiWI (C^GTAC_G)
BsmI (GAATG_CN^)
BspCNI (CTCAGNNNNNNN_NN^)
BspDI (AT^CG_AT)
BspHI (T^CATG_A)
BspQI (GCTCTTCN^NNN_)
BsrDI (GCAATG_NN^)
BsrGI (T^GTAC_A)
BstAPI (GCAN_NNN^NTGC)
BstBI (TT^CG_AA)
BstEII (G^GTNAC_C)
BstZ17I (GTA|TAC)
Bsu36I (CC^TNA_GG)
BtsI (GCAGTG_NN^)
ClaI (AT^CG_AT)
CspCI (_NN^NNNNNNNN NNNCAANNNNNGTGGNNNNNN NNNN_NN^)
DdeI (C^TNA_G)
DraI (TTT|AAA)
EcoRV (GAT|ATC)
FseI (GG_CCGG^CC)
FspI (TGC|GCA)
HindIII (A^AGCT_T)
HinfI (G^ANT_C)
HpaI (GTT|AAC)
EarI (CTCTTCN^NNN_)
EcoP15I (CAGCAGNNNNNNNNN NNNNNNNNNNNNNNNNN^NN_)
EcoRI (G^AATT_C)
KasI (G^GCGC_C)
KpnI (G_GTAC^C)
MfeI (C^AATT_G)
MluI (A^CGCG_T)
MlyI (GAGTCNNNNN|)
NarI (GG^CG_CC)
NdeI (CA^TA_TG)
NheI (G^CTAG_C)
NruI (TCG|CGA)
NsiI (A_TGCA^T)
PacI (TTA_AT^TAA)
PciI (A^CATG_T)
PflFI (GACN^N_NGTC)
PleI (GAGTCNNNN^N_)
PmeI (GTTT|AAAC)
PshAI (GACNN|NNGTC)
PsiI (TTA|TAA)
PvuI (CG_AT^CG)
RsrII (CG^GWC_CG)
SalI (G^TCGA_C)
SapI (GCTCTTCN^NNN_)
SbfI (CC_TGCA^GG)
SexAI (A^CCWGG_T)
SfiI (GGCCN_NNN^NGGCC)
SfoI (GGC|GCC)
SgrAI (CR^CCGG_YG)
SnaBI (TAC|GTA)
SpeI (A^CTAG_T)
SspI (AAT|ATT)
SwaI (ATTT|AAAT)
TfiI (G^AWT_C)
Tsp45I (^GTSAC_)
Tsp509I (^AATT_)
TspRI (_NNCASTGNN^)
Tth111I (GACN^N_NGTC)
XbaI (T^CTAG_A)

5. Plasmid map of pNV11 [from http://yeast.lab.nig.ac.jp/cgi-bin/nig/print_file.cgi?file=byp/BYP803.pdf]

Name: pNV11

Constructed by: Satoshi Nomoto (野本 理)

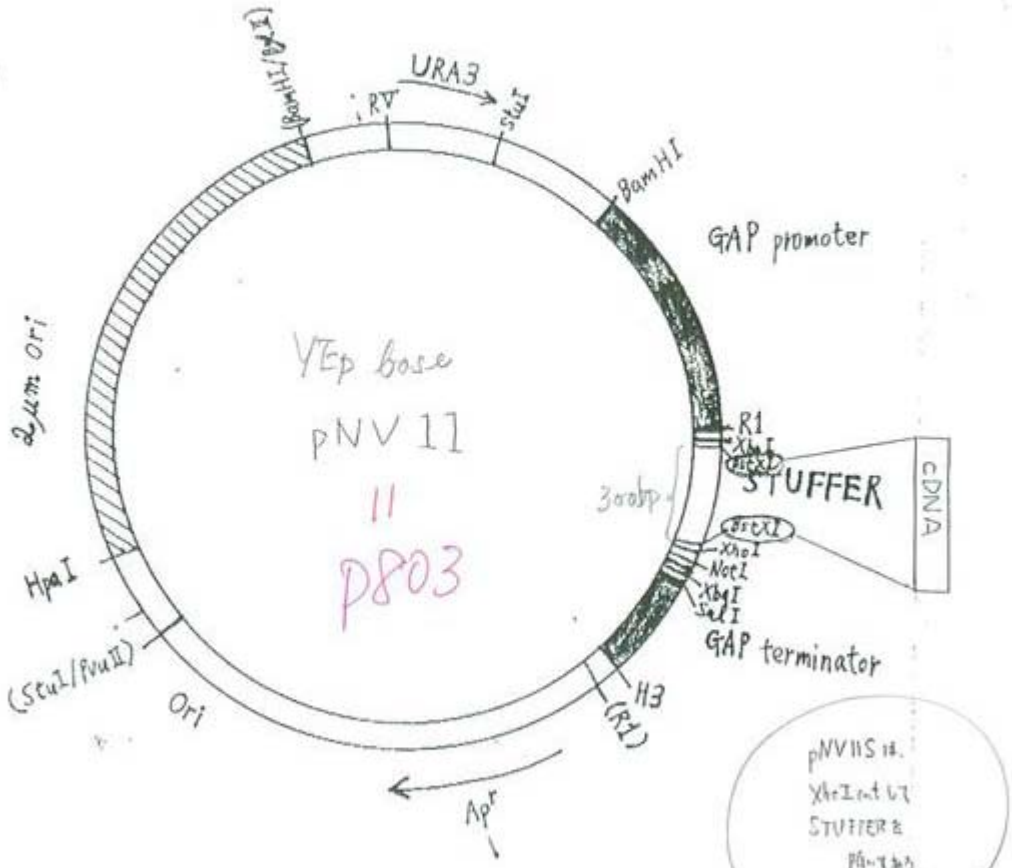
Date:

Size: ~6.8 kb

Vector: pKT10

Selection marker: Ap^R, URA3

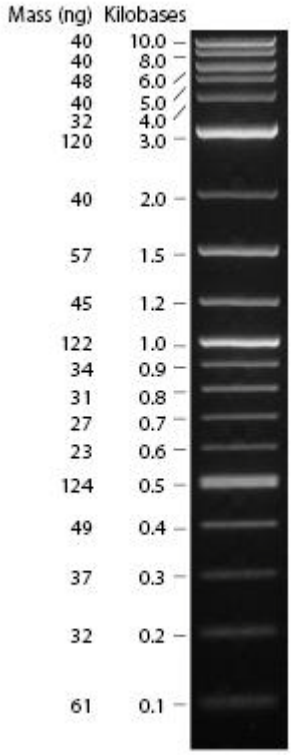
Unique restriction sites:



6. T-coffee alignment result (T_COFFEE, Version 8.93 (Thu Aug 5 2010))

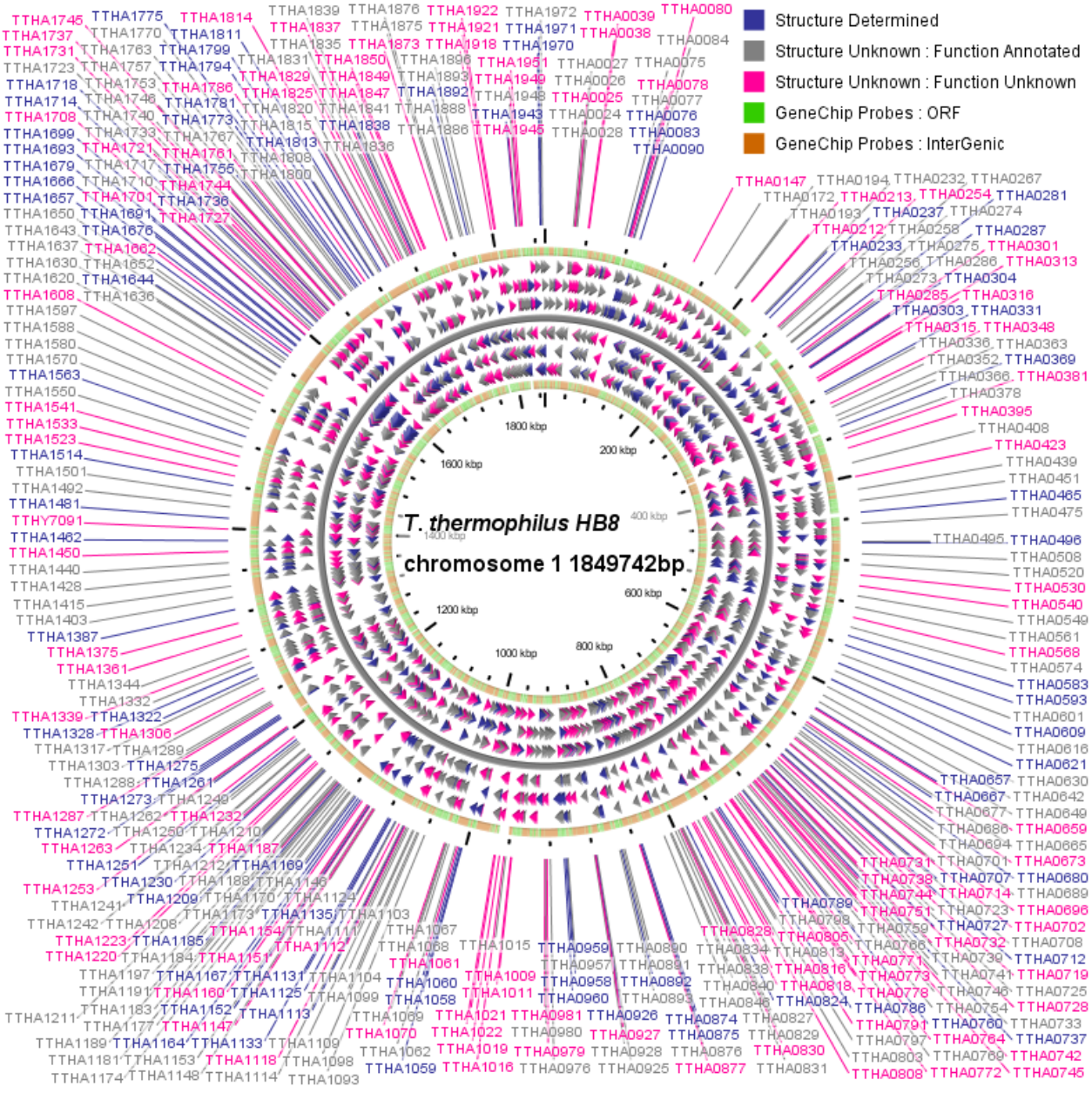
```
 SCORE=99                                                                BAD AVG GOOD
cerevisiae   :  99       thermus      :  99       cons         :  99

 S.cerevisiae   mlrftncscktfvkssyklnirrmnssfrtetdafgeih
T.thermophilus -------------------------meyrierdtmgevr
cons                                     .:* * *::**::

S.cerevisiae   vpadkywgaqtqrsfqnfkiggarermplplvhafgvlk
T.thermophilus vpadkywgaqtqrslenfrigtdrfrmpleiiraygmlk
cons           *************.:**:**   * ****  :::*:*:**

S.cerevisiae   ksaaivneslggldpkiskaiqqaadevasgklddhfpl
T.thermophilus kaaaranlelgelpeeiakaiiqaaeevvqgkwddhfpl
cons           *:**  .*  .** *   :*:*** ***:**..** ******

S.cerevisiae   vvfqtgsgtqsnmnanevisnraieilggkigskqvhpn
T.thermophilus vvfqtgsgtqtnmnvnevianraseilgkplgskyvhpn
cons           **********:***.****:*** ****   :*** ****

S.cerevisiae   nhcnqsqssndtfptvvhiaaslqiqnelipeltnlkna
T.thermophilus dhvnrgqssndtfptamyvavalalhqrlypaveglirt
cons           :* *:.*********.:::*.:* :::.* * : .* .:

S.cerevisiae   leakskefdhivkigrthlqdatpltlgqefsgyvq
T.thermophilus ftakaqafdqivkvgrthlmdavpitlgqeigswaa
cons           : **:::    ***:***** **.*:*****:..:.

S.cerevisiae   qvengiqrvahslktlsflaqggtavgtglntkpgfdvk
T.thermophilus qlkttlaavkemekglynlaiggtavgtglnahprfgel
cons           *::. :  *  .   * *  ** **********::* *.

S.cerevisiae   iaeqisketglkfqtapnrfealaahdaivecsgalntl
T.thermophilus vakylaeetglpfrvaenrfaalaahdelvnvmgairtl
cons           :*: :::**** *:.* *** ****** :*:   **:.**

S.cerevisiae   acslfkiaqdirylgsgprcgyhelmlpenepgssi
T.thermophilus agalmkigndvrwlasgpyagigeitipanepgssi
cons           * :*:**.:*:*:*.*    .*  *: :* *******

S.cerevisiae   mpgkvnptqnealtqvcvqvmgnnaaitfagsqgqfeln
T.thermophilus mpgkvnptqvealtmvvvrvygndhtvafagsqgnfqln
cons           ********* **** * *:* **: :::******:*:**

S.cerevisiae   vfkpvmivnllnsirlitdaaysfrvhcvegikane
T.thermophilus vykpvmaystlesinlladavasfdahlaqgiepnl
cons           *  ***   . *:**.*::**. ** .*  .:**:.*

S.cerevisiae   prihelltkslmlvtalnpkigydaaskvaknahkkgit
T.thermophilus erieehlqknpmlatalnkaigydkaaeivkkalkekkt
cons            **.* * *. **.****   **** *:::.*:* *:   *

S.cerevisiae   lkesalelgvltekefdewvvpehmlgp---k
T.thermophilus lkqaalelgylteeefdrivvpmrlakphega
cons           **::***** ***:***. *** ::   *
```

7. 2-log DNA ladder

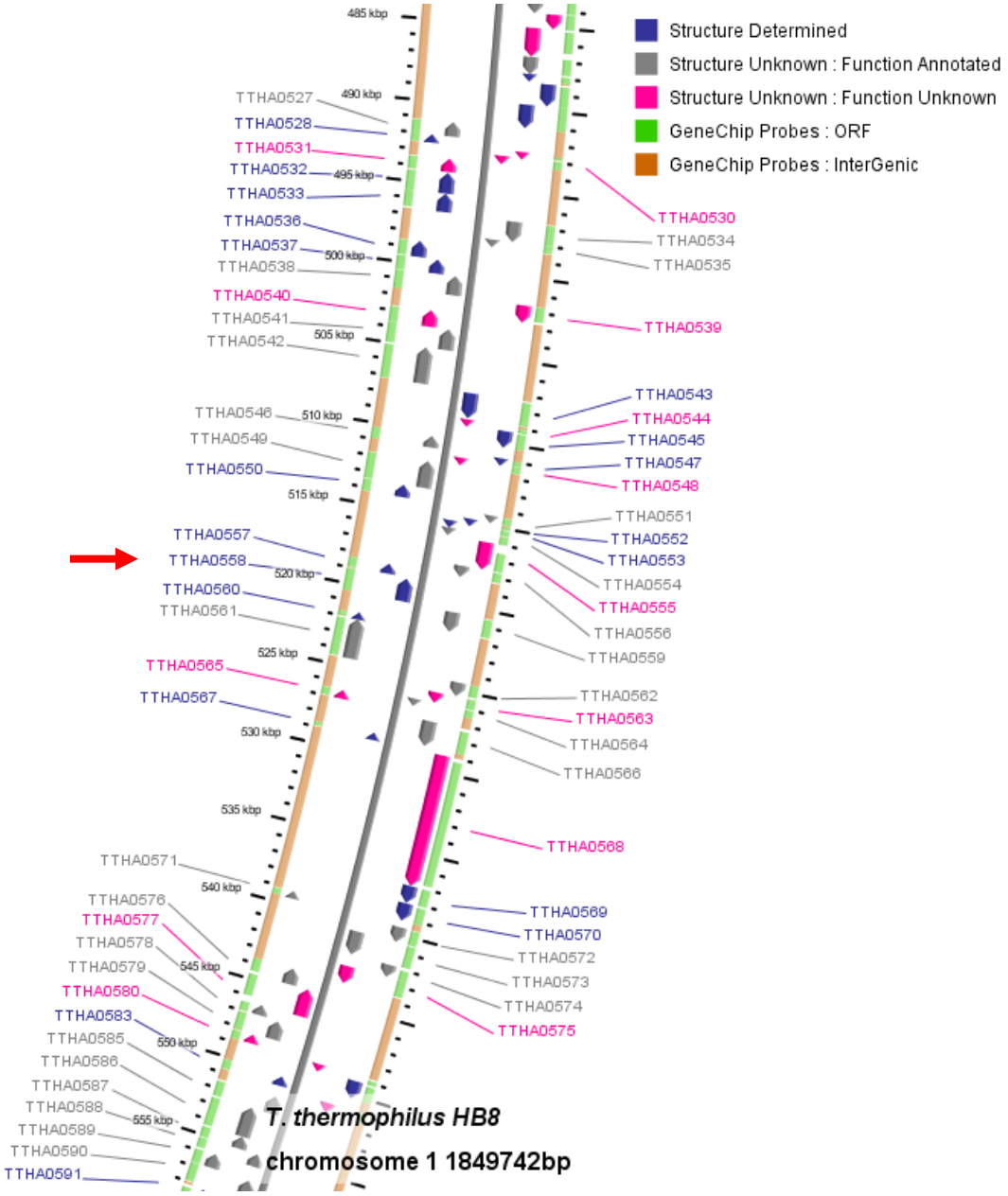


2-log DNA Ladder visualized by EtBr staining on a 1% TBE agarose gel. Mass values are for 1µg/lane. Modified from http://www.neb.com/nebecomm/products/productn3200.asp

8. *Thermus thermophilus* genome [from Harima Riken Institute]

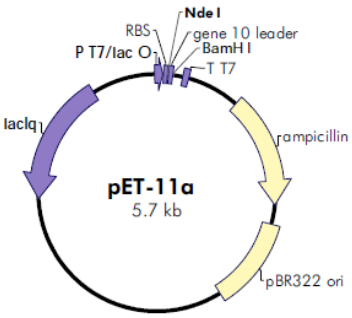


9. *Thermus thermophilus*, chromose 1, zoom on the fumarate hydratase gene

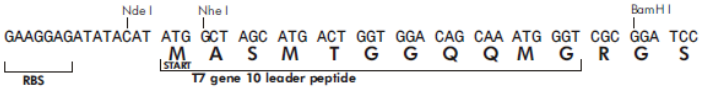


10. **pET-11a information, from http://www.synthesisgene.com/vector/pET-11a.pdf**

T7 promoter with *lac* operator 1–43
ribosome binding site 74–80
Nde I cloning site 86–91
T7 gene 10 leader 89–121
BamH I cloning site 125–130
T7 terminator 199–245
ampicillin resistance (*bla*) ORF 657–1514
pBR322 origin 1665–2332
***lacI*^{q} repressor ORF** 4212–5168



pET-11a Cloning Site Region
sequence shown (74–130)



11. Creation of S. cerevisiae RBS consensus sequence logo

Steps followed to create RBS sequence list

1. On UCSC table browser, Select clade, genome , assembly, group: "Genes and Gene Prediction Track"; track: "CCDC". Filter
2. Output format: "selected fields from primary and related tables", click on "get output"; Select the fields
3. Copy & paste the list of CDS position (without the header lines).
4. Reformat the table browser output into .bed
5. On the table browser main page, add custom track. Submit file.
6. Get output as sequence. Add 10 extra bases upstream and 10 extra downstream, download sequence.
7. Generate the sequence logo as follows: seqlogo -c -Y -f hs_cds_start.seq -F GIF -o hs_cds_start -k 1 M

Perl Script

```
#!/usr/bin/perl

while(<STDIN>) {

chomp; #reads one line after the other

my @col = split /\t/, $_; #creation of first array

my @exon = split /,/, $col[3]; #creation of second array, containing only the specific position

my $end = $#exon; #$end contains the number of exon in the second array

my $start = 1; # we don't want the first exon

for my $i ($start..$end) {

print "$col[1]\t$exon[$i]\t$exon[$i]\t$col[0]\t0\t$col[2]\n";

}

}
```