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RECOMBINANT PROTEIN PRODUCTION IN *Bacillus* SPECIES, ISOLATION AND METHIONINE PRODUCTION IN METHIONINE-PRODUCING MICROORGANISMS

RECOMBINANT PROTEIN PRODUCTION IN *Bacillus* SPECIES, ISOLATION AND METHIONINE PRODUCTION IN METHIONINE-PRODUCING MICROORGANISMS

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor in Philosophy in Poultry Science

By

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ABSTRACT

Linoleic acid isomerase (LAI) is the enzyme that converts linoleic acid to conjugated linoleic acids (CLAs) which appear to be capable of lowering cancer risk and enhancing immunity. There is an interest in developing commercial processes for the production of single isomers of CLA by biotransformation of LA using microbial cells and enzymes. However, the evaluation of these strains suggest that growth and LA isomerase production levels by these anaerobes are insufficient to support economic commercial production of single CLA isomers. A better alternative is to clone the isomerase gene and to generate new production strains using recombinant technology. *Bifidobacterium* LAI genes were cloned into pET24a(+) as an expression vector and transferred into *E. coli* BL21 (DE3) as the expression host while *Propionibacterium acnes* LAI were cloned into *Bacillus* species as the expression hosts. Unfortunately, the expressed *Bifodobacterium* LAI formed inclusion bodies and did not exhibit detectable enzymatic activities. Propeptide *Staphylococcus hyicus* lipase fused with *P. acnes* LAI expressed in *B. megaterium* YYBm1 was secreted into the surrounding medium. Based on MALDI-TOF MS results showed that this propeptide *S. hyicus* lipase still attached with the secreted LAI and might inhibit activity. However, other propeptides (*B. subtilis nprE, B. subtilis amyE*, *B. megaterium nprM*) did not protect LAI from proteolytic degradation.

Methionine is the first limiting amino acid in poultry feed and must be supplemented to the poultry feed mixture. Many studies attempted to isolate methionine producing microoragnisms from environments and genetically modified by using chemical mutagens. However, genetically-modified organisms are considered unacceptable for use in organic food production. Therefore, wild type strains with methionine-producing ability are necessary for the organic poultry industry. In this study, numerous bacteria were isolated from natural

environments that appeared to produce high methionine; however, all of them were identified as pathogens. In addition, bacteria excreted methionine into the surrounding medium and it was concluded that this could be impractical for large scale recovery of methionine. Conversely, isolated yeast strain K1 yielded high methionine content, compared to other yeasts. Based on large subunit rRNA sequences, these isolated strains were identified as *Pichia kudriavzevii/Issatchenkia orientalis*. *P. kudriavzevii/I. orentalis* is recognized as a ″generally recognized as safe″ (GRAS) organism. Yeast strain K1 may be suitable as a source of methionine for dietary supplements in organic poultry feed.

This dissertation is approved for recommendation
to the Graduate Council.

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CHAPTER ONE

INTRODUCTION

Gram-positive bacteria appear to be more promising hosts for large-scale production of heterologous proteins. *Bacillus* species are attractive hosts because they possess a naturally high secretion capacity, and they export proteins directly into the extracellular medium since the cytoplasm of a Gram-positive bacterium is surrounded by a single membrane system (Terpe, 2006). For heterologous secretion, proteins also require a targeting signal in the form of a signal peptide. The signal sequence is incorporated into the vector at the N-terminus of the target protein; however, only some heterologous proteins are successfully secreted. Currently, no universal signal peptide is suitable for production of all heterologous proteins (Brockmeier et al., 2006). Some instances showed that signal peptides alone were not sufficient to secrete proteins with complex structures. The propeptide of the *S. hyicus* lipase appears to prevent the degradation of secreted heterologous proteins unrelated to lipase (Meens et al., 1993; 1997; Puohiniemi et al., 1992).

Conjugated linoleic acid (CLA) production is of interest by many researchers since these compounds have various biological benefits such as inhibiting the initiation of carcinogenesis and tumorigenesis (Pariza et al., 2001; Pariza, 2004). Linoleic acid isomerases (LAI) found in microorganisms catalyze the conversion of linoleic acid to CLA. There is an interest in developing commercial processes for the large-scale production of single isomers of CLA by biotransformation of LA using microbial cells and enzymes (Kim et al., 2000; Rainio et al., 2001; Ando et al., 2004). However, the evaluation of these strains suggested that growth and LA isomerase production levels by these anaerobes are insufficient to support economic commercial

production of single CLA isomers (Peng et al., 2007). A better alternative is to clone the isomerase gene and to generate new production strains using recombinant technology.

Methionine is a nutritionally essential amino acid required in the diet of humans and livestock, including poultry. Chickens are unable to produce methionine and therefore must obtain it through their diets. Generally, methionine is one of the first limiting amino acids in poultry nutrition and typically in most diets this amino acid has to be added to the poultry feed (Gomes and Kumar, 2005). Currently, methionine is produced by chemical processes or hydrolyzing proteins (Kumar and Gomes, 2005). However, chemical synthesis is expensive and produces a mixture of D- and L-methionine. In addition, these processes require hazardous chemicals such as acrolein, methyl mercaptan, ammonia, and cyanide (Fong et al., 1981). However, methionine from protein hydrolysis must be separated from the complex mixture (Kumar and Gomes, 2005). Furthermore, synthetic methionine is currently allowed as an additive to organic poultry feed by the U.S. Department of Agriculture's National Organic Program (NOP) however NOP is only extending its use until October 1st 2012 (USDA, Agricultural Marketing Service, http://www.paorganic.org/wp-content/uploads/2011/10/USDA-Methionine.pdf).

Many studies have attempted to isolate methionine producing microoragnisms from environments and genetically modified by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Several studies have reported that methionine-analog resistance in bacterial strains correlated to higher methionine production due to an alteration in the regulation of L-methionine biosynthesis (Adelberg, 1958; Chattopadhyay et al., 1991; Musilkova and Fencl, 1964;Yamada et al., 1982 Tani et al., 1988). Although the mutation was successful in generating methionine overproducing microorganisms, any genetically-modified organisms are considered unacceptable for use in

organic food production. Therefore, wild type strains with methionine-producing ability are necessary for the organic poultry industry.

The objectives of this research were cloning *Bifobacterium* LAI into *E. coli* BL21 (DE3) and *Propionibacterium acnes* LAI in *Bacillus* species. For methionine production in microorganisms, bacteria and yeasts were isolated from environmental samples and methionine contents in these strains were quantified.

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CHAPTER TWO

LITERATURE REVIEW

BACTERIAL EXPRESSION SYSTEMS FOR HETEROLOGOUS PROTEIN PRODUCTION

Introduction

Microbial products continue to be major sources of dietary and health products in the daily lives of humans and include products such as vitamin B_{12}, glucose blood test, antibiotics, and antivirus medicines. These products include alcohols from anaerobic metabolism, acids from incomplete oxidation, primary and secondary metabolites, enzymes, biocatalysts, and more recently, heterologous proteins. Enzymes produced by microorganisms have been used in food, fermentation, textile, paper industries. For example, α-, β-amylases for starch modification have been utilized by the baking industry (Eppinger et al., 2011; Vary, 1994). Schallmey et al. (2004) reported that the world market for industrial enzymes was approximately 1.6 billion $ US. For the economic production of heterologous proteins, bacterial hosts are usually the systems of choice.

Escherichia coli as host

Production of specific proteins from original organisms, such as human hormone proteins, is impractical at the industrial level and expression hosts serve as possible solutions for these problems. Compared to other expression systems, bacteria, particularly *Escherichia coli*, are more favorable because they offer a proven economical route for protein production due to their ability to grow rapidly and yield a high density of cells on inexpensive substrates (Terpe, 2006). In addition, these hosts possess well-characterized genetics that require minimal technical

expertise to establish at a laboratory level and eventually on an industrial scale (Zerbs et al., 2009).

Among expression systems, *E. coli* is considered to be the first choice since numerous vectors, readily available engineered strains, and minimal technical requirements are already in place. In addition, production of large numbers of cells is rapid due to short doubling times (Brondyk, 2009). Although the *E. coli* system is a well established method for expressing bioactive proteins, there are a number of limitations. Posttranslation is essential in some proteins for bioactivity; however, many bacterial systems are not able to modify protein posttranslationally. In these cases, bacterial systems should not be used for heterologous production (Terpe, 2006). Other disadvantages of protein expression in the cytoplasm of *E. coli* are that these proteins form inclusion bodies and accumulate lipopolysaccharides (LPSs). These LPSs are well-known as endotoxins, which are pyrogenic in humans or other mammals (Terpe, 2006). *E. coli* also has problems in protein folding and forming disulfide bonds which is insufficient for many recombinant proteins (Brondyk, 2009).

The primary method to minimize inclusion body formation and maximize the formation of soluble, properly folded proteins in the cytoplasm is lowering the incubation temperature to 15 to 30 ^{o}C during the expression period (Brondyk, 2009). The reduced temperature reduces the rate of transcription, translation, refolding and thus increases proper folding (Brondyk, 2009). Adding a soluble fusion tag at either the N-terminus or C-terminus can promote solubility of some recombinant proteins (Brondyk, 2009).

T7 RNA polymerase system

One of the most widely used expression systems using *E. coli* as the host is the T7 RNA polymerase system (Table 2.1 and Fig. 2.1) (Terpe, 2006). For protein production, a recombinant

plasmid is transferred to an *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase. These *E. coli* hosts are lysogens of bacteriophages DE3, a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase. This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter. By a two- point mutation at the -10 region, *lacUV5* promoter has more promoter strength and less dependence on cyclic AMP and receptor protein CAP. In addition, the third point mutation causes this promoter to be less sensitive to glucose. Even in the presence of glucose, the expression of this system is induced by addition of lactose analog isopropyl-β-D-1-thiolgalactopyranoside (IPTG) to the culture medium or by using an autoinduction medium (Terpe, 2006).

Bacilli as host

An ideal host should have the ability to secrete large amounts of recombinant proteins with only low quantitatives of host proteins and not generate detectable proteolytic activities in the supernatant. Because of the endotoxin production and inclusion body formation problems associated with Gram-negative bacterium *E. coli*, Gram-positive bacteria appear to be more promising hosts for large-scale production of recombinant proteins. Gram-positive bacteria *Bacillus subtilis* and some of its close relatives are widely used for the industrial production of enzymes for the detergents, food, and beverage industries (Eppinger et al., 2011). *Bacillus* species are the dominant bacterial hosts for industrial enzyme producers because *B. subtilis* and *B. licheniformis* are on the Food and Drug Administration's GRAS (generally regarded as safe)

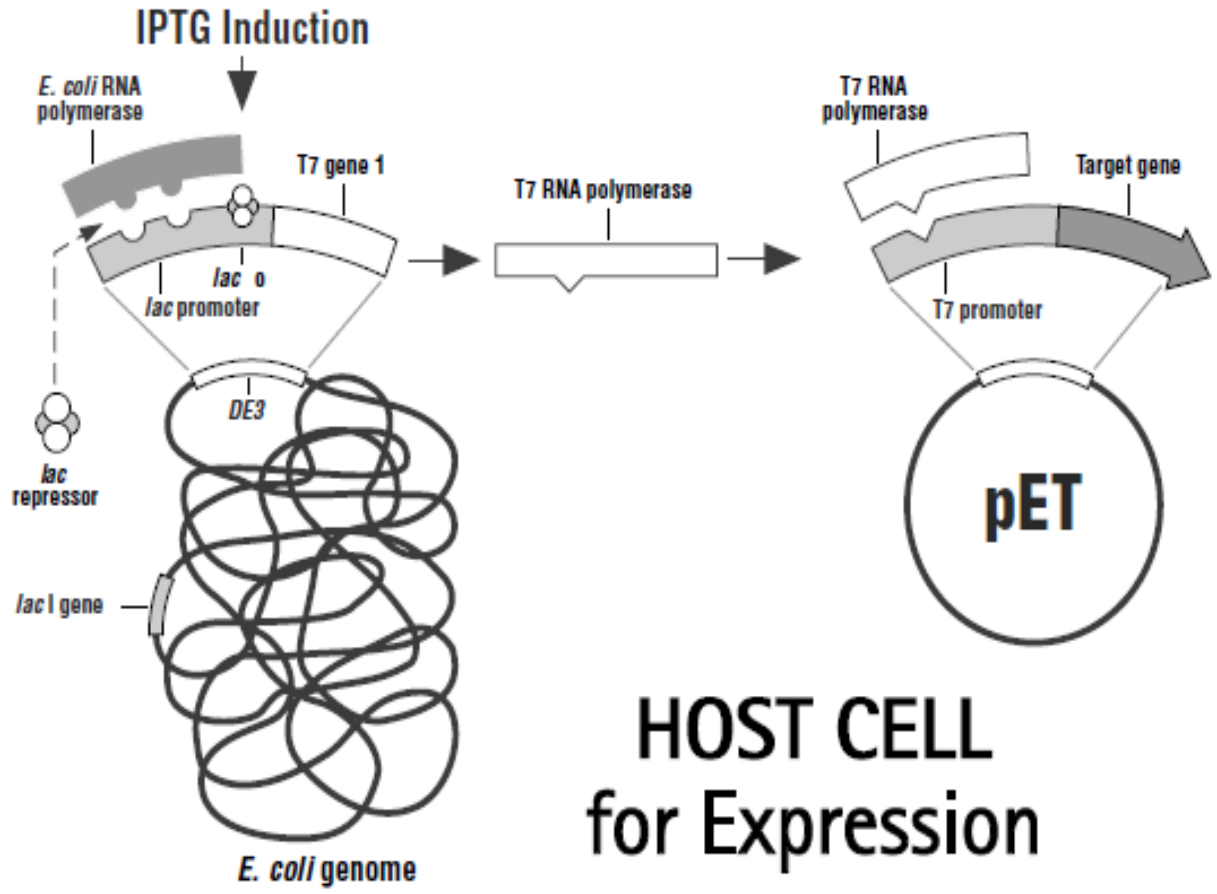


Fig. 2.1. In this system, plasmids contain a *lac* operator sequence just downstream of the T7 promoter. They also carry the natural promoter and coding sequence for the *lac* repressor (*lacI*). The T7*lac* and *lacI* promoters are divergent. The *lac* repressor acts both at the lacUV5 promoter in the *E. coli* BL21 (DE3) chromosome to repress transcription of the T7 RNA polymerase gene by the host polymerase and at the T7*lac* promoter in the vector to block transcription of the target gene by any T7 RNA polymerase that is made (Novagen, pET system manual, 11th Edition, 2006).

list and some selected strains can secrete large quantities (20 to 25 g/L) of extracellular enzymes (Schallmey et al., 2004).

Intracellular accumulations can lead to problems related to the formation of insoluble protein inclusion bodies, incorrect protein folding, and inefficient disulfide bond formation (Schallmey et al., 2004). From a commercial point of view, the purification of proteins from the culture medium rather than from the cytoplasm is considerably more cost-effective, often leads to improved structural authenticity, and reduces the likelihood of the co-purification of endotoxins and other potential contaminants (Pohl and Harwood, 2010). For protein secretion, most researchers employ *Bacillus* and related bacteria. In contrast to Gram negative bacterial hosts such as *E. coli*, *Bacillus* strains have the general advantage that the outer membrane does not possess LPSs. Furthermore, *Bacillus* spp. are attractive hosts because they possess a naturally high secretion capacity, and they export proteins directly into the extracellular medium since the cytoplasm of a Gram-positive bacterium is surrounded by a single membrane system, in contrast to the double membrane found in Gram-negative bacteria. Gram-positive bacteria have the ability to secrete proteins directly into the culture medium at high concentrations.

Although *Bacillus* species, compared to *E. coli*, have many advantages, *Bacillus* species, particularly *B. subtilis*, secrete proteases which rapidly degrade heterologous proteins (Nijland and Kuipers, 2008). By deleting neutral (*nprE*) and alkaline protease (*aprE*) genes, *B. subtilis* mutant still retained extracellular protease activity at 4% of the total activity of wild type and caused some foreign protein degradation (Wang et al., 1989). To avoid this problem, Wong and his colleague developed the extracellular protease negative *B. subtilis* WB800 strain but unfortunately this strain is not commercially available (Table 2.1) (Braaz et al., 2002). In the

case of *B. megaterium*, Wittchen and Meinhardt (1995) deleted the neutral protease *nprM* gene for suitable heterologous expression host.

Brevibacillus choshinensis

In contrast to *B. subtilis*, wild-type *Brevibacillus* (*Bre.*) *choshinensis* (formerly *Bacillus brevis*) produces very low extracellular protease and exhibits high productivity of heterologous proteins (Udaka and Yamagata 1993). *Bre. choshinensis* SP3 has been utilized as a host for heterologous expression since this strain has the ability to secrete large amounts of recombinant proteins. Currently, *Bre. choshinensis* SP3 is supplied by Takara, Tokyo, Japan and it has been used successfully to produce heterologous proteins that originated from prokaryotic and eukaryotic organisms (Table 2.1). By using this strain, Yamagata et al.(1989) produced 0.15 g/L of human epidermal growth factor.

Bacillus megaterium

B. megaterium is a Gram-positive and non-pathogenic bacterium. Compared to *B. subtilis*, this organism has a number of favorable features as an expression host, including low extracellular protease activity, structural and segregational stability of plasmids, and the ability to grow on more than 62 carbon sources including waste products from the meat industry and corn syrups (Vary, 1994; Vary et al., 2007). *B. megaterium*, in contrast to *B. subtilis*, does not possess any detectable alkaline proteases that can degrade heterologous products (Bunk et al., 2010). In 2011 the two genomes of the biotechnologically important *B. megaterium* QM B1551 and DSM319 were completely sequenced (Eppinger et al., 2011). While *B. megaterium* QM B1551 carries 7 plasmids, DSM319 is a plasmid-less strain. The chromosome of DSM319 is a circular molecule of 5,097,447 bp with an average G+C content of 38.2% and encodes 5272 genes (Eppinger et al., 2011).

Table 2.1. Selected bacterial expression hosts.

Strain	Feature	References or Suppliers
E. coli BL21 (DE3)	Widely used expression strain which lacks the *lon* and *opmT* proteases and contains a copy of RNA the T7 RNA polymerase gene under the control of the *lacUV5* promoter. These modifications enable stable expression of proteins using T7 promoter driven constructs	EMD Millipore Corporation, Billerica, MA, USA, Novagen, Darmstadt, Germany
E. coli Rosetta-gami	K-12 derivatives with mutations in the *trxB* and *gor* genes which greatly facilitate cytoplasmic disulfide bond formation in the cytoplasm. This strain enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AUA, AGG, AGA, CGG, CUA, CCC, and GGA	EMD Millipore Corporation, Billerica, MA, USA, Novagen, Darmstadt, Germany
Bacillus subtilis WB800	Extracellular protease-negative strain	Braaz et al.(2002)
Bacillus megaterium MS941	DSM319 derivative with inactivation of the major extracellular protease *nprM*. This strain showed only 1.4% of the wild-type total extracellular protease activities.	Wittchen and Meinhardt (1995), Molecular Biotechnology, Goettingen, Germany
Brevibacillus choshinensis SP3	Wild-type strain which produces negligible amount of extracellular protease.	Takara, Tokyo, Japan
Lactococcus lactis NZ9000 *clpP-htrA*	Strain deficient in both its major proteases, intracellular (ClpP) and extracellular (HtrA)	Cortes-Perez et al., 2006
Staphylococcus carnosus	Wild type strain with complete absence of proteolytic activity	Terpe (2006)

Table 2.2. Selected expression vectors.

Vector	Feature	References or Suppliers
pET series	Widely used expression systems for inducible expression of protein using a T7 promoter construct	EMD Millipore Corporation, Billerica, MA, USA., Novagen, Darmstadt, Gernany
pBAD series	Tightly regulated expression systems with expression controlled by the presence of arabinose. These vectors are wildly used in *E. coli*, *Salmonella enterica* and related bacteria.	Guzman et al.(1995), Invitrogen, Carlsbad, CA, USA
Gateway vectors	A series of vectors that use a recombinantional strategy to enable transfer of DNA fragments between different cloning vectors	Invitrogen, Carlsbad, CA, USA
Ligation independent cloning vectors	A series of vectors that use an anneal strategy to enable parallel cloning of coding region fragments	Aslanidis and de Jong (1990)
pNC series	The shuttle vectors between *E. coli* and *B. choshinensis* containing promoters working in *Brevibacillus* and signal peptides for protein secretion	Takara, Tokyo, Japan
pMM1520 series	The shuttle vector between *E. coli* and *Bacillus* species carrying xylose-inducible promoter.	Molecular Biotechnology, Goettingen, Germany

B. megaterium DSM319 has been shown to be an excellent host for heterologous production due to the possession of only one major protease, stability of inserted plasmids, and no wild-type plasmids (Vary et al., 2007). To generate DSM319 for heterologous protein production, Wittchen and Meinhardt (1995) achieved knockout of the major extracellular protease *npr M* (neutral protease) gene and recovered 1.4% of the extracellular proteolytic activity of the wild type and strain MS941 is well suited for extracellular heterologous protein production. Unlike *B. megaterium*, the mutant strain of *B. subtilis* required deletion of three genes (*nprE*, *aprE*, and *epr*) in order to produce approximately 1% of the extracellular proteolytic activity of the wild type (Wang et al., 1989). *B. megaterium* strain MS941 is a commercial strain supplied by Molecular Biotechnology (Table 2.1). Strain MS 941 allowed up to 1.2 g/L functional proteins to be recovered from the medium (Stammen et al., 2010; Eppinger et al., 2011). Inactivation of the *xylA* gene for xylose metabolism in *B. megaterium* MS941 led to development of the strain YYBm1, which does not require metabolism of the inducer for gene activation (Yang et al., 2006).

The *spac* promoter

A widely used system in *Bacillus* species is the *spac* promoter (Terpe, 2006). This promoter has been constructed by fusing the 5'-sequence of a promoter from the *B. subtilis* phage SPO1 and the 3'-sequence of the *E. coli lac* promoter including the operator (Pohl and Harwood, 2010). The expression of *spac* promoter is dependent on the inactivation of a constitutively expressed lactose repressor LacI by IPTG addition. IPTG is a lactose analog that can bind to LacI repressor and then changes LacI conformation to prevent *lac* promoter binding. Finally, RNA polymerase can initiate transcription from phage SPO1 promoter. The spac promoter can function in plasmid and chromosomal locations. This system can direct the

synthesis of a protein to a significant proportion of total cellular protein. However, this promoter is not sufficiently strong and IPTG is too expensive for large-scale fermentation. Based on this promoter located in plasmid, Chen et al. (2009) was able to achieve production of 30.6 mg/L of cecropin A, a chimeric antimicrobial peptide.

The xylose-inducible promoter

Another widely used system in *Bacillus* species is the xylose-inducible promoter based on the XylR repressor (Table 2.1 and 2.2). This promoter originates from *B. subtilis* and related organisms. The *xylR*-controlled promoter exhibits high levels of expression and has the advantage that the inducer xylose is relatively inexpensive (Bunk et al., 2010). The *xylA* promoter is located upstream on the operon coding for *xylABT* genes (Biedendieck et al., 2010). The gene encoding the repressor protein XylR is divergently oriented upstream of the operon with the promoter regions of *xylR* and of the *xyl ABT*-operon overlapping. In the absence of xylose, XylR binds to the two tandem overlapping operator sequences located in *xylA* promoter and prevents transcription of the *xylABT*-operon. In the presence of xylose, the sugar binds to the repressor XylR. This results in a conformational change of XylR which prevents promoter binding. By using this system, the RNA polymerase efficiently recognizes the promoter, and gene expression is induced approximately 350-fold. (Vary et al., 2007).

In 1991, based on a xylose-inducible promoter, Rygus and Hillen (1991) developed plasmid pWH1520, which could serve as a shuttle vector between *E. coli* and *B. megaterium*. Currently, Molecular Biotechnology, Goettingen, Germany, provides the plasmid pMM1520 series (Table 2.1 and Fig. 2.3) developed from plasmid pWH1520 which allows for the simple cloning of target genes by the use of 15 different DNA restriction enzyme cleavage sites (Malten

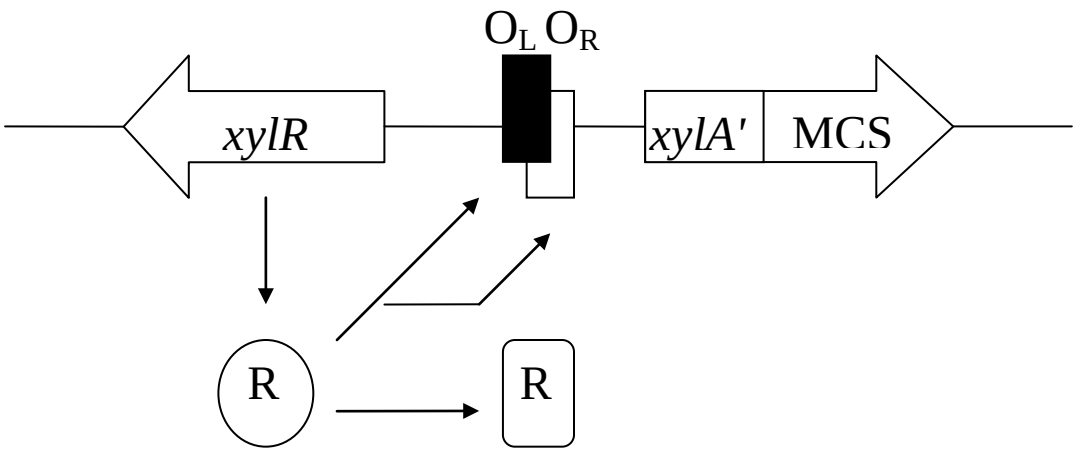


Fig. 2.2. The control elements of the *B. megaterim* expression vector pMM1520 (modified from Biedendieck et al., 2010). The *xylR* gene encodes the xylose repressor protein. The *xyl* operator region is denoted by O_LO_R indicating the two overlapping binding sites for *xylR*. The first 15 bp of *xylA*, denoted as *xylA'*, are fused to a short DNA sequence multiple cloning sites (MCS).

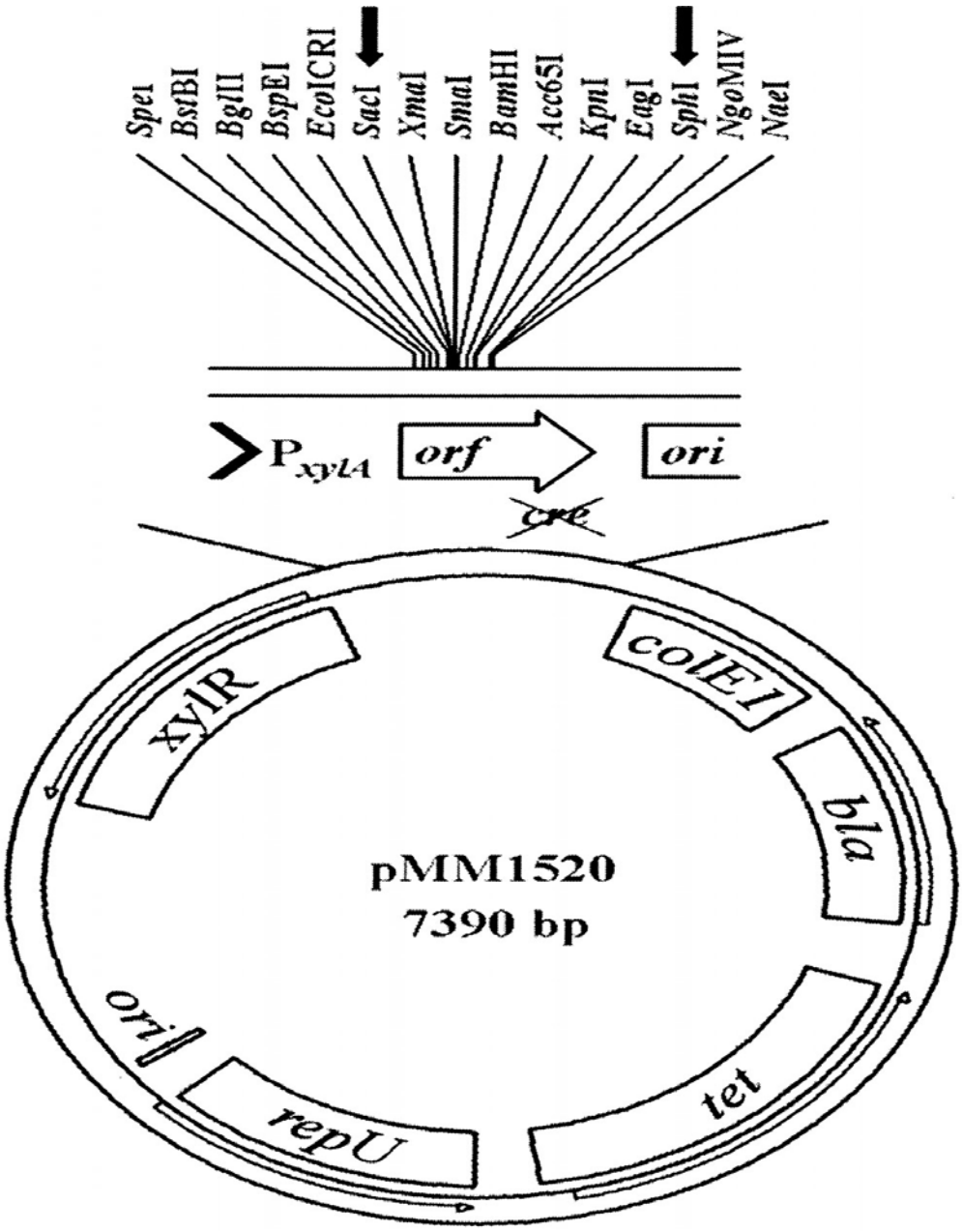


Fig. 2.3. Structure of the *B. megaterium* expression vector pMM1520 (Malten et al., 2005). Elements for xylose-inducible recombinant gene expression in *B. megaterium* are P_{xylA} and the gene encoding the xylose repressor (*xylR*). Elements for plasmid replication in *Bacillus* are the origin of replication *oriU* from *B. cereus*, *repU* from *B. cereus*, the tetracycline resistance gene (*tet*) from *B. cereus*. Elements for plasmid replication in *E. coli* are the origin of replication *colE1* from *E. coli*, the β-lactamase gene (*bla*) for ampicillin resistance from *E. coli*.

et al., 2005). It contains the origin of replication *colE1* and β-*lactamase* gene for replication and selection in *E. coli* (Biedendieck et al., 2010). In addition, it carries the origin of replication *oriU*, the *repU*, and a tetracycline resistance gene for replication and selection in *B. megaterium*. The expression for secreted heterologous production of these plasmids is based on the xylose-inducible promoter (Bunk et al., 2010). Based on this xylose-inducible protein production system, novel expression vectors for intra- and extracellular protein production have been developed by placing a His6- or Strep II-tag at N or C-terminus and signal peptides (Biedendieck et al., 2007).

Using *B. megaterium* MS941 with pMM1520 derivative plasmids has proven to be useful for efficient secretion of heterologous proteins such as a dextransucrase (DsrS) from *Leuconostoc mesenteroides*, a *Lactobacillus reuteri* levansucrase (Lev), the *B. megaterium* penicillin G amidase (PGA), and a *T. fusca* hydrolase (Biedendieck et al., 2007; Malten et al., 2005, 2006; Yang et al., 2006, 2007). By optimizing codon usage, the promoter region, and the ribosome-binding site, Stammen et al. (2010) achieved production of secreted *T. fusca* hydrolase at 7.7 mg/L which is sufficient for commercial purposes.

Prepeptide or signal peptide

In general, *Bacillus* species secrete numerous extracellular homologous proteins with their own signal peptides into their surrounding growth medium. Homologous proteins are natural proteins from bacterial production. For example, alkaline protease production from *B. clausii* was successful for supplementing into detergents. Conversely, heterologous proteins are foreign products from gene transferring from one organism into the other organisms as the expression hosts. For heterologous secretion, proteins also require a targeting signal in the form of a signal peptide. The signal sequence is incorporated into the vector at the N-terminus of the

target protein; however, only some heterologous proteins have been successfully secreted. Currently, no universal signal peptide is suitable for production of all heterologous proteins (Brockmeier et al., 2006).

Secretion systems of *Bacillus*

In *Bacillus* species, there are two pathways to secrete proteins across the cytoplasmic membrane: Sec-dependent (Sec) and twin-arginine translocation (TAT) (Pohl and Harwood, 2010). Most studies concentrate on Sec pathway since this pathway is the main route for protein secretion. Consequently the current review focuses only on the Sec pathway. The signal peptide is the transient extension to the amino terminus of the protein and is removed by enzymes signal peptidases. The signal peptides in Sec pathway are usually between 20 to 30 amino acids in length, possess a positive charged amino terminal (N) region, which is followed by a hydrophobic (H) central region and a short cleavage (C) region containing the target site for signal peptidase (Fig. 2.4). After secretion, the signal peptide is removed by Type I signal peptidases. The signal peptides have similar consensus cleavage sequences (AXA). The signal peptide is removed during the later stages of the process (Harwood and Cranenburgh, 2008). Substrate proteins are cleaved by two classes of signal peptidases. Type I signal peptidases cleave the most abundant class of secretory substrates.

In *E. coli*, the signal recognition particle (SRP) pathway chaperoning or targeting system is complemented by the SecB and SecA chaperones that interact with a subset of SRP-independent secretory proteins. Unlike *E. coli*, *Bacillus* species lack a homolog of the secretion-specific chaperone SecB, and consequently the SPR pathway is the only recognized intracellular pathway for presecretory protein recognition, chaperoning, and targeting. The SPR is an RNA-protein complex that interacts with hydrophobic regions of signal peptides of nascent proteins,

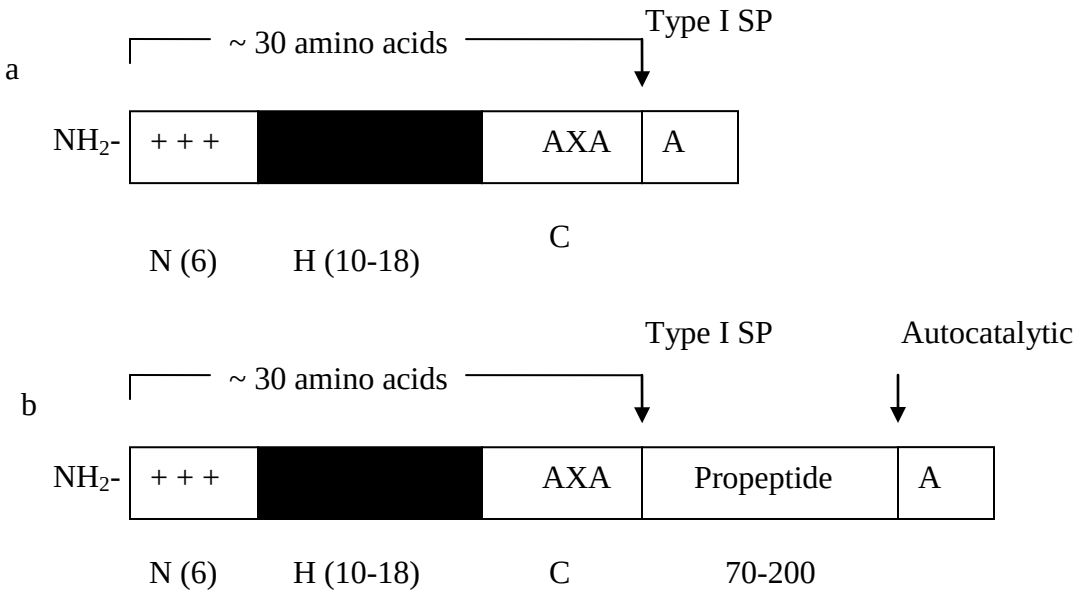


Fig. 2.4. The basic design of *Bacillus* signal peptides and propeptides. (a). Sec-dependent signal peptide cleaved by a Type I signal peptidase (SP); (b) The signal peptide and propeptide (prepropeptide) at the N-terminal end of a secretory protein requiring the propeptide for folding on the *trans* side of the cytoplasmic membrane. The signal peptide is removed by a Type I enzyme and propeptide either autocatalytically or by a coexisting protease (Harwood and Cranenburgh, 2008).

and delivers them to a membrane-bound docking protein (FtsY). Finally the protein complex passes the target protein to the secretory translocase (Harwood and Cranenburgh, 2007).

In *Bacillus* species, the SRP consists of an RNA molecule that provides the backbone for the attachment of two proteins: Ffh and HBsu (Fig. 2.5). The Sec-dependent translocases of *B. subtilis* consists of SecY, SecE, SecG protein to form the core of a heterotrimeric integral membrane pore that interacts with SecA, the motor component that drives translocation (Fig.2.6). The other functions of SecA work as a chaperone and lead secretory substrates for their site of synthesis in the cytoplasm to the Sec translocase. SecDF complex with YrbF is important for SecA cycling. Proteins emerge from the Sec-dependent translocase on the *trans* side of the cytoplasmic membrane in a relatively unfolded state. To this end, the secreted proteins are rapidly folded, facilitated by a combination of intrinsic and extrinsic factors, such as chaperones and folding factors.

The signal peptides are highly variable, rapid evolving structures (Heijne, 1990). Numerous attempts have been made to maximize the secretion of heterologous proteins by identifying optimal signal peptides. However, for a specific signal peptide for one target protein, it is often found not to be optimal for another. The successful secreted proteins depend on characteristics of both the signal peptide and the mature protein; however, this mechanism is not fully elucidated (Brockmeier et al., 2006; Nijland and Kuipers, 2008). Therefore, the prediction of optimal signal peptides for heterologous protein secretion is difficult and cannot be based solely on the signal pepide itself (Nijland and Kuipers, 2008).

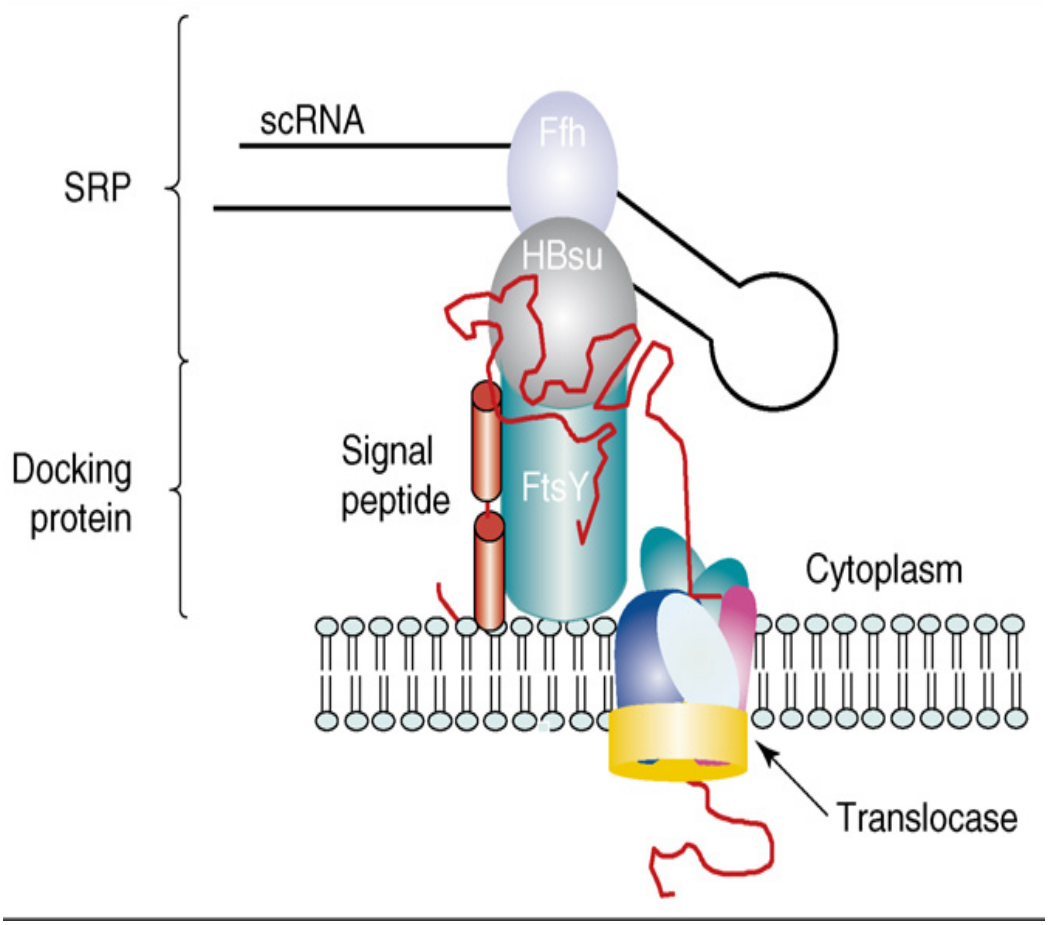


Fig. 2.5. The structure of the signal-recognition particle of *B. subtilis* (Harwood and Cranenburgh, 2008). Details of the interactions among the components of the SRP, the docking protein (FtsY) and the translocase. The SRP is an RNA molecule (small cytoplasmic or scRNA) attached with Ffh and HBsu proteins.

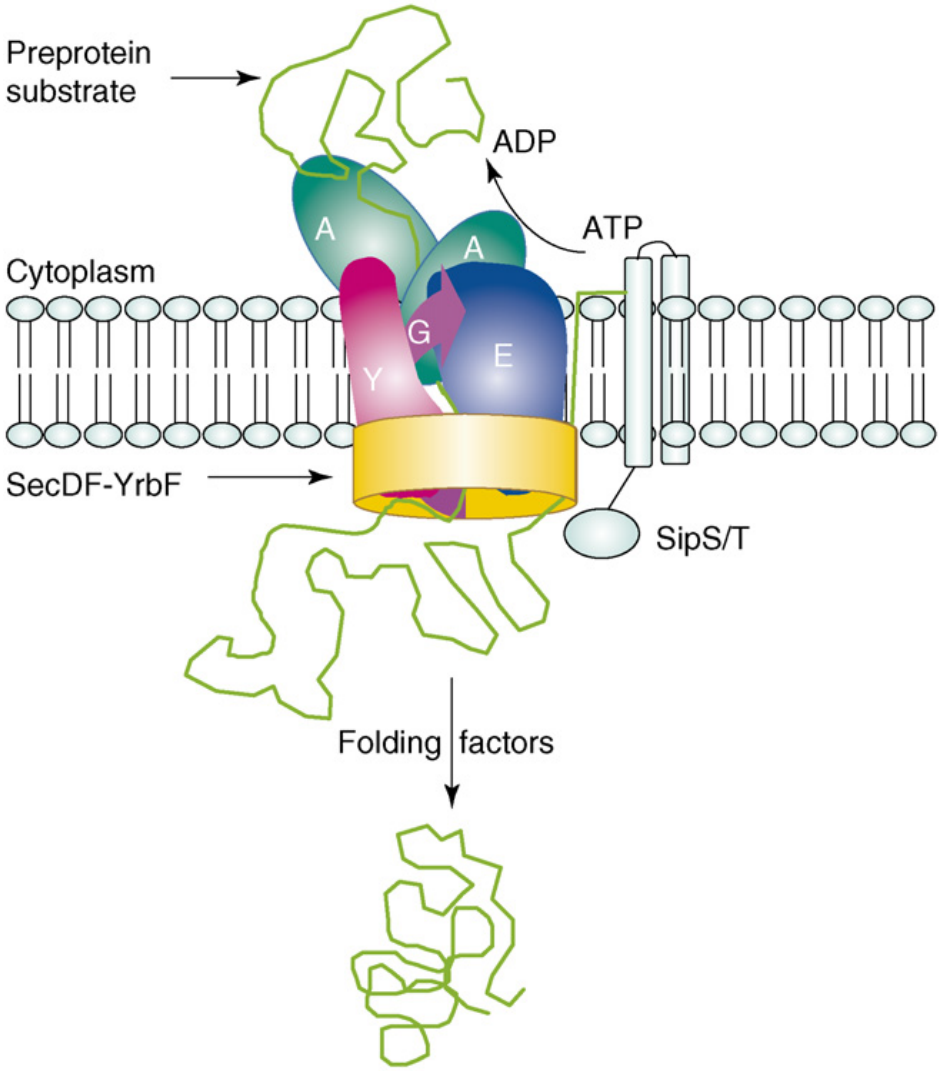


Fig. 2.6. The structure of the *B. subtilis* Sec-dependent secretory protein translocase. The preprotein substrate is transported through the translocase consisting of the SecA dimmer, heterotrimeric pore (SecYEG), the heterotrimeric SecDF-YrbF complex and the main type I signal peptidases (SipS and SipT). After translocation, the preprotein is folded into its correct formation by propeptides, isomerase and divalent cations as folding factors (Harwood and Cranenburgh, 2008).

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Propeptide

For heterologous protein production, there are some instances where signal peptides alone are considered not sufficient to secrete proteins with complex structures. For example, none of the tested signal peptides alone enabled human growth hormone secretion in *Staphylococcus carnosus* (Sturmfels et al., 2001). The protein was found only in strains expressing the fusions of human growth hormone with signals and propeptides.

In *Bacillus* species, the precursors of some secreted proteins contain propeptides which represent significant folding factors. The length and function of propeptides are variable. The cleavable propeptide is part of the precursors of some secreted proteins and is located at the N-terminal on the primary translation product between a typical signal peptide (prepeptide) and the N-terminus of the mature protein (Fig. 2.4) (Sarvas et al., 2004). The length is between 70 to 200 amino acid residues (Harwood and Cranenburgh, 2007). Propeptides are not directly involved in protein translocation per se but are essential in the post-translational folding required to achieve the active and stable form of their cognate secreted protein (Sarvas et al., 2004; Harwood and Cranenburgh, 2007). During the secretion of a preproprotein, the signal peptide is cleaved in the usual manner during or immediately after translocation. Only after the proteolytic self-cleavage and degradation of the propeptide, is full enzymatic activity achieved.

The roles of propeptide of serine proteases are to provide intermediate complex stability as intramolecular chaperones and nucleus generation for the completion of the folding pathway (Sarvas et al., 2004). This accelerates folding of proteins. In the absence of propeptides, serine proteases do not fold properly and cannot be secreted. In addition, it forms a stable molten globule-like folding intermediate during folding. The propeptide binds to the corresponding preprotein and guides or catalyses its rapid folding by stabilizing the secondary structure at the

site of binding, thereby generating a nucleus for folding. In addition, the other role of propeptides of serine proteases is as a proteolytic inhibitor. During the final step in the folding of a protease, propeptides are removed autocatalytically by a proenzyme. Degradation of the propeptide maintains enzymatic stability and protease-resistant conformation; finally this degradation facilitates the release of an enzymatically active form.

Demleitner and Gotz (1994) demonstrated that N- terminal region of prepropeptide region originating from *Staphylococcus hyicus* lipase was important for lipase activity while the C-terminal portion assisted lipase secretion and proteolytic stability. Compared to serine protease propeptides removed by proteolytic self-cleavage, the *S. hyicus* lipase propeptide is degraded by a metalloprotease (Sarvas et al., 2004). The lipase propeptide plays a role both in secretion and its post-translocational stability. By deleting the propeptide, the rate of secretion decreases and the enzyme remains inactive. Surprisingly, the propeptide of the *S. hyicus* lipase prevented the degradation of secreted heterologous proteins unrelated to lipase. For example, the *S. hyicus* lipase propeptide protected the *E. coli* outer membrane protein OmpA, against degradation when expressed in *S. carnosus* or *B. subtilis* (Meens et al., 1993; 1997; Puohiniemi et al., 1992). This pattern of production was dramatically altered by extending the N-terminus of OmpA with the propeptide of *S. hyicus* lipase which significantly increased the amount of OmpA in the culture medium with little or no evidence of degradation products (Meens et al., 1997). The absence of degradation reflects the physiological function of the propeptide. Meens et al. (1997) concluded that the propeptide facilitates the release of unfolded proteins from the translocase and/or passage through the cell wall, protecting them from proteases at the membrane-cell wall interface.

A very different conclusion was drawn from studies by Sturmfels et al. (2001). Fusion of various staphylococcal propeptides to human growth hormone appeared to maintain the proprotein in a secretion competent form in the cytoplasm before engagement with the translocase. Since the propeptide of *S. hyicus* lipase is cleaved by extracellular proteases in the natural host strain, in some recombinant proteins, the propeptide still remained fused with the mature proteins due to the absence of proteolytic activity in the expression hosts.

In conclusion, the bacterial systems provide rapid expression methods (days), inexpensive bioproduction media, high density biomass, simple process to scale-up, and minimal technical requirements (Brondyk, 2009). In spite of these advantages, bacterial systems have a number of important limitations for expression of heterologous proteins that should be considered, particularly eukaryotic proteins such as chaperones and post-translational modification proteins (Terpe, 2006).

LINOLEIC ACID ISOMERASE

There is an interest in developing commercial processes for the production of single isomers of conjugated linoleic acid (CLA) by biotransformation of LA using microbial cells and enzymes (Kim et al., 2000; Rainio et al., 2001; Ando et al., 2004). However, the evaluation of these strains suggested that growth and LA isomerase production levels by these anaerobes are insufficient to support economic commercial production of single CLA isomers (Peng et al., 2007). A better alternative is to clone the linoleate isomerase gene and to develop new production strains using recombinant technology.

CLA (18:2), an octadecadienoic acid with two conjugated double bonds, has a variety of positional and geometric isomers. Numerous publications have demonstrated various biological and physiological benefits of CLA in animal and human health, such as inhibiting the initiation

of carcinogenesis and tumorigenesis, reducing atherosclerosis, improving hyperinsulinemia, altering the low density lipoprotein/high density lipoprotein cholesterol ratio, and reducing body fat, while increasing muscle mass (Pariza et al., 2001; Pariza, 2004). Although CLA is present in virtually all foods, especially dairy products and other foods derived from ruminant animals, this important nutrient may not be available in sufficient quantities in the human diet (Pariza, 2004). CLA is currently available as a dietary supplement, and it is produced by alkaline isomerization of linoleic acid (LA, c9,c12 octadecadienoic acid, 18:2) or vegetable oils rich in LA esters. Chemical isomerization results in predominantly c9,t11 and t10,c12 isomers (40–45% each) with a variety of other isomers (Reaney et al., 1999; Sehat et al., 1998). Recent studies have demonstrated separate and/or synergistic actions of the two biologically active isomers, c9,t11 and t10, c12 CLA (Pariza, 2004). Therefore, single CLA isomers or isomers formulated at specific ratios represent the preferred compositional form.

Processes to prepare single isomers from chemically synthesized mixtures are not practical on a large scale because of high cost of purification and difficulties in achieving high purity (Berdeaux et al., 1998; Chen et al., 1999; Hass et al., 1999). LA isomerases catalyze the conversion of LA into single CLA isomers, c9,t11 or t10,c12 CLA, depending on the specific enzyme (Fig. 2.7). Tove's group described an LA C12 isomerase that catalyzed the conversion of LA into c9,t11 CLA, an intermediate step in the biohydrogenation of LA to stearic acid in anaerobic rumen bacterium *Butyrivibrio fibrisolvens* (Kepler and Tove, 1967; Kepler et al., 1966, 1971). The LA C12 isomerase has since been detected in a variety of bacteria (Coakley et al., 2003; Peng et al., 2007; Rosson et al., 2001; Verhulst et al., 1985). Biotransformation of LA using microbial cells and enzyme extracts has been explored for the production of c9,t11 CLA (Ando et al., 2004; Rainio et al., 2001). *Propionibacterium acnes* and *Megasphaera elsdenii*

were reported to contain a LA C9 isomerase converting LA to t10,c12 CLA (Kim et al., 2002; Verhulst et al., 1987).

Since February 2008 *B. breve* LAI sequence (accession number AX647943) (Stanton et al., 2002) and *P. acnes* LAI sequence (accession number AX062088) (Deng et al., 2007) were available in GenBank, *Bifidobacterium* and *P. acnes* LAI were chosen to be cloned in this study. However, *Bifidobacteium* LAI had never been cloned for heterologous production while *P. acnes* LAI activity expressed in *E. coli* DL21 (DE3) was very low (Deng et al., 2007). In addition, at that time there were two finished genome sequences, *B. longum* NC004307 (accession number AE014295) (Schell et al., 2002) and *B. adolescentis* ATCC 15703 (accession number AP009256) in GenBank. By comparison between three *Bifidobacterium* sequences, the primer sets for *Bifidobacterium* LAI amplification were designed. Moreover, the products of *Bifidobacterium* and *P. acnes* LAI are different. While *B. breve* LAI converts linoleic acid to c9,t11 CLA (Stanton et al., 2002), *P. acnes* LAI produces t10,c12 CLA (Deng et al., 2007). By using these systems, each single CLA isomer will be able to generate.

The aim of this part of the dissertation was to clone linoleate isomerase gene from *Bifidobacterium* species and *Propionibacterium acnes* into *E. coli* BL21 (DE3) and *Bacillus* species, respectively. The pET system with *E. coli* BL21 (DE3) were selected for protein expression in this study since this system claimed that the target proteins were produced in more than 50% of total protein (Novagen, pET system manual, 11th Edition, 2006). Since protein expression in *E. coli* BL21 (DE3) formed inclusion bodies and *Bacillus* species were subsequently chosen as other expression hosts. However, the detection of this enzyme activity is laborious and the enzyme activity may be low. The prepropeptides from *S. hyicus* lipase, *B. subtilis nprE,* and *B. megeterium nprM* were experimentally evaluated for promoting

heterologous secretion by using hydrolase from *T. fusca* and *E. coli* alkaline phosphatase PhoA as models. The expression vectors in this study were based on pMM1525 (Stammen et al., 2010). The -35 region and the ribosome-binding site on these expression vectors were systematically optimized for protein production maximization (Stammen et al., 2010). Prepropeptide from *S. hyicus* lipase enhanced *E. coli* alkaline phosphatase PhoA secretion in *B. subtilis* (Kouwen et al., 2010) while *B. subtilis nprE,* and *B. megeterium nprM* are the major extracellular proteases in *B. subtilis,* and *B. megeterium nprM,* respectively. *B. megaterium* YYBm1 was chosen to be the host in this study since the major extracellular protease *nprM* and xylose metabolism *xylA* genes were deleted.

METHIONINE IN THE POULTRY INDUSTRY

Methionine is a nutritionally essential amino acid required in the diet of humans and livestock, including poultry. Chickens are unable to produce methionine and therefore must obtain it through their diets. Generally, methionine is one of the first limiting amino acids in poultry nutrition and typically in most diets this amino acid has to be added to the poultry feed (Gomes and Kumar, 2005). In the U.S., approximately 90% of poultry feed is composed of corn and soybean which both are insufficient to meet methionine requirements of the bird (Baker, 2009). The organic poultry industry faces an even tougher challenge regarding methionine supplementation in organic poultry feed. Currently, formulated organic poultry feed contains insufficient amounts of methionine when fed which results in reducing growth rates of broilers (Rodenberg et al., 2008) as well as lowered egg weight in laying hens (Elwinger et al., 2008).

Methionine is an essential amino acid and must be supplemented in most diets for normal growth and function of the body. Methionine supplementation in poultry production is known to

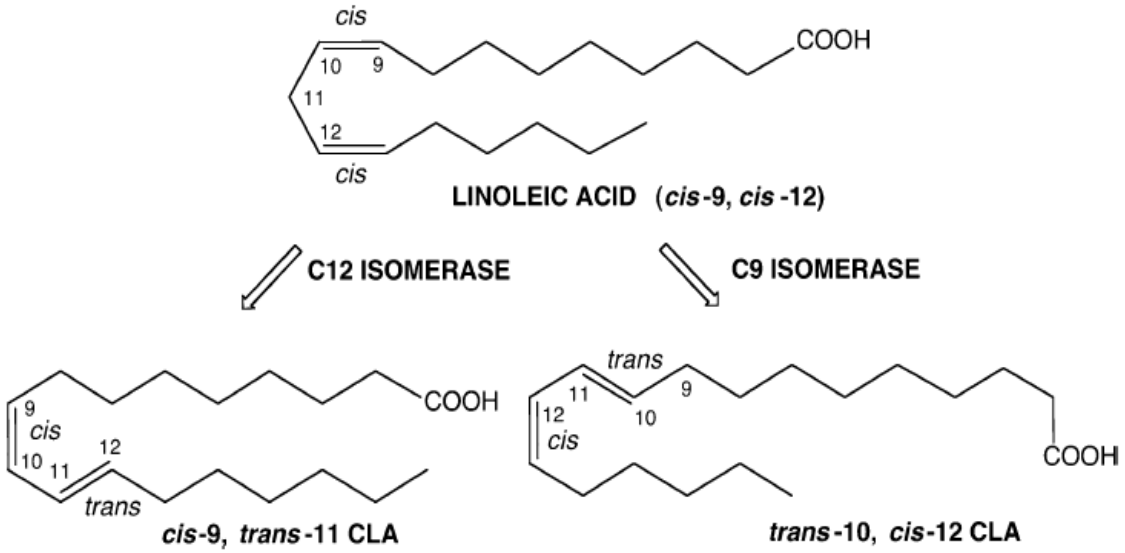


Fig. 2.7. Enzymatic conversion of LA to c9,t11 CLA by LA C12 isomerase and to t10,c12 CLA by LA C9 isomerase (Deng et al., 2007).

enhance feed efficiency, increase protein synthesis, and improve immune systems (Bunchasak, 2009). Methionine supplementation also prevented broiler chicks from developing neurological symptoms when raw grass pea seeds (*Lathyrus sativus*) were used as protein and energy sources in the diet (Fikre et al., 2010). In addition, methionine in diets demonstrated an improved oxidative stability, an increase in color stability, and a decrease in drip loss in chicken meat (Wang et al., 2009). Sufficient methionine levels in the diet have been shown to be necessary for sustaining normal immunocompetence and achieving maximum egg production in laying hens in subtropical conditions (Poosuwan et al., 2010). Bunchasak and Silapasorn (2005) reported that laying hens under tropical conditions fed a low-protein diet (14 % crude protein) supplemented with 0.44% methionine improved egg production and egg weight. In the same study bird mortality was reduced and egg shell thickness was improved when these hens were supported with methionine in feed. Conversely, insufficient methionine in organic feed showed a higher incidence of breast blisters in broilers (Rodenburg et al., 2008) or cannibalism in Hyline hens (Elwinger et al., 2008). The reduction of methionine content also decreased in the percentage of large and extra large eggs in Brown laying hens (Safaa et al., 2008).

Currently, methionine is produced by chemical processes or hydrolyzing proteins (Kumar and Gomes, 2005). However, chemical synthesis is expensive and produces a mixture of D- and L-methionine. In addition, these processes require hazardous chemicals such as acrolein, methyl mercaotan, ammonia, and cyanide (Fong et al., 1981). However, methionine from protein hydrolysis must be separated from the complex mixture (Kumar and Gomes, 2005). Furthermore, synthetic methionine is currently allowed as an additive to organic poultry feed by the U.S. Department of Agriculture's National Organic Program (NOP) however NOP is only

extending its use until October 1st 2012 (USDA, Agricultural Marketing Service, http://www.paorganic.org/wp-content/uploads/2011/10/USDA-Methionine.pdf).

Methionine production by microorganisms

A number of microorganisms capable of producing amino acids have been isolated and the production of amino acids has become an important aspect of industrial microbiology. Amino acids such as L-lysine, L-glutamic acid, L-threonine, and L-isoleucine have been produced successfully by fermentation (Hermann, 2003). Numerous studies have attempted to isolate and mutate microorganisms for overproduction of methionine but commercial methionine production from microorganisms is not available due to the highly branched pathway with complicated metabolic control in methionine biosynthesis (Kumar and Gomes, 2005).

Even though microorganisms use different biosynthetic routes, the pathways of methionine biosynthesis in various microorganisms have many common features (Fig. 2.8). First, aspartate is converted to aspartyl phosphate by aspartate kinase (EC 2.7.2.4, reaction 1) and then oxidized by aspartaldehyde semialdehyde dehydrogenase (EC 1.2.1.11, reaction 2) to form aspartate semialdehyse. The latter is oxidized by homoserine dehydrogenase (EC 1.1.1.3, reaction 3). Aspartate semialdehyde is subsequently converted to lysine. In one route, homoserine is converted to dihydropicolinate by dihydropicolinate synthase (reaction 8 and 9) and subsequently threonine and isoleucine production. In another pathway, homoserine undergoes condensation with acetyl CoA to produce O-acetyl homoserine by homoserine acetyl transferase (EC 2.3.1.31, reaction 4). Some yeasts, fungi, and bacteria can directly synthesize homocysteine from O-acetylhomoserine via the direct sulfhydrylation pathway by utilizing sulfide (S^{2-}) as the sulfur donor. Cystathionine is synthesized from O-acetyl homoserine and cysteine by cystathionine γ-synthase (EC 4.2.99.9, reaction 5). After hydrolysis of cystathionine

to homocysteine, pyruvate, and ammonia by cystathionine b-lyase (EC 4.4.1.8, reaction 6), methionine is formed by the methylation of homocysteine by methionine synthase. Two forms of methionine synthases are involved in the final methylation reactions. Vitamin B_{12} (cobalamin)-dependent methionine synthase (EC 2.1.1.13, *metH*) utilizes N^5- methyl-tetrahydrofolate or its polyglutamyl derivative as the methyl group donor, while the cobalamin-independent form (EC 2.1.1.14, *metE*), utilizes N^5-methyl-tetrahydropteroyl-triglutamate.

All microorganisms possess mechanisms to regulate enzymes such that excess amino acids production is avoided. For example, in *Corynebacterium glutamicum*, the activity of enzyme homoserine O-transacetylase was not inhibited by L-methionine, S-adenosylmethionine or S-adenoylhomocysteine (Kase and Nakayama, 1974). However, the synthesis of the enzyme was strongly repressed by L-methionine. The methionine biosynthesis is also regulated at the transcriptional level. In *E. coli*, MetJ repressor interacting with S-adenosylmethionine binds at *met* box, an eight-base consensus sequence, and subsequently leads to repression of the *met* genes, except *metH* (Weissbach and Brot, 1991). In general, *met* genes have at least two to five contiguous *met* boxes located at operator sequences. However, the MetR activator in *E. coli* stimulates the expression of *metE* and *metH*, encoding methionine synthases.

Numerous studies have attempted to mutate microorganisms for methionine overproduction by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine UV irradiation. Several studies have reported that methionine-analog resistance in mutants correlated to higher methionine production due to an alteration in the regulation of L-methionine biosynthesis (Adelberg, 1958; Brigidi et al., 1988; Chattopadhyay et al., 1991; Dunyak and Cook, 1985; Komatsu et al., 1974; Morzycka et al., 1976; Musilkova and Fencl, 1964; Tani et al., 1988; Yamada et al., 1982). Rowbury (1965) reported that resistance to norleucine, a methionine analog, in microorganisms

is associated with a failure of methionine to repress any of the methionine biosynthetic enzymes by feedback effect. Based on this concept, two methionine analogs (ethionine and norleucine) are typically used to screen for methionine overproduction in either mutants or wild type microorganisms from various natural sources.

The major cause of inhibition appears to be that methionine analogs mimic the means by which methionine regulates its own production (Gomes and Kumar, 2005). Methionine analogs can effectively function as true feedback inhibitors without participating in other functions in the cells. These analogs may bind to the product site of the enzyme or may bind effectively to the repressor and consequently shutdown the pathway for the synthesis of methionine. Analogs inhibit growth by starving the cell for methionine. Therefore, methionine analogs act as pseudo-feedback inhibitors or repressors, thereby inhibiting or repressing the synthesis of methionine. Only strains having resistance to analogs may overproduce methionine. These strains are able to resist the analogs either because of an alteration in the structure of the enzyme or an alteration in the enzyme formation system. Natural methionine analog resistant strains are insensitive to methionine accumulation and thereby will overproduce methionine. Using methionine analogs to screen the methionine overproducing microorganism could be an efficient and robust method for the identification of commercial strains since these strains lack methionine feedback inhibition.

Methionine detection

For methionine quantification in animal feed, chemical methods including high performance liquid chromatography (HPLC) are commonly used (Chalova et al., 2010). By using the chemical methods, feeds are treated with acid digestion and the proteins in samples are completely digested. However, liberation of methionine is different from protein digestion under physiological conditions. Feed-derived methionine, which is available to animals to assimilate,

can be more accurately estimated by animal or microbial assays which correspond more directly to the physiological needs of animals (Parson et al., 1997). Although considered the biological standard, animal assays are laborious, expensive, and time consuming (Chalova et al., 2007; Cork et al., 1997; Froelich et al., 2005).

In contrast to animal assays, microbial assays appear to be easier and more affordable for routine analysis. This method is based on microorganisms response to feed nutrients by increasing the population number of organisms (Froelich and Ricke, 2005). In contrast to animal assays, microbiological assays require smaller quantities of nutrients and respond in less time. Rapid development and recent improvements in molecular techniques allow for constructing successful and accurate amino acid biosensors via more precise genetic targeting of specific genes in microbial cells (Chalova et al., 2010). Among all microorganisms, *E. coli* is one of the most highly investigated bacteria for the purposes of biosensor fabrication. It is easy to cultivate, with simple nutritive requirements and rapid growth (Ingraham et al., 1983). Based on *E. coli* auxotroph, threonine, tryptophan, lysine, and glutamine quantification have all been successful. (Erickson et al., 2000; Hitcheins et al., 1989; Tessaro et al., 2012). Since *E. coli* is an intestinal bacterium of most animals and humans, the assimilation of amino acids would be similar to animals (Chalova et al., 2009). After feed ingredients treated with enzymes, Erickson et al. (1999) demonstrated a correlation of 0.94 between lysine bioavailability determined by using an *E. coli* lysine auxotroph and previously published chick bioassay data. An *E. coli* biosensor developed by Chalova et al. (2007) proved to be as accurate as the corresponding chick bioassay for lysine bioavailable quantitation in diverse feed ingredients.

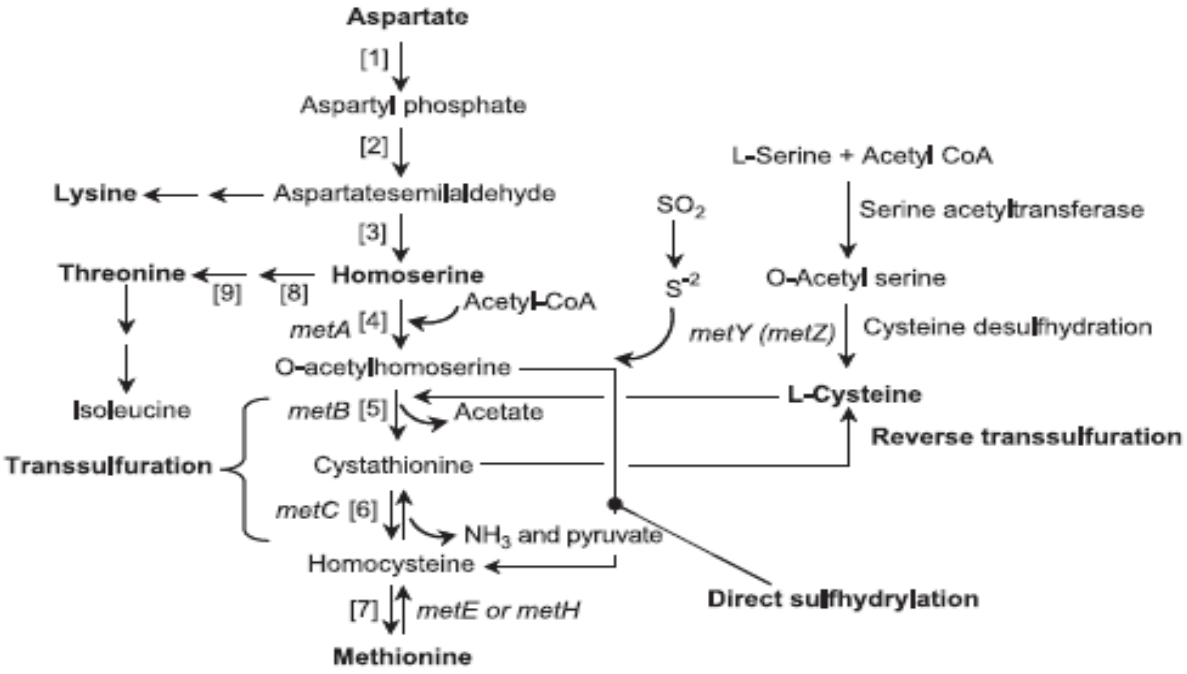


Fig. 2.8. Methionine biosynthetic pathway in *Corynebacterium glutamicum* (Kuma and Gomes, 2005). (1) Aspartate kinase, (2) aspartaldehyde dehydrogenase, (3) homoserine dehydrogenase, (4) homoserine O-acetyltransferase, (5) cystathionine γ-synthase, (6) cystathionine g-lyase, (7) homoserine S-methyltransferase (vitamin B_{12} independent or *metH*-encoded, vitamin B_{12} dependent), (8) homoserine kinase, (9) threonine synthase

E. coli methioine auxotrophs have been constructed (Hitchins et al., 1989). However, these strains were mutated by using chemical mutagens and isolated based on the methionine requirement (Adelberg et al., 1965). As a result, the mutation is not target specific and various non-methionine related genes can be affected. Revertants or compensatory mutations may occur to abolish the desired functionality (Wright et al., 1997). In the case of methionine, the auxotrophic requirements for this amino acid are not specific and can also be satisfied by a variety of compounds including methioninyl peptides, α-hydroxy methionine, *N*-acetylmethionine, and the α-keto analog α-keto-λ-methiol butyrate (Mulligan et al., 1982). When a chemically generated *E. coli* methionine auxotroph (ATCC 23798) was used, Froelich et al. (2002) observed no differences based on substrate affinities of an *E. coli* methionine auxotroph to methionine and methionine hydroxy analog, respectively. Estimated maximum growth rate of the *E. coli* auxotroph when grown on both substrates was also found to be similar. To avoid problems as mentioned above, in this study partial *metC* deletion was constructed from wild-type *E. coli* K12. Without methionine supplement at 1μg/ml, this mutant should be unable to grow.

Microbial sources for organic poultry industry

Many studies have attempted to isolate methionine producing microorganisms from environments or by genetic modification by using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. Several studies have reported that methionine-analog resistance in bacterial strains correlated to higher methionine production due to an alteration in the regulation of L-methionine biosynthesis (Adelberg, 1958; Chattopadhyay et al., 1991; Musilkova and Fencl, 1964;Yamada et al., 1982 Tani et al., 1988). Although the mutation was successful in producing methionine overproducing microorganisms, any genetically-modified organisms are considered unacceptable for use in

organic food production (Electric Code of Federal Regulations: U.S. National Organic Program). Therefore, wild type strains with methionine-producing ability are necessary for the organic poultry industry. The aims of this part of the PhD dissertation were to isolate methionine-producing microorganisms and to quantify of methionine production from these strains; therefore, two methionine analogs were used to screen methionine overproduction in wild-type yeasts.

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CHAPTER THREE

LINOLEIC ACID ISOMERASE EXPRESSION IN *E. coli* BL21 (DE3) AND *Bacillus* SPECIES

ABSTRACT

Linoleic acid isomerase (LAI) is the enzyme responsible for converting linoleic acid (c9,c12, 18:2) to c9,t11 or c9,c12 conjugated linoleic acids. Conjugated linoleic acids are believed to lower cancer risk and enhance immunity. In this study, *Bifodobacterium* LAI gene was cloned into *E. coli* BL21 (DE3) by using pET24a(+) as the expression vector while *Propionibacterium acnes* LAI gene fused with pSSBm97 derivatives was expressed in *Bacillus* species. Even though the protein expressed of *Bifidobacterium* LAI was found in *E. coli*, no activity was detectable. The enzyme was possibly in an inactive form or activity was below detection. By changing cysteine residues to alanine, *P. acnes* LAI activity was present in *B. megaterium* YYBm1 but activity was not improved. From prepropeptide *B. subtilis amyE* fused with *P. acnes* LAI at N-terminus, the results showed that the proteins were unstable. Also, by transferring plasmids carrying prepropeptide *Staphylococcus hyicus* lipase and prepropeptide *B. subtilis amyE* fused with *P. acnes* LAI into *B. licheniformis* NRRLB-14212, LAI was not found due to possible proteolytic degradation.

KEY WORDS: *Bacillus, Bifidobacterium,* heterologous expression, linoleic acid isomerase,

INTRODUCTION

Conjugated linoleic acid (CLA, 18:2) is an octadecadienoic acid with two conjugated double bonds. These compounds possess abilities to lower cancer risk, enhance immunity and reduce body fat (Pariza et al., 2001; Pariza, 2004). CLA is currently marketed as a dietary supplement and is produced by alkaline isomerization of linoleic acid (LA) or vegetable oils containing triglyceride esters of LA (Peng et al., 2007); however, chemical synthesis produces a mixture of CLA (Reaney et al., 1999; Sehat et al., 1998) and the processes required to separate the respective single isomer are expensive (Berdeaux et al., 1998; Chen et al., 1999; Hass et al., 1999).

In contrast to chemical processes, biological processes originating from microorganisms can provide production of a single isomer of CLA (Deng et al., 2007). The LA C12 isomerase has since been detected in a variety of bacteria (Coakley et al., 2003; Peng et al., 2007; Rosson et al., 2001; Verhulst et al., 1985). Biotransformation of LA using microbial cells and enzyme extracts has been explored for the production of c9,t11 CLA (Ando et al., 2004; Rainio et al., 2001). *Propionibacterium acnes* was reported to contain a LA C9 isomerase converting LA to t10,c12 CLA (Deng et al., 2007).

There is an interest in developing commercial processes for the production of single isomers of CLA by biotransformation of LA using microbial cells and enzymes (Kim et al., 2000; Rainio et al., 2001; Ando et al., 2004). However, the evaluation of these strains suggested that growth and linoleic acid isomerase (LAI) production levels by these anaerobes are insufficient to support economic commercial production of single CLA isomers (Peng et al., 2007). A better alternative is to clone the LAI gene and to generate new production strains using recombinant technology.The aim of this study was to clone the linoleate isomerase gene from

Bifidobacterium species and *Propionibacterium acnes* into *E. coli* BL21 (DE3) and *Bacillus* species.

MATHERIALS AND METHODS

DNA manipulation for the construction of plasmids.

The bacterial strains used in this study were described in Table 3.1. Kanamycin sulfate and cysteine hydrochloride were purchased from Sigma (St. Louis, MO). All restriction endonucleases and PCR master mix and T4 DNA ligase were purchased from Promega (Madison, WI).

General methods.

All *Bifidobacterium* strains were grown anaerobically (anaerobic jars with a gas pack in MRS broth supplemented with 0.05% (w/v) L-cysteine (98% pure; Sigma) and incubated at 37^{o}C overnight. Genomic DNA was prepared from *Bifidobacterium* strains by using QIAamp DNA Stool Mini Kit. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Cloning of LA gene into pET24a(+).

Linoleic acid isomerase (LAI) gene from *B. breve* (accession number AX647943) and two finished genome sequences, *B. longum* NC004307 (accession number AE014295) and *B. adolescentis* ATCC15703 (accession number AP009256), were aligned by using T-coffee (Notredame et al., 2000). Primers (Table 3.2) were designed according to the potential LA sequences. PCR conditions (30 cycles) were: initial denaturation 95 ^{o}C, 120 s; denaturation, 95 ^{o}C, 30 s, annealing, 45 ^{o}C, 30 s, extension 72 ^{o}C, 120 s, final extension, 72 ^{o}C, 7 min. PCR conditions were identical to all primer sets, except the annealing temperature (40 ^{o}C) for primers Bifido1F and Bifido1RHis. The 1990-bp PCR products were confirmed by agarose gel

electrophoresis. Insertion of all PCR products via the corresponding XhoI and NdeI restriction sites in pET24a(+) led to plasmid pET2 to pETH5 (Table 3.3). The plasmids pET2 to pETH5 were transformed to *E. coli* BL21 (DE3) by electroporation. Individual colonies from kanamycin agar plates were selected.

For *Propionibacterium acnes* LAI, the new DNA sequence was designed by JCat software (http://www.jcat.de/) (Grote et al., 2005) and was synthesized by Integrated DNA Technologies, Coralville, IA. The *P. acnes* LAI was used as the template and primers LF to A5F were used to change from 5 cysteine positions to alanine (Table 3.4). The PCR products were flanked by SpeI-EagI restriction sites, was digested with these enzymes, and subsequently inserted into pLPPL after SpeI-EagI digestion, creating the plasmids pA1, pA2, pA3, pA4, pA5, pA6.

For prepropeptide *B. subtilis amyE* fused with *P. acnes* LAI, PCR product was amplified by using primers amyEF and amyER (amyEF: 5'tat atg taca ATG TTT GCA AAA CGA TTC AAA ACC TC -3'; AmyER: 5' tat aag atc tac tagt CTC ATT CGA TTT GTT CGC CGT-3'). The PCR products were flanked by BsrGI-SpeI restriction sites, was digested with these enzymes, and subsequently was inserted into pLPPL, pA1, pA2, pA3, pA4, pA5, pA6 after BsrGI-SpeI digestion, creating the plasmids pE0, pE1, pE2, pE3, pE4, pE5, pE6.

Protoplast *B. megaterium* YYBm1 cells were transformed with the appropriate expression plasmids using a polyethylene glycol-mediated procedure described by Christie et al. (2008) while plasmids were transferred into *B. licheniformis* NRRLB-14212 by electroporation as described in Xue et al. (1999).

Enzymatic activity measurements.

For *E. coli* BL21 (DE3), 50 ml of LB broth containing kanamycin (50 µg/ml) was inoculated (1:100, v/v) with a freshly grown overnight culture of strains hosting an isomerase expression plasmid. After growning at 37 ^{o}C for 3 hours, cultures were induced with 1 mM isopropylthio-β-D-galactoside (IPTG) for 2 hours.

E. coli cultures were harvested by centrifugation at 10,000 xg for 10 min at 4 ^{o}C. Cells were suspended in BugBuster (Novagen, Darmstadt, Germany). The subcellular localization of heterologous protein production was separated following the protocol described in pET system manual 11th edition (Novagen, Darmstadt, Germany) and the protein samples were analyzed by SDS-PAGE.

All *Bacillus* plasmid strains were grown in baffled shake flasks at 30 ^{o}C in Luria-Bertani (LB) medium at 200 rpm. Recombinant expression of genes under transcriptional control of the xylose-inducible promoter was induced by the addition of 0.5% (w/v) xylose at OD578 of 0.4. The secreted proteins were separated from cells by centrifugation at 10,000 xg, 4^{o}C for 10 min. After separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane and detected with 6X his tag antibody (Abcam, Cambridge, MA) and horseradish peroxidase–anti-rabbit immunoglobulin G conjugates.

Linoleaste isomerase activity was carried out as described by Peng et al. (2007). The preparation of fatty acid methyl esters (FAME) was described in Lewis et al. (2000) and FAME was detected by gas chromatography with tridecanoic acid as the internal standard.

RESULTS AND DISCUSSION

LA expression in *E. coli* BL21 (DE3) as the host

Among expression systems, *E. coli* is considered to be the first choice since numerous vectors, readily available engineered strains, and minimal technical requirements are already in place. In addition, this system is rapid due to short doubling times for the generation (approximately 20 min per generation) (Brondyk, 2009). In this study, *E. coli* BL21 (DE3) was chosen as the host. In this system it was suggested that the target protein was synthesized to an equivalent of more than 50% of the total cell protein within a few hours after induction (pET system manual 11th edition, 2006). By using SDS-PAGE, the results showed that the expression of LAI in *E. coli* was tightly controlled by IPTG (Fig. 3.1). The soluble cytoplasm proteins in *E. coli* carrying plasmids were collecting and detected by SDS-PAGE (Fig. 3.2 and 3.3). The results demonstrated that these proteins were unstable in the cytoplasm and some strains, particularly strain H3 and H5, did not produce soluble cytoplasm proteins. The soluble and insoluble cytoplasm proteins were collected to identify the localization of proteins (Fig. 3.4). The results demonstrated that 6X His tag adversely affected the solubility of LAI, particularly LAI originating from *B. longum* ATCC15707 (clones 2 versus H2, Fig.3.4). Since expressed proteins were sequestered in inclusion bodies, the lower temperature incubation might enhance enzymatic folding. At 21.5 ^{o}C incubation, the results from SDS-PAGE did not show improved folding of the enzyme (Fig. 3.5). Deng et al. (2007) reported that the folding of *P. acnes* LAI expressed in *E. coli* was interfered by the C-terminal 6X His tag. The activities of LAI from these clones were undetectable due to possible improper folding of enzyme or very low activities. Deng et al. (2007) reported that *P. acnes* LAI expressed in *E. coli* BL21 (DE3) was only 1 nmol/min/ml. The primary method to minimize inclusion body formation and maximize the formation of

soluble, properly folded proteins in the cytoplasm is lowering the incubation temperature to 15 to 30 ^{o}C during the expression period because the reduced temperature reduces the rate of transcription, translation, refolding and thus increase proper folding (Brondyk, 2009). In this study, the incubation temperature was reduced to 21.5 ^{o}C; however, this method did not enhance the enzymatic activity. In addition, protein accumulated in inclusion bodies in this study is one of the disadvantages of protein expression in *E. coli* (Terpe, 2006).

Because of the inclusion body formation problems associated with Gram-negative bacterium *E. coli*, Gram-positive bacteria appear to be more promising hosts for large-scale production of recombinant proteins. *B. megaterium* YYBm1 and *B. licheniformis* NRRLB-14212 were chosen for the hosts. By deleting *nprM*, the major extracellular protease, and *xylA* genes, the gene for xylose metabolism in *B. megaterium* DSM319, *B. megaterium* YYBm1 is well suited for extracellular protein production (Stammen et al., 2010). *B. licheniformis* was chosen due to its ability to secrete large quantities of extracellular enzymes (Schallmey et al., 2004).

B. megaterium YYBm1 carrying pLPPL showed that this host produced secreted *P. acnes* LAI; however, no activity was detectable. Based on *P. acnes* LAI amino acid sequence, there are 5 cysteine residues (Fig. 3.6). All five cysteine residues were changed to alanine by using primers LF to A5F (Table 3.4) with *P. acnes* LAI as the template but no activity in *B. megaterium* YYBm1 carrying pA1 to pA6 was detectable. Liu and Escher (1999) reported that the bioluminescence activity of the secreted *Renilla* luciferase could be improved after selective removal of sulfhydryl groups by substitution of cysteine residues. Since wild type *Renilla* luciferase protein contains an odd number of cysteine residues in amino acid sequence, they proposed that a free cysteine residue and/or unfavorable disulfide bond in secreted *Renilla*

luciferase could affect its bioluminescence activity and alanine, an amino acid considered to be one of the most neutral, was used for this purpose.

Based on matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), the results showed that propeptide *S. hyicus* lipase still attached with *P. acnes* LAI and might impede enzymatic activity. In the next step, propeptide *B. subtilis* amplase (*amyE*) was chosen since this propeptide enhanced the secreted human interferon-α in *B. subtilis* as the host. In addition, the propeptide *B. subtilis amyE* is only 8 amino acid length, compared to 207 amino acid length in propeptide *S. hyicus* lipase. The plasmids pE0 to pE6 were transferred into *B. megaterium* YYBm1. Based on Western blot detected with 6X his tag antibody, no secreted proteins were found in these strains. The results demonstrated that the protein fused with propeptide *B. subtilis* amyE was unstable in *B. megaterium* YYBm1 as the host (data not shown), compared to human interferon-α in *B. subtilis*.

Since attempts with *B. megaterium* YYBm1 were unsuccessful, *B. licheniformis* NRRLB-14212 was examined as a possible expression host. The plasmids pLPPL and pE0 were transferred into *B. licheniformis* NRRLB-14212 by electroporation. The plasmid pE0 constructed in *E. coli* could not be successfully transferred into the expression strain *B. licheniformis*, indicating a lethal effect. This result agrees with Brockmeier et al. (2006) that 25 of 173 prepeptides could not be transferred into *B. subtilis* TEB1030. The secreted protein in *B. licheniformis* NRRLB-14212 carrying pLPPL could not be detected by Western blot (data not shown). Possibly, *B. licheniformis* NRRLB-14212, a wild type strain, has extracellular proteases or propeptide *S. hyicus* lipase was unable to protect *P. acnes* LAI from proteolytic degradation.

In conclusion, *Bifidobacterium* LAI expressed in *E. coli* BL21 (DE3) did not function due to insoluble formation in inclusion bodies. Amino acid modification of *P. acnes* LAI

expressed in *B. megatreium* YYBm1 did not improve the activity. Also, propeptide of *B. subtilis amyE* and both propeptides could not protect *P. acnes* LAI from proteolytic degradation in *B. megaterium* YYBm1 and *B. licheniformis* NRRLB-14212 as the hosts, respectively.

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Table 3.1. Bacterial strains used in this study

Bacterial strain	Description	Source
E. coli BL21 (DE3)	This strain lacks the lon and opmT proteases and contains a copy of RNA the T7 RNA polymerase gene under the control of the lacUV5 promoter. These modifications enable stable expression of proteins using T7 promoter driven constructs	Novagen, Darmstadt, Germany
Bifidobacterium sp.		
B. longum ATCC15700	Wild type	Robert Preston Story, Jr.
B. breve ATCC15700	Wild type	ATCC, Manassas, VA
B. adolescentis ATCC15703	Wild type	ATCC, Manassas, VA
B. infantis ATCC25962	Wild type	ATCC, Manassas, VA
Bacillus sp.		
B. subtilis	Wild type	Center for Food Safety, University of Arkansas
B. licheniformis NRRLB-14212	Wild type	Center for Food Safety, University of Arkansas
B. megaterium YYBm1	This strain is deficient in the major extracellular protease NprM and xylose metabolism XylA.	Stammnen et al. (2010)

Table 3.2. Oligonucleotides used in this study

Primer name	Sequence	Application
Bifido1F	5'-CAG A*CA TAT G*TA CTA CAG CGG CAA YTA T-3'	Forward primer containing an NdeI site for cloning LA from *B. breve* ATCC 15700
Bifido1R	5'-CTA TCT CGA GTC AGA TYA CRY GGT ATY CGC GTA-3'	Reverse primer containing an XhoI site for cloning LA from *B. breve* ATCC 15700
Bifido R1His	5'- CTA T*CT CGA G*GA TYA CRY GGT ATY CGC GTA-3'	Reverse primer containing an XhoI site for cloning LA from *B. breve* ATCC 15700 with a C-terminal His_6-tag
longumF1	5'-CAGA CAT ATG TAC TAC AGC AGC GGC AAT-3'	Forward primer containing an NdeI site for cloning LA from *B.longum* ATCC 15707 or *B. infantis* ATCC 25962
longumR1	5'-CTAT *CTC GAG* TCA GAT TAC GCG GTA TTC GCG-3'	Reverse primer containing an XhoI site for cloning LA from *B.longum* ATCC 15707 or *B. infantis* ATCC 25962
longumR1His	5'-CTAT CTC GAG GAT TAC GCG GTA TTC GCG-3'	Reverse primer containing an XhoI site for cloning LA from *B.longum* ATCC 15707 or *B. infantis* ATCC 25962 with a C-terminal His_6-tag
adolesF1	5'-CAGA CAT ATG TAC TAT TCC AAC GGC AAT-3'	Forward primer containing an NdeI site for cloning LA from *B. adolescentis* ATCC 15703
adolesR1	5'-CTAT CTC GAG TCA GAT CAC GCC GTA TTC CTT-3'	Reverse primer containing an XhoI site for cloning LA from *B. adolescentis* ATCC 15703
adolesR1His	5'-CTAT CTC GAG GAT CAC GCC GTA TTC CTT-3'	Reverse primer containing an XhoI site for cloning LA from *B. adolescentis* ATCC 15703 with a C-terminal His_6-tag

Table 3.3. Plasmids used in this study

plasmid	Description	Source
pCR® 4-TOPO®	Cloning vector for PCR products, Amp^r, Kan^r	Invitrogen
pET24a(+)	Expression vector with an N-terminal His_6-tag, Kan^r	Novagen
pET2	pET24a(+) with NdeI-XhoI LA gene from *B.longum* ATCC 15707	This work
pETH2	pET24a(+) with NdeI-XhoI LA gene with His_6-tag from *B.longum* ATCC 15707	This work
pET3	pET24a(+) with NdeI-XhoI LA gene from *B. breve* ATCC 15700	This work
pETH3	pET24a(+) with NdeI-XhoI LA gene with His_6-tag from *B. breve* ATCC 15700	This work
pET4	pET24a(+) with NdeI-XhoI LA gene from *B. adolescentis* ATCC 15703	This work
pETH4	pET24a(+) with NdeI-XhoI LA gene with His_6-tag from *B. adolescentis* ATCC 15703	This work
pET5	pET24a(+) with NdeI-XhoI LA gene from *B. infantis* ATCC 25962	This work
pETH5	pET24a(+) with NdeI-XhoI LA gene with His_6-tag from *B. infantis* ATCC 25962	This work
pLPPL	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pPPlip; P_{xylA}-(-35^+ rbs^+)-$prepeptide_{lipA}$-$propeptide_{lipA}$- LAI *P. acnes*	This work
pA1	pLPPL derivative, C46A	This work
pA2	pLPPL derivative, C154A	This work
pA3	pLPPL derivative, C286A	This work
pA4	pLPPL derivative, C344A	This work
pA5	pLPPL derivative, C412A	This work
pA6	pLPPL derivative, C46A, C154A, C286A, C344A, C412A	This work
pE0	Prepropeptide *B. subtilis amyE* inserted into BsrGI and SpeI sites of pLPPL; P_{xylA}-(-35^+ rbs^+)-$prepeptide_{amyE}$-$propeptide_{amyE}$-LAI *P. acnes*	This work
pE1	pE0 derivative, C46A	This work
pE2	p E0 derivative, C154A	This work
pE3	p E0 derivative, C286A	This work
pE4	pE0 derivative, C344A	This work
pE5	pE0 derivative, C412A	This work
pE6	pE0 derivative, C46A, C154A, C286A, C344A, C412A	This work

Table 3.4. Oligonucleotides used for LAI engineering in this study

Primer	Sequence 5’ to 3’
LF	tat aag atc t act agt ATGTCTATTTCTAAAGATTCTCG
LR	tat aag atc t CGG CCG TTA GTG ATG GTG
A1R	GAG AGT GAG CTT TAC CAC CTA CGT GAT CTG TAC G
A1F	GGT GGT AAA GCT CAC TCT CCA AAC TAC CAC G
A2R	CAG CTT CAG CAC CGT TTA AAG CTA AGA ATT CAT CGA A
A2F	TTA AAC GGT GCT GAA GCT GCT CGT GAT TTA TG
A3R	TTA CTA AAG CAG CAT CTA CCA TGT ATT GTT GGT
A3F	GTA GAT GCT GCT TTA GTA AAA GAA TAC CCA ACA ATT TCT GG
A4R	TTT GAC GAG CTT CTT CTT GTG TTT TAT CAG CGT AAT C
A4F	AAG AAG AAG CTC GTC AAA TGG TAT TAG ATG ATA TGG AAA
A5R	AGT AGT GAG CTA CTT CAT CGA AGT TAC CGA AAG AC
A5F	ATG AAG TAG CTC ACT ACT CTA AAG ATT TAG TAA CAC GTT

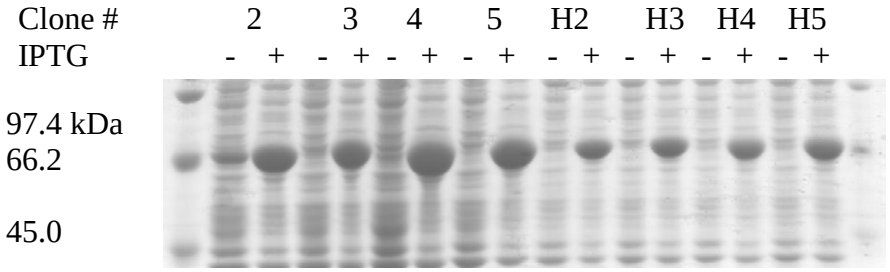


Fig. 3.1 Total cell protein fraction isolated from *E. coli* clones containing LAI gene originated from *Bifidobacterium* strains. 2: LAI gene originated from *B. longum* ATCC1570; 3: LAI gene originated from *B. breve* ATCC15700; 4: LA gene originated from *B. adolescentis* ATCC15703; 5: LAI gene originated from *B. infantis* ATCC25962; H: LAI gene fused with 6X His tag.

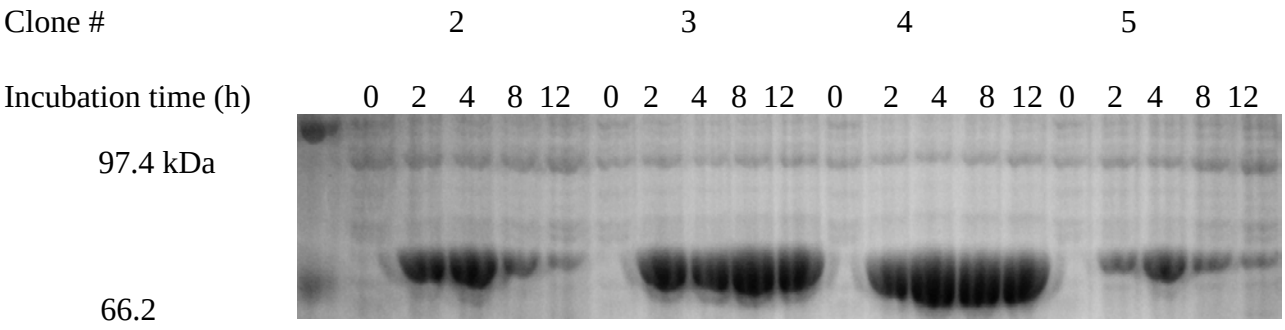


Fig. 3.2 Soluble cytoplasmic protein fraction isolated from *E. coli* clones containing LAI gene originated from *Bifidobacterium* strains. 2: LAI gene originated from *B. longum* ATCC1570; 3: LAI gene originated from *B. breve* ATCC15700; 4: LAI gene originated from *B. adolescentis* ATCC15703; 5: LAI gene originated from *B. infantis* ATCC25962.

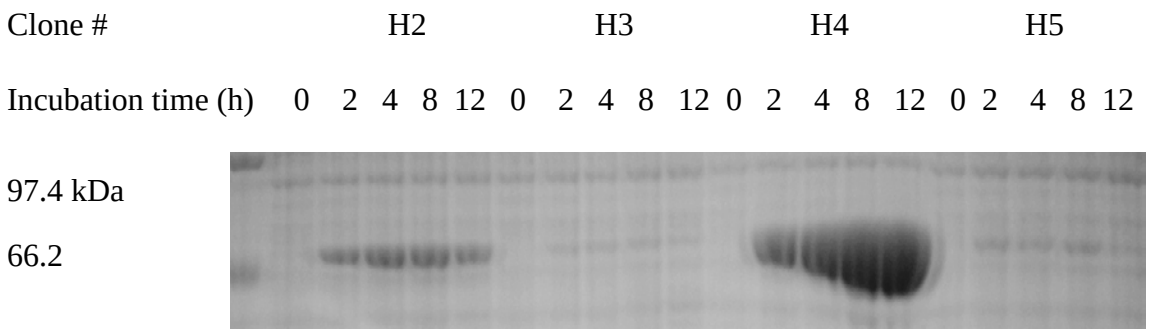


Fig. 3.3 Soluble cytoplasmic protein fraction isolated from *E. coli* clones containing LAI gene originated from *Bifidobacterium* strains. 2: LAI gene originated from *B. longum* ATCC1570; 3: LAI gene originated from *B. breve* ATCC15700; 4: LAI gene originated from *B. adolescentis* ATCC15703; 5: LAI gene originated from *B. infantis* ATCC25962; H: LAI gene fused with 6X His tag.

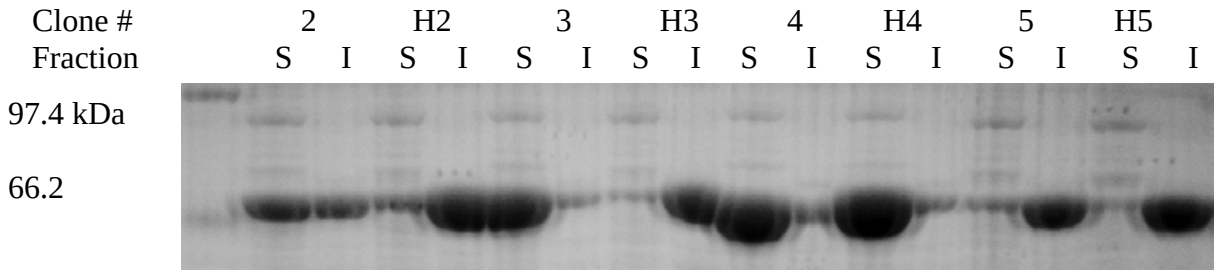


Fig. 3.4. Soluble (S) and insoluble (I) cytoplasmic protein fraction from *E. coli* clones containing LAI gene originated from *Bifidobacterium* strains after adding IPTG 4 hours at 25 ^{o}C incubation. 2: LAI gene originated from *B. longum* ATCC1570; 3: LAI gene originated from *B. breve* ATCC15700; 4: LAI gene originated from *B. adolescentis* ATCC15703; 5: LAI gene originated from *B. infantis* ATCC25962; H: LAI gene fused with 6X His tag.

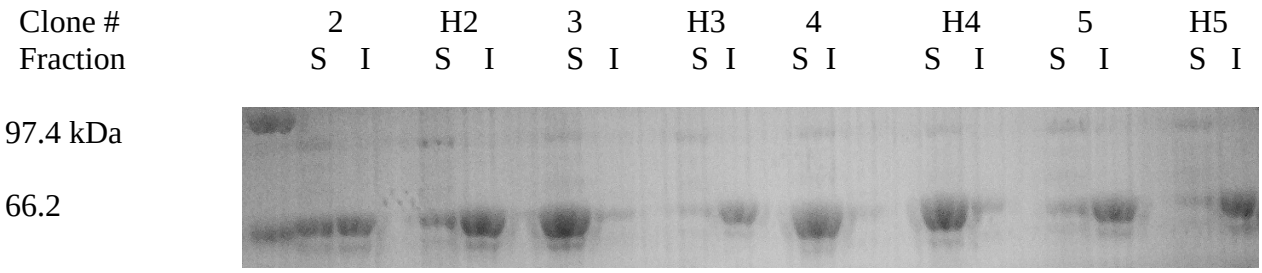


Fig 3.5. Soluble (S) and insoluble (I) cytoplasmic protein fraction isolated from *E. coli* clones containing LAI gene originated from *Bifidobacterium* strains after adding IPTG 4 hours at 21.5 ^{o}C incubation. 2: LAI gene originated from *B. longum* ATCC1570; 3: LAI gene originated from *B. breve* ATCC15700; 4: LAI gene originated from *B. adolescentis* ATCC15703; 5: LAI gene originated from *B. infantis* ATCC25962; H: LAI gene fused with 6X His tag.

M S I S K D S R I A I I G A G P A G L A A G M Y L E Q A G F H D Y T I L E R T D H V G

G K **C** H S P N Y H G R R Y E M G A I M G V P S Y D T I Q E I M D R T G D K V D G P K

L R R E F L H E D G E I Y V P E K D P V R G P Q V M A A V Q K L G Q L L A T K Y Q G

Y D A N G H Y N K V H E D L M L P F D E F L A L N G **C** E A A R D L W I N P F T A F G

Y G H F D N V P A A Y V L K Y L D F V T M M S F A K G D L W T W A D G T Q A M F E

H L N A T L E H P A E R N V D I T R I T R E D G K V H I H T T D W D R E S D V L V L T

V P L E K F L D Y S D A D D D E R E Y F S K I I H Q Q Y M V D A **C** L V K E Y P T I S G

Y V P D N M R P E R L G H V M V Y Y H R W A D D P H Q I I T T Y L L R N H P D Y A D

K T Q E E **C** R Q M V L D D M E T F G H P V E K I I E E Q T W Y Y F P H V S S E D Y K

A G W Y E K V E G M Q G R R N T F Y A G E I M S F G N F D E V **C** H Y S K D L V T R F

F V

Fig. 3.6. *P. acnes* LAI amino acid sequence. The underlines show the positions of cysteine.

M F A K R F K T S L L P L F A G F L L L F Y L V L A G P A A A S A↓E T A N K S N E

Fig. 3.7. prepropeptide *B. subtilis amyE* amino acid sequence. The arrow shows the cutting site by Type I signal peptidase between prepeptide (signal peptide) and propeptide.

CHAPTER FOUR

CONTRIBUTION OF THE PRE- AND PRO-REGIONS OF *Staphylococcus hyicus* LIPASE, *Bacillus subtilis* NEUTRAL PROTEASE, AND *Bacillus megaterium* THE MAJOR EXTRACELLULAR PROTEASE TO SECRETION OF HETEROLOGOUS PROTEINS BY *Bacillus megaterium* AND *Bacillus licheniformis*

ABSTRACT

The secretion of heterologous proteins by using *Bacillus* species is a well-established system. However, inefficient translocation across the plasma membrane of heterologous proteins is a major problem when *Bacillus* species are employed as the expression hosts. A recent study showed that prepropeptide *Staphylococcus hyicus* lipase enhanced the secretion of heterologous proteins in *B. subtilis*. In this study, prepropeptides of *S. hyicus* lipase, *B. subtilis nprE*, and *B. megaterium nprM* were investigated by using hydrolase from *T. fusca* (Tfh) and *E. coli* alkaline phosphatase PhoA as models. The results show that the secreted Tfh and PhoA activities were lower when the proteins fused with propeptides, compared to those without propeptides. Only propeptide *S. hyicus* lipase protected *Propionibacterium acnes* linoleic acid isomerase from proteolytic degradation and did not impede the translocation. However, no activity of isomerase was detectable. MALDI-TOF MS of linoleic acid isomerase showed that the propeptide was still attached with secreted protein. In addition, the results of the propeptide *S. hyicus* lipase fused with *B. subtilis nprE* demonstrated that enzymatic activities were interfered with by the attached propeptide *S. hyicus* lipase.

KEY WORDS: *Bacillus subtilis, Bacillus megaterium, Bacillus licheniformis,* heterologous protein, prepropeptide, *Staphylococcus hyicus*

INTRODUCTION

Heterologous production in bacterial systems, particularly *E. coli*, provide an economical route for protein production due to their ability to grow rapidly and yield high cell concentrations on inexpensive substrates (Terpe, 2006). However, inclusion body formation, incorrect protein folding, and inefficient bond formation might occur by intracellular production in *E. coli* (Schallmey et al., 2004). To avoid these problems, *Bacillus* species as the hosts are an alternative way to secrete heterologous proteins into surrounding medium (Pohl and Harwood, 2010).

By using *Bacillus* species as the hosts, inefficient translocation across the plasma membrane could be one of the bottlenecks in the secretion of heterologous proteins (Li et al., 2004). A recent study showed that prepropeptide *Staphylococcus hyicus* lipase enhanced the secretion of heterologous proteins in *B. subtilis* (Kouwen et al., 2010). Compared to *B. subtilis*, *B. megaterium* strains have the advantage of highly stable, freely replicating plasmids (Vary et al., 1994). In addition, a recent study developed expression plasmids for maximizing heterologous protein production (Stammen et al., 2010).

Here, we investigate the suitability of several prepeptides and prepropeptides for the heterologous production and secretion by *B. megaterium* YYBm1 and *B. licheniformis* NRRL B-14212. Prepropeptides from *S. hyicus* lipase, *B. subtilis nprE*, and *B. megeterium nprM* were experimentally evaluated for promoting heterologous secretion by using hydrolase from *T. fusca* and *E. coli* alkaline phosphatase PhoA as models. The prepropeptide from *S. hyicus* lipase was chosen because it had been previously demonstrated to be successful for enhancing *E. coli* alkaline phosphatase PhoA secretion in *B. subtilis* (Kouwen et al., 2010) while *B. subtilis nprE*, and *B. megeterium nprM* are the major extracellular proteases in *B. subtilis*, and *B. megeterium*, respectively.

MATERIALS AND METHODS

DNA manipulation for the construction of plasmids.

The synthetic oligonucleotides used in this work are listed in Table 4.S1 in the supplemental material. The plasmids constructed or employed are listed in Table 4.1. *E. coli* 10G (Lucigen, Middleton, WI) was used for all cloning purposes. *B. megaterium* YYBm1 (Stammen et al., 2010), strain inactivated *xylA* gene for xylose metabolism and the major extracellular protease gene *nprM* was deleted, and *B. licheniformis* NRRL B-14212 were used as protein production hosts. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; and tetracycline 10 μg/ml.

The basic expression plasmid of this study was pSSBm97 (Stammen et al., 2010). The oligonucleotides lipF, lipR, and lipR2 were used as primers for the PCR to amplifiy pre- and prepropeptide *S. hyicus lipA* lipase by using oligonucleotide PL1 to Pu12 as the template. For pre- and prepropeptide *B. subtilis nprE* neutral protease amplification, the oligonucleotide nprEF, nprER, nprER2 were used as primers and *B. subtilis* genomic DNA was used as the template. The oligonucleotides nprMF, nprMR, nprMR2 were used as primers for the PCR to amplifiy pre- and prepropeptide *B. megaterium nprM* gene by using *B. megaterium* DSM319 as the template. Insertion of all PCR products via the corresponding BsrGI and SpeI restriction sites led to pPL, pPPL, pPE, pPPE, pPM, and pPPM, respectively.

PhoA gene was amplified from *E. coli* K12 by using primers PhoF and PhoR. PCR product was cloned into pCR®4-TOPO and later into pSSBm97, pPL, pPPL, pPE, pPPE, pPM, and pPPM after SpeI-EagI digestion, resulting in construction of pA97, pAPL, pAPPL, pAPE, pAPPE, pAPM, pAPPM, respectively.

For *P. acnes* linoleate isomerase, the new DNA sequence was designed by JCat software (http://www.jcat.de/) (Grote et al., 2005) and was synthesized by Integrated DNA Technologies, Coralville, IA. The linoleate isomerase fragment was flanked by SpeI-EagI restriction sites, was digested with these enzymes, and was subsequently inserted into pSSBm97, pPL, pPPL, pPE, pPPE, pPM, and pPPM after SpeI-EagI digestion, creating the plasmids pL97, pLPL, pLPPL, pLPE, pLPPE, pLPM, pLPPM, respectively.

The oligonucleotides enprEF and enprER were used as *nprE* gene amplification by using *B. subtilis* genome as the template and PCR product was cloned into pCR®4-TOPO. Insertion of the nprE PCR products via the corresponding SpeI and EagI restriction sites in plasmids pPL and pPPL led to pEPL and pEPPL, respectively. Protoplast *B. megaterium* YYBm1 cells were transformed with the appropriate expression plasmids using a polyethylene glycol-mediated procedure described by Christie et al. (2008) while plasmids were transferred into *B. licheniformis* NRRLB-14212 by electroporation as described in Xue et al. (1999).

Protein production in *Bacillus megaterium* and *B. licheniformis*.

All *B. megaterium* and *B. licheniformis* plasmid strains were grown in baffled shake flasks at 30 or 37 ^{o}C in Luria-Bertani (LB) medium, TM medium (Takara Bio manual), 2SY medium (Takara Bio manual), MPB medium (Chiang et al., 2010), or MSR medium (Chiang et al., 2010) at 200 rpm. Recombinant expression of genes under transcriptional control of the xylose-inducible promoter was induced by the addition of 0.5% (w/v) xylose at OD578 of 0.4.

Localization of heterologous protein expression

The subcellular localization of heterologous protein production was determined using the protocol described in Kouwen et al. (2010). After separation by SDS-PAGE, proteins were

transferred to a nitrocellulose membrane and detected with 6X his tag antibody (Abcam, Cambridge, MA) and horseradish peroxidase–anti-rabbit immunoglobulin G conjugates.

Proteomics

Cells of *B. megaterium* were grown at 30 °C at 200 rpm. Cells were separated from the growth medium by centrifugation. The secreted proteins in the growth medium were collected for SDS-PAGE, gels were stained with Coomassie brilliant blue, and the respective protein bands were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS).

Enzymatic activity measurements.

The hydrolase activity of the enzyme Tfh was measured as described by Stammen et al. (2010). The enzymatic release of *p*-nitrophenol was photometrically detected at 410 nm. One enzyme unit was defined as the amount that caused the release of 1 μmol *p*-nitophenol per minute under the given assay condition. The enzymatic activity was calculated using a molar absorption coefficient of 15,000 M^{-1} cm^{-1}.

Alkaline phosphatase (PhoA) activity was carried out as described by Darmon et al. (2006). PhoA activities, expressed in U/ml/unit of optical density at OD600, were determined by measuring changes in OD405 nm as a function of the time of incubation (in minutes) and the OD600. To do this, the following formula was used: [2/3 x (OD405 x 352)]/(*t* x OD600), where *t* is the time of incubation. Cells were separated from the growth medium by centrifugation.

Protease activity was measured as described in Mansfeld and Ulbrich-Hofmann (2007). One unit of proteolytic acitivity was defined as the amount of enzyme yielding an increase of 0.001 in the absence at 275 nm per min at 30 ^{o}C under standard reaction condition. All experiments were repeated at least three times. Linoleaste isomerase activity was carried out as

described by Peng et al. (2007). The preparation of fatty acid methyl esters (FAME) was described in Lewis et al. (2000) and FAME was detected by GC with tridecanoic acid as the internal standard.

Statistical analyses

Where standard deviations are presented in the respective figures, these values represent the average of at least triplicate measurements. The statistical tests for treatment effects were performed using an analysis of variance (ANOVA) procedure of Statistixs 9.1 Analytical Software (SAS Institute, Cary, NC). Means were further separated using least-significant difference multiple comparisons.

RESULTS

Fusion of Tfh and PhoA with prepropeptides from *S. hyicus* lipase, *B. subtilis nprE*, and *B. megaterium nprM*

Kouwen et al. (2010) demonstrated that the prepropeptide of *S. hyicus* lipase enhanced the secretion of PhoA in *B. subtilis*. The prepropeptides of *B. subtilis nprE* and *B. megaterium nprM* were chosen because both are major extracellular proteases in these organisms. In this study, all plasmids originated from pSSBm97 (Stammen et al., 2010). Plasmid pSSBm97 contains an optimal the -35 region and the ribosome-binding site, enhancing heterologous production in *B. megaterium* YYBm1 (Stammen et al., 2010).

The secreted amounts of active Tfh were assessed by measuring hydrolase activity in growth medium of cells containing the seven different prepropeptides (Fig. 4.1). Analysis of the medium fractions showed that the highest Tfh activity was detected in growth medium of cells carrying *yocH* as the prepeptide (pSSBm97), while the levels of Tfh activity were lower ($P < 0.05$) in the medium of cells expressing other prepropeptides. Surprisingly, the levels of active

Tfh in the medium of cells expressing prepropeptide *S. hyicus* lipase and *B. subtilis nprE* were significantly lower than those cells containing only prepeptides.

For secreted PhoA levels in *B. megaterium*, seven prepropeptides were fused with PhoA at the N-terminus. Analysis of the medium fractions showed that the levels of PhoA of cells expressing prepropeptides were lower than cells of expressing only their pair prepeptides (Fig. 4.2); however, the levels between their pair prepeptides and prepropeptides were not significantly different. In this case, the lowest level of active PhoA in the medium fraction was cells carrying *yocH* as prepeptide (pA97).

Linoleate isomerase production in *B. megaterium* and *B. licheniformis*

Linoleate isomerase in *P. acnes* is the enzyme that isomerizes the double bond at the C9 position in linoleic acid (*c*9,*c*12, 18:2) to form *t*10,*c*12 conjugated linoleic acid (Deng et al., 2007). The conjugated linoleic acids have been demonstrated to exhibit anti-cancer, anti-diabetic, and immune-enhancing properties (Pariza, 2004). This gene was expressed in *E. coli* BL21 (DE3) as the host; unfortunately, the recombinant enzyme formed an inclusion body (Deng et al., 2007). Secreted protein production might solve the formation of insoluble protein inclusion bodies from intracellular accumulations (Schallmey et al., 2004).

The major goal of this investigation was the extracellular linoleate isomerase production by *B. megaterium* and *B. licheniformis*. By using the 6X His tag antibody detection, only cells expressing prepropeptide *S. hyicus* lipase produced linoleate isomerase (Fig. 4.3). To analyze whether the lack of *P. acnes* linoleate isomerase production after fusions with prepropeptides, except with prepropeptide *S. hyicus* lipase, was due to a failure in secretion or to instability of the protein, the total cell samples were probed with the 6X his tag-specific antibody (Fig. 4.4). Only a distinct band from *B. megaterium* carrying pLPPL was observed, suggesting that the protein

was reasonably stable. No corresponding protein occurred in the other cells with prepropeptides, indicating that these proteins were less stable.

To observe the efficient translocation across protoplast membrane, the cells expressing prepropeptide *S. hyicus* lipase fused with linoleate isomerase were divided into medium, total cell, cell wall, protoplast, protoplast incubated with trypsin, and protoplast incubated with trypsin and Triton X-100 fractions. The proteins from medium, total cell, cell wall, and protoplast fractions were all the same size (Fig. 4.5), representing no processing of translocated linoleate isomerase after crossing the membrane. The protein in proplasts was degraded when adding trypsin, suggesting that the protein was effectively translocated across the protoplast membrane.

In order to maximize linoleate isomerase production, the cells carrying pLPPL were cultured under various conditions. The results from the 6X His tag antibody detection showed that MPB enhanced more linoleate isomerase production than other media (Fig. 4.6). In addition, a different set of forms of linoleate isomerase larger than the major band could represent an aggregated form of linoleate isomerase or linoleate isomerase bound to other proteins. In addition to the major protein from cells culturing in MSR medium, minor bands of lower mass reacted with the antibody, suggesting that they represented shortened species of the fusion protein. By culturing at 37 ^{o}C in MPB medium, the supernatant of cells expressing prepropeptide *S. hyicus* lipase exhibited smear bands, indicating proteolytic degradation, compared to supernant from cells cultured at 30 ^{o}C (Fig. 4.7). The 6X His tag antibody detection showed that the linoleate isomerase was initially detectable 4 hours and increased production up to 8 hours after xylose addition (Fig. 4.8). After 8 hours induction, the amounts of protein were reduced due to possible proteolytic degradation.

To observe linoleate isomerase activity, *B. megaterium* YYBm1 carrying pLPPL was cultured in MPB medium and the supernatant was collected at 8 hours after xylose addition. The supernatant was incubated with linoleate as described in Peng et al. (2007). FAME was prepared as described in Lewis et al. (2000); however, no activity was detected.

Since no activity was found when *B. megaterium* YYBm1 was used as the host, *B. licheniformis* and *B. subtilis* were chosen due to their abilities to secrete large quantities of extracellular enzymes (Schallmey et al., 2004). The plasmids L97 to pLPPL were transferred into *B. licheniformis* NRRL B-14212 by electroporation; however, plasmid pLPPE transformation was unsuccessful. Unfortunately, no plasmid transformation was successful in *B. subtilis*. *B. licheniformis* carrying these plasmids were cultured in LB medium at 37 ^{o}C and the supernatant was collected at 6 hours after xylose supplement; unfortunately, no protein was detected by using the 6X His tag antibody.

Protease production in *B. megaterium*

Since prepropeptide *S. hyicus* lipase fusion impeded Tfh (Fig. 4.1) and PhoA activities (Fig. 4.2) but supported secreted linoleate isomerase production (Fig. 4.3), *B. subtilis nprE* gene was chosen for the further evaluation of this propeptide on secreted protein in *B. megaterium*. By measuring active protease in the medium fractions collected from *B. megaterium* expressing pre- and prepropeptide *S. hyicus* fused with *nprE* gene, the results showed that the secreted NprE did not require propeptide for translocation; however, its activity was inhibited by the propeptide fusion (Fig. 4.9). In addition, the results from the secreted NprE proteins detected by the 6X His tag antibody showed that cells expressing prepropeptide *S. hyicus* lipase produced a larger form than proteins from cells expressing prepeptide (data not shown).

Peptide analysis by MALDI-TOF MS

B. megaterium carrying pLPPL was cultured in MPB medium at 30 ^{o}C and the sample was collected at 8 hours after xylose induction. The major protein band in SDS-PAGE was excised and digested with trypsin. Subsequently, the peptide sample was analyzed by MALDI-TOF MS. From the peptide mass fingerprint obtained from the MALDI-TOF MS (Fig. 4.10), the intense peaks were selected and subjected to MS/MS ion search. The MS/MS data were analyzed both by running MASCOT (http://www.matrixscience.com) as well as manual analysis in order to identify the protein. MS/MS spectra were searched with GPS software using 95% confidence interval threshold ($P < 0.05$), with which a minimum Mascot score of > 61 was considered imperative for further analysis. From the peptide analysis, the results demonstrated that propeptide *S. hyicus* lipase was still attached with the linoleate isomerase (Fig. 4.11).

DISCUSSION

In this study, two enzymes, Thf and PhoA, were chosen to be model proteins to study the influence of prepropeptides on export efficiency in *B. megaterium*. Tfh has been shown to be successful for generating secreted protein in *B. megaterium* YYBm1 up to 7,200 U per liter (Stammen et al., 2010). PhoA from *E. coli* was the other model protein because it contains two disulfide bonds which is one of the limitations for heterologous production in *Bacillus* species (Kouwen and van Dijl, 2009).

From Tfh and PhoA activity measurement, the results showed that prepeptide *yocH* and prepeptide *nprE* were the best choices for secreted protein production. Currently, every secreted heterologous proteins requires prepeptide optimization (Brockmeier et al., 2006). In some cases, the efficiency of heterologous protein secretion with each prepeptide depends on each strain. By comparing between two strains of *B. lichniformis*, most prepeptides, except *SacC*, showed

related secretion efficiency (Degering et al., 2010). Surprisingly, the Tfh and PhoA activities in medium of three-pair propeptide addition were lower compared to the cells proteins expressing without propeptides.

Linoleate isomerase is the enzyme for modifying linoleic acid to conjugated linoleic acids which are anti-carcinogenic compounds (Deng et al., 2007). This enzyme formed inclusion bodies when expressing in *E. coli* BL21 (DE3) (Deng et al., 2007). The goal of this investigation was to secrete active linoleate isomerase in *B. megaterium*. Sturmfels et al. (2001) demonstrated propeptide lipase was necessary for human growth hormone translocation process at *S. carnosus* membrane and Kouwen et al. (2010) demonstrated that propeptide *S. hyicus* lipase significantly enhanced the secretion of PhoA by *B. subtilis*.

In general, heterologous proteins are vulnerable to wall-associated proteases during the slow protein-folding process (Braun et al., 1999). The results in this study showed that the propeptide protected linolated isomerase from proteolytic degradation in *B. megaterium* cells. Demleitner and Gotz (1994) reported that the second half of this propeptide maintained lipase stability in *S. carnosus* cells and proposed that the heterologous protein without propeptide was degraded in intracellular or at the membrane site during translocation. Kouwen and van Dijl (2009) proposed that propeptide might function like chaperone to form protein correctly, and therefore, enzyme was not degraded inside the cells. Meens et al. (1997) concluded that the propeptide facilitates the release of unfolded proteins from the translocase and/or passage through the cell wall, protecting them form proteases at the membrane-cell wall interface.

In this study, MPB medium appeared to maximize linoleate isomerase production, similar to Rluc production in *B. subtilis* (Kakeshita et al., 2011). When the host cells *Bacillus subtilis* grew on the MSR medium, production of recombinant protein Rluc was unsuccessful. A

detectable amount of Rluc was found when *B. subtilis* growing on MPB medium. It should be noted that the composition of the MSR and MPB media are similar except that glucose in the former is substituted with casamino acid in the latter. It suggests that the nitrogen source instead of carbon is flavorable for the production of Rluc by *B. subtilis*.

The results of linoleate isomerase activity, NprE activity, NprE detection by the 6X His tag antibody, peptide analysis of by MALDI-TOF MS showed that propeptide was still attached with the enzymes and might inhibit enzyme activities. In *S. hyicus*, the prepeptide is cleaved after the propeptide-enzyme complex is released into the culture medium (Sarvas etal., 2004). The propeptide is subsequently removed by a metalloprotease, and full enzymatic activity is achieved (Sarvas et al., 2004; Yabuta et al., 2001). Ayora et al. (1994) demonstrated that ShpII, a neutral metalloprotease in *S. hyicus*, is necessary for the propeptide removal. The propeptide-PhoA complex produced by *B. subtilis* 168 (*trpC2*) showed that the propeptide was removed when the complex was released into the medium (Kouwen et al., 2010); however, the propeptide-OmpA complex produced by *B. subtilis* DB104 (*his*, *nprR2*, *nprE18*, Δ*aprA3*) was unprocessed after releasing into the medium (Meens et al., 1997). NprE is an extracellular major metalloprotease in *B. subtilis* (He et al., 1991) and might remove the propeptide from the propeptide-PhoA complex. From *B. megaterium* DSM319 genome analysis, an extracellular metalloprotease InhA was reported (Eppinger et al., 2011); however, the propeptide removal was not found in this study.

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Table 4.1. Plasmids used in this study

Plasmid	Description	Source
pSSBm97	P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{yocH}$-*tfh*	Stammen et al., (2010)
pPlip	prepeptide of *S. hyicus lipA* lipase inserted into BsrGI and SpeI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-*tfh*	This study
pPPlip	prepropeptide of *S. hyicus lipA* lipase inserted into BsrGI and SpeI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-propeptide$_{lipA}$-*tfh*	This study
pPnprE	prepeptide of *B. subtilis nprE* neutral protease inserted into BsrGI and SpeI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-*tfh*	This study
pPPnprE	prepropeptide of *B. subtilis nprE* neutral protease inserted into BsrGI and SpeI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-propeptide$_{nprE}$-*tfh*	This study
pPnprM	prepeptide of *B. megaterium nprM* neutral protease inserted into BsrGI and SpeI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprM}$-*tfh*	This study
pPPnprM	prepropeptide of *B. megaterium nprM* neutral protease inserted into BsrGI and SpeI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-propeptide$_{nprM}$-*tfh*	This study
pCR®4-TOPO	Plasmid used for cloning PCR product	Invitrogen
pA97	*E. coli phoA* alkaline phosphatase inserted into SpeI and EagI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{yocH}$-*PhoA*	This study
pAPL	*E. coli phoA* alkaline phosphatase inserted into SpeI and EagI sites of pPlip; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-*PhoA*	This study
pAPPL	*E. coli phoA* alkaline phosphatase inserted into SpeI and EagI sites of pPPlip; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-propeptide$_{lipA}$-*PhoA*	This study
pAPE	*E. coli phoA* alkaline phosphatase inserted into SpeI and EagI sites of pPnprE; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-*PhoA*	This study
pAPPE	*E. coli phoA* alkaline phosphatase inserted into SpeI and EagI sites of pPPnprE; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-propeptide$_{nprE}$-*PhoA*	This study

Table 4.1. (continued)

Plasmid	Description	Source
pAPM	*E. coli phoA* alkaline phosphatase inserted into SpeI and EagI sites of pPnprM; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprM}$-*PhoA*	This study
pAPPM	*E. coli phoA* alkaline phosphatase inserted into SpeI and EagI sites of pPPnprM; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-propeptide$_{nprM}$-*PhoA*	This study
pL97	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pSSBm97; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{yocH}$-*pal*	This study
pLPL	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pPlip; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-*pal*	This study
pLPPL	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pPPlip; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-propeptide$_{lipA}$-*pal*	This study
pLPE	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pPnprE; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-*pal*	This study
pLPPE	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pPPnprE; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-propeptide$_{nprE}$-*pal*	This study
pLPM	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pPnprM; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprM}$-*pal*	This study
pLPPM	*P. acnes* linoleate isomerase inserted into SpeI and EagI sites of pPPnprM; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{nprE}$-propeptide$_{nprM}$-*pal*	This study
pEPL	*B. subtilis nprE* inserted into SpeI and EagI sites of pPlip; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-*nprE*	This study
pEPPL	*B. subtilis nprE* inserted into SpeI and EagI sites of pPPlip; P_{xylA}-(-35^{+} rbs^{+})-prepeptide$_{lipA}$-propeptide$_{lipA}$-*nprE*	This study

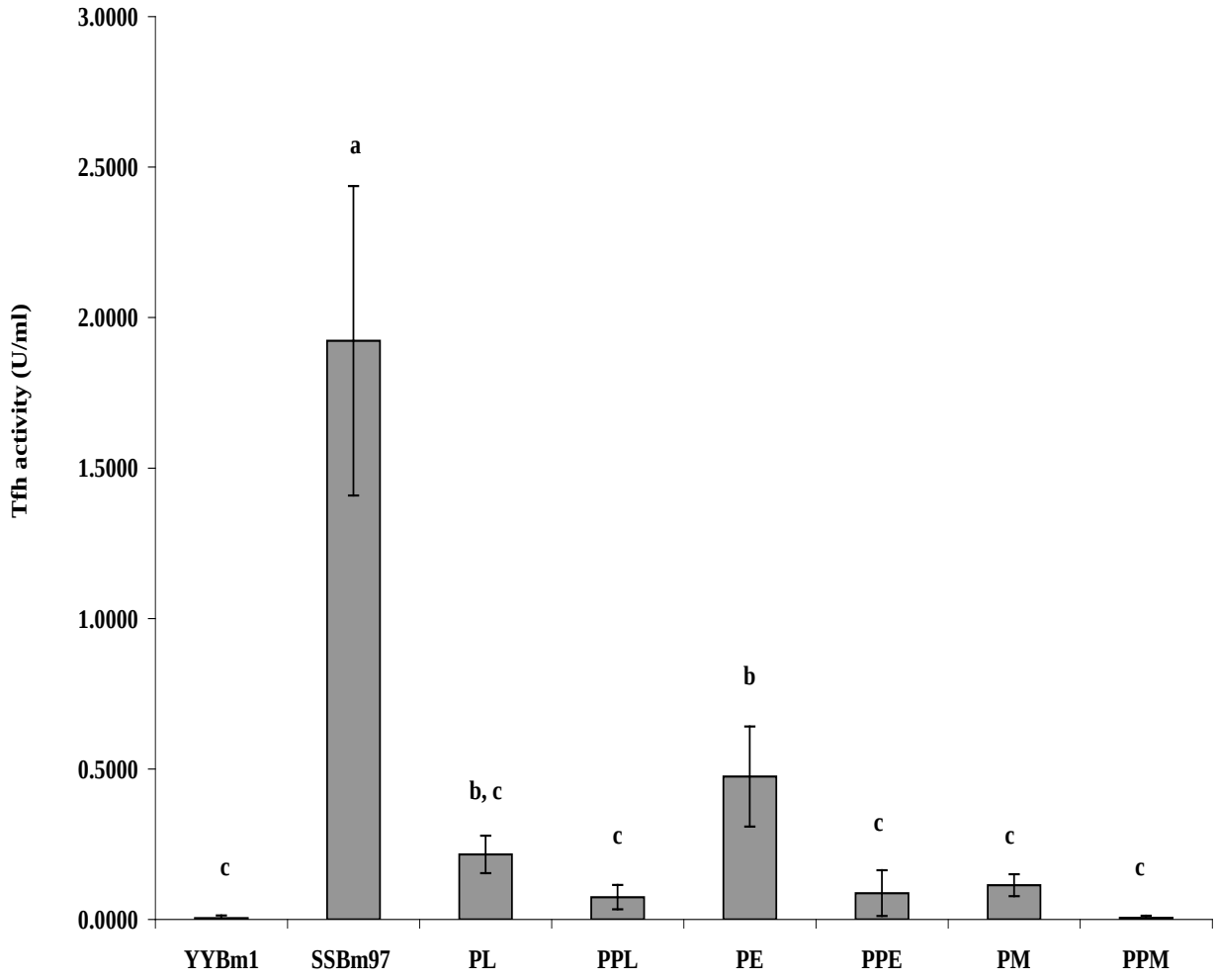


Fig. 4.1. Secretion of active Tfh. Strain YYBm1 without plasmid was used as a negative control. Samples were taken 6 hours after the xylose supplement. The activities are expressed in U/ml. ^{a,b,c} Means within the same sample measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between enzyme activities detected for each construct.

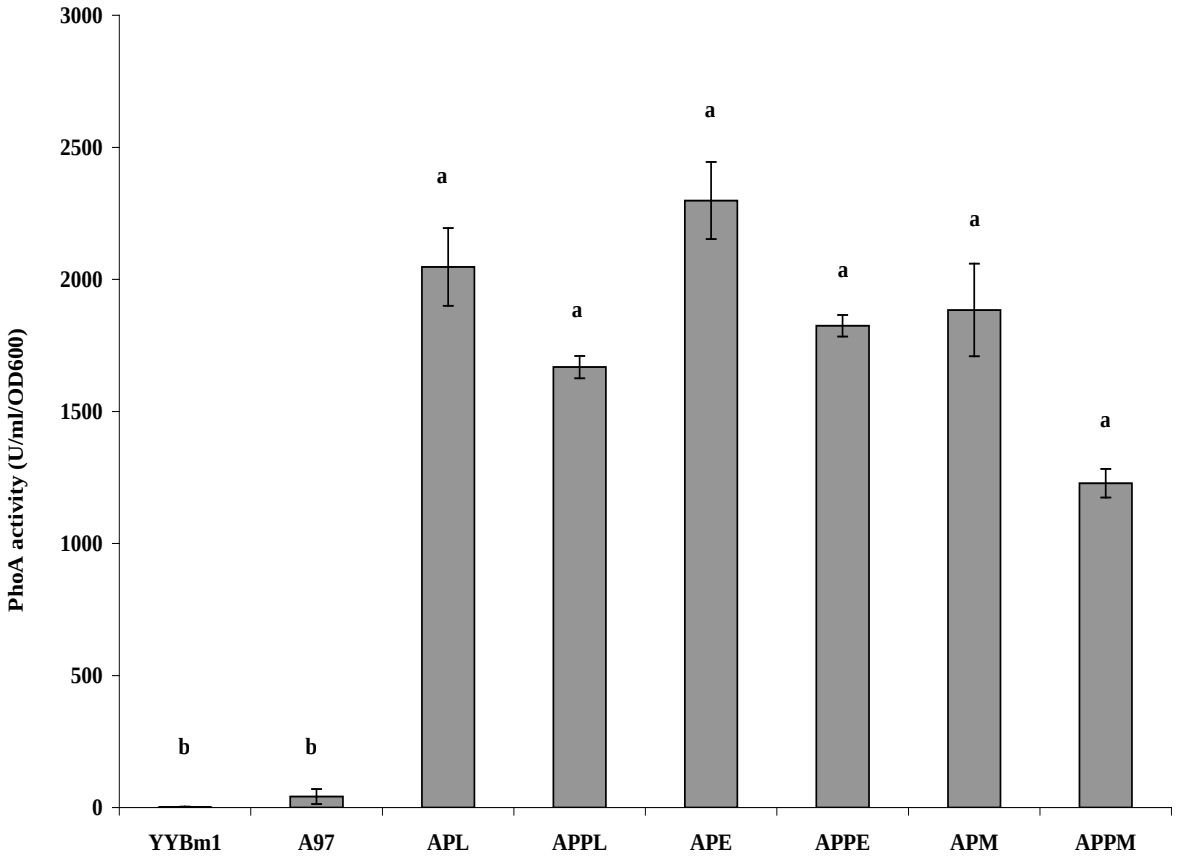


Fig. 4.2. Secretion of active PhoA. Strain YYBm1 without plasmid was used as a negative control. Samples were taken 6 hours after the xylose supplement. The activities are expressed in U/ml. [a,b] Means within the same sample measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between enzyme activities detected for each construct.

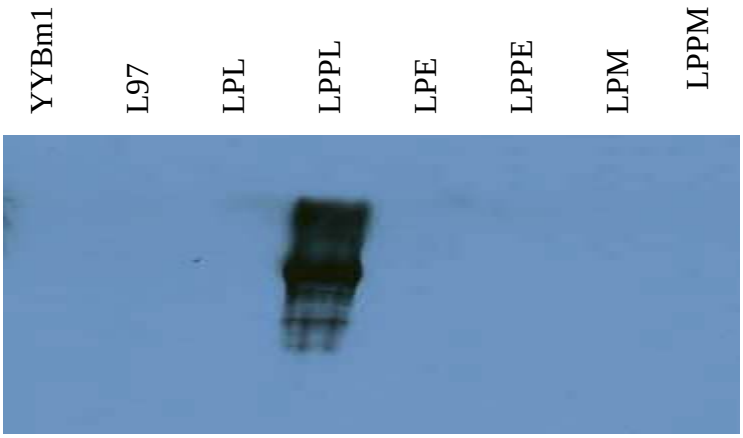


Fig. 4.3. Secretion of linoleate isomerase in *B. megaterium*. Cells carrying plasmids were cultured in LB at 37 ^{o}C and were collected at 6 hours after xylose induction. Proteins from 3 ml of cell-free supernatants from *B. megaterium* cultures carrying plasmids were precipitated with trichloroacetic acid. Samples were used for SDS-PAGE and Western blotting. The 6X His tag antibody was used to detect the proteins.

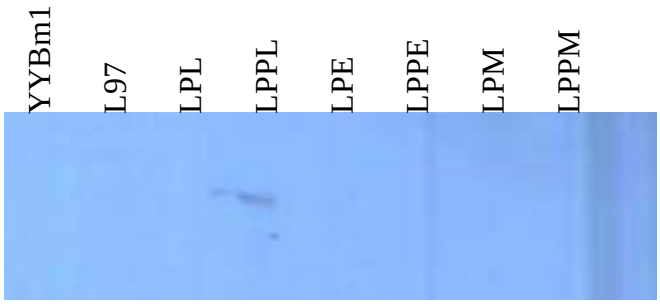


Fig. 4.4. Total cell samples of linoleate isomerase in *B. megaterium*. Cells carrying plasmids were cultured in LB at 37 ^{o}C and were collected at 6 hours after xylose induction. Proteins from 50 μl of cells were collected by centrifugation. Samples were used for SDS-PAGE and Western blotting. The 6X His tag antibody was used to detect the proteins.

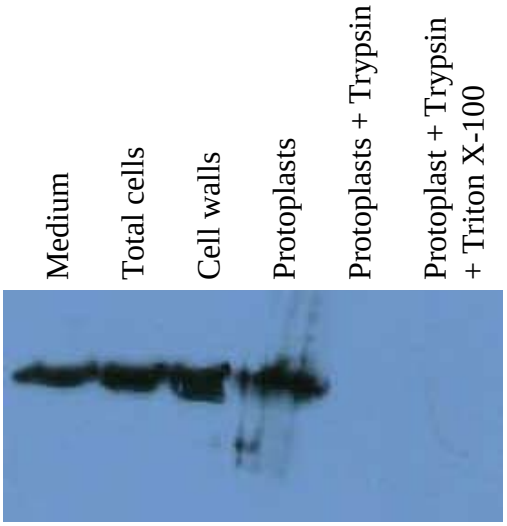


Fig. 4.5. Secretion and subcellular localization of linoleate isomerase in *B. megaterium*. Cells carrying pLPPL were cultured in LB at 37 ^{o}C and were collected at 6 hours after xylose induction. The sample was divided into medium, total cells, cell walls, protoplasts. Protoplasts were incubated for 30 min in the presence of 1 mg/ml of trypsin with or without 1% Triton X-100. Samples were used for SDS-PAGE and Western blotting, and 6X His tag antibody was used to detect the proteins.



Fig. 4.6. Secretion of linoleate isomerase in *B. megaterium*. Cells carrying pLPPL were cultured in LB, 2SY, TM, MPB, and MSR media at 37 ^{o}C and were collected at 6 hours after xylose induction. Proteins from 6 ml of cell-free supernatants from *B. megaterium* cultures carrying plasmids were precipitated with trichloroacetic acid. Samples were used for SDS-PAGE and Western blotting. The 6X His tag antibody was used to detect the proteins.

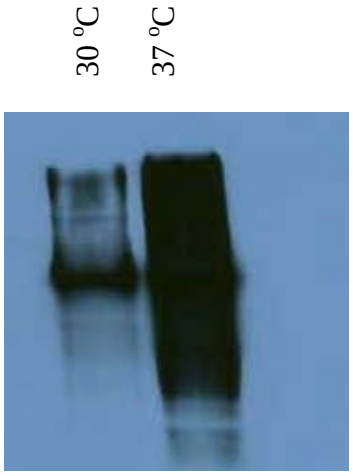


Fig. 4.7. Secretion of linoleate isomerase in *B. megaterium*. Cells carrying pLPPL were cultured in MPB medium at 30 and 37 °C and were collected at 6 hours after xylose induction. Proteins from 4.5 ml of cell-free supernatants from *B. megaterium* cultures carrying plasmids were precipitated with trichloroacetic acid. Samples were used for SDS-PAGE and Western blotting. The 6X His tag antibody was used to detect the proteins.

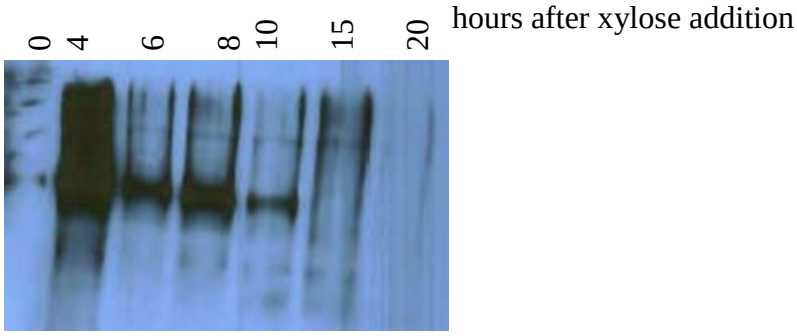


Fig. 4.8. Secretion of linoleate isomerase in *B. megaterium*. Cells carrying pLPPL were cultured in MPB medium at 30 ^{o}C and were collected at 0, 4, 6, 8, 10, 15, and 20 hours after xylose induction. Proteins from 4.5 ml of cell-free supernatants from *B. megaterium* cultures carrying plasmids were precipitated with trichloroacetic acid. Samples were used for SDS-PAGE and Western blotting. The 6X His tag antibody was used to detect the proteins.

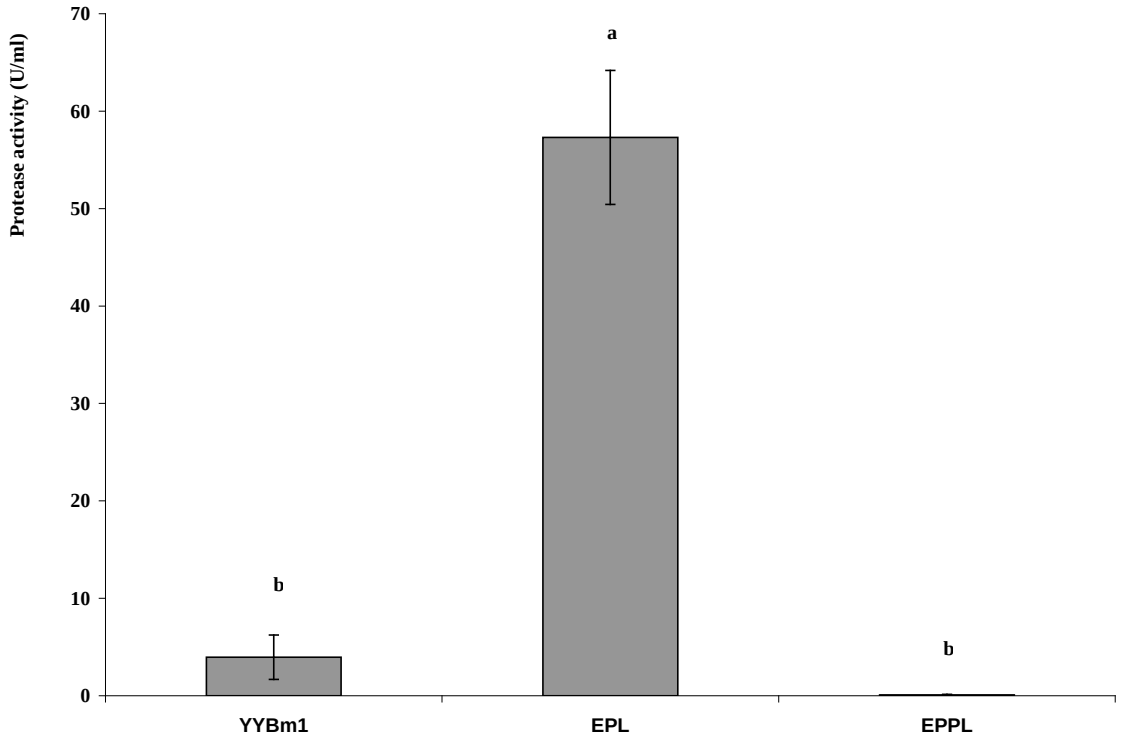


Fig. 4.9. Secretion of active protease. Strain YYBm1 without plasmid was used as a negative control. Samples were taken 6 hours after the xylose supplement. The activities are expressed in U/ml. [a,b] Means within the same sample measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between enzyme activities detected for each construct.

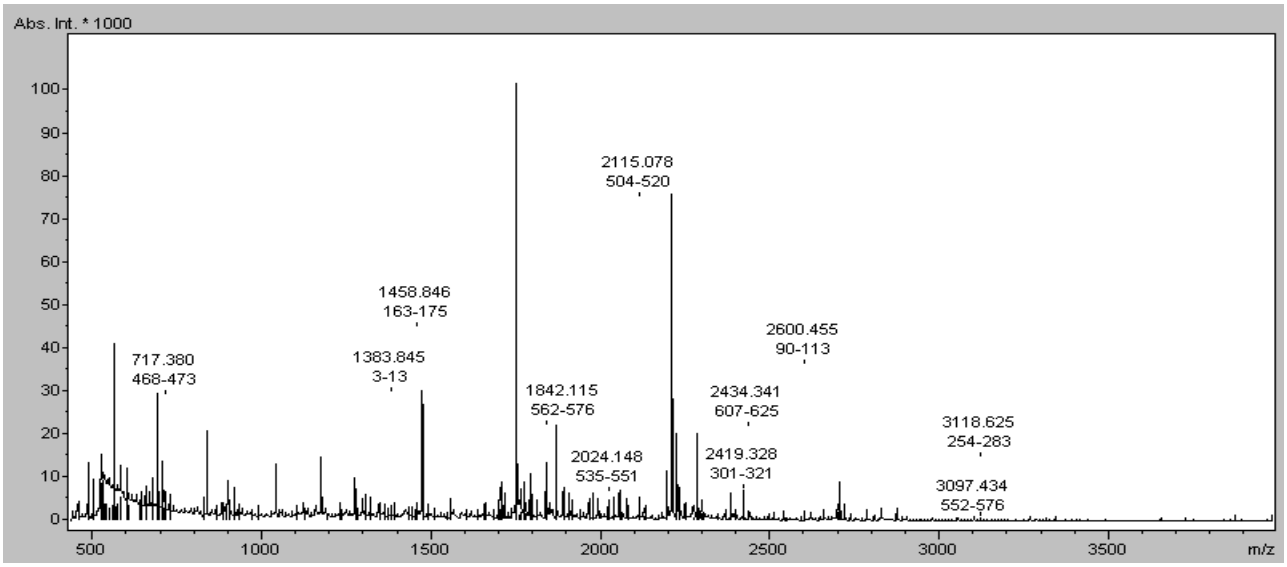


Fig. 4.10. A typical peptide mass fingerprint of trypsin digested proteins from *B. megaterium* carrying pLPPL.

Prepeptide sequence
M K E T K H Q H T F S I R K S A Y G A A S V M V A S C I F V I G G G V A E A

Propeptide sequence
N D S T T Q T T T P L E V A Q T S Q Q E T H T H Q T P V T S L H T A T P E H V D D S
K E A T P L P E K A E S P K T E V T V Q P S S H T Q E V P A L H K K T Q Q Q P A Y K
D K T V P E S T I A S K S V E S N K A T E N E M S P V E H H A S N V E K R E D R L E
T N E T T P P S V D R E F S H K I I N N T H V N P K T D G Q T N V N V D T K T I D T V
S P K D D R I D T A Q P K Q V D V P K E N T T A Q N K F T S Q A S D K K P T

Linoleate isomerase sequence
M S I S K D S R I A I I G A G P A G L A A G M Y L E Q A G F H D Y T I L E R T D H V G
G K C H S P N Y H G R R Y E M G A I M G V P S Y D T I Q E I M D R T G D K V D G P
K L R R E F L H E D G E I Y V P E K D P V R G P Q V M A A V Q K L G Q L L A T K Y
Q G Y D A N G H Y N K V H E D L M L P F D E F L A L N G C E A A R D L W I N P F T
A F G Y G H F D N V P A A Y V L K Y L D F V T M M S F A K G D L W T W A D G T Q
A M F E H L N A T L E H P A E R N V D I T R I T R E D G K V H I H T T D W D R E S D
V L V L T V P L E K F L D Y S D A D D D E R E Y F S K I I H Q Q Y M V D A C L V K E
Y P T I S G Y V P D N M R P E R L G H V M V Y Y H R W A D D P H Q I I T T Y L L R N
H P D Y A D K T Q E E C R Q M V L D D M E T F G H P V E K I I E E Q T W Y Y F P H
V S S E D Y K A G W Y E K V E G M Q G R R N T F Y A G E I M S F G N F D E V C H Y
S K D L V T R F F V

Fig.4.11. Amino acid sequences of prepropeptide *S. hyicus* lipase and *P. cenes* linoleate isomerase in plasmid LPPL. The underlines show the amino acid sequences obtained from MALDI-TOF MS results.

SUPPLEMENTARY MATERIAL

Table S1. Oligonucleotides used in this study

Primer	Sequence 5’ to 3’
PL1	TTC ACG TTT TTC CAC ATT TGA AGC ATG ATG TTC AAC AGG TGA CAT CTC ATT TTC TGT TGC
PL2	TTT ATT TGA TTC AAC CGA CTT TGA TGC TAT CGT TGA CTC TGG TAC CGT TTT ATC CTT ATA
PL3	CGC CGG TTG TTG CTG TGT TTT TTT ATG TAA CGC AGG TAC TTC CTG TGT ATG CGA TGA AGG
PL4	TTG AAC TGT CAC TTC GGT TTT TGG TGA CTC TGC TTT TTC AGG TAA AGG TGT TGC TTC TTT
PL5	AGA GTC ATC AAC ATG TTC AGG TGT TGC AGT ATG TAA TGA TGT AAC AGG TGT TTG ATG TGT
PL6	ATG TGT TTC TTG CTG CGA CGT TTG AGC GAC TTC TAG TGG TGT CGT TGT TTG TGT TGT CGA
PL7	ATC ATT TGC CTC TGC CAC GCC CCC ACC GAT GAC AAA TAT ACA TGA TGC GAC CAT AAC CGA
PL8	CGC GGC ACC ATA AGC CGA CTT ACG GAT AGA AAA TGT GTG
PL9	TTG ATG TTT TGT TTC TTT CAT GAC CTT GTG TTC TCC TCC TCT
PL10	TGT TGG TTT TTT GTC GCT CGC TTG TGA TGT AAA TTT ATT TTG TGC CGT TGT ATT TTC TTT
PL11	AGG AAC GTC GAC TTG TTT CGG TTG CGC CGT ATC TAT TCT GTC ATC TTT CGG TGA AAC GGT
PL12	GTC TAT CGT TTT CGT ATC AAC ATT AAC GTT TGT TTG TCC ATC CGT TTT TGG ATT TAC GTG
PL13	CGT ATT ATT GAT GAT TTT ATG GCT AAA TTC ACG GTC CAC TGA TGG
PL14	CGG TGT TGT CTC ATT AGT CTC CAA TCT ATC TTC ACG TTT TTC CAC
PU1	AGA GGA GGA GAA CAC AAG GTC ATG AAA GAA ACA AAA CAT CAA CAC ACA TTT TCT ATC CGT
PU2	AAG TCG GCT TAT GGT GCC GCG TCG GTT ATG GTC GCA TCA TGT ATA TTT GTC ATC GGT GGG
PU3	GGC GTG GCA GAG GCA AAT GAT TCG ACA ACA CAA ACA ACG ACA CCA CTA GAA GTC GCT CAA
PU4	ACG TCG CAG CAA GAA ACA CAT ACA CAT CAA ACA CCT GTT ACA TCA TTA CAT ACT GCA ACA
PU5	CCT GAA CAT GTT GAT GAC TCT AAA GAA GCA ACA CCT TTA CCT GAA AAA GCA GAG TCA CCA
PU6	AAA ACC GAA GTG ACA GTT CAA CCT TCA TCG CAT ACA CAG GAA GTA CCT GCG TTA CAT AAA
PU7	AAA ACA CAG CAA CAA CCG GCG TAT AAG GAT AAA ACG GTA CCA GAG TCA ACG ATA GCA TCA
PU8	AAG TCG GTT GAA TCA AAT AAA GCA ACA GAA AAT GAG ATG
PU9	TCA CCT GTT GAA CAT CAT GCT TCA AAT GTG GAA AAA CGT GAA

Table S1. (continued)

Primer	Sequence 5’ to 3’
PU10	GAT AGA TTG GAG ACT AAT GAG ACA ACA CCG CCA TCA GTG GAC CGT GAA TTT AGC CAT AAA
PU11	ATC ATC AAT AAT ACG CAC GTA AAT CCA AAA ACG GAT GGA CAA ACA AAC GTT AAT GTT GAT
PU12	ACG AAA ACG ATA GAC ACC GTT TCA CCG AAA GAT GAC AGA ATA GAT ACG GCG CAA CCG AAA
lipF	TAT A**TG TAC A**AT GAA AGA AAC AAA ACA TCA ACA CAC AT
lipR	TAT AAG ATC T**AC TAG T**AT TTG CCT CTG CCA CGC C
lipR2	TAT AAG ATC T**AC TAG T**TG TTG GTT TTT TGT CGC TCG
nprEF	TAT A**TG TAC A**AT GGG TTT AGG TAA GAA ATT GTC TG
nprER	TAT AAG ATC T**AC TAG T**AG CAG CCT GAA CAC CT
nprER2	TAT AAG ATC T**AC TAG T**AT GTT CTA CTT TAT TTT GCT GTT TTA AAA
nprMF	TAT A**TG TAC A** ATGAAAAAGAAAAAACAGGCTTTAAAGG
nprMR	TAT AAG ATC T**AC TAG T**ATG TGC AAA AGC AAA TGA TGA AG
nprMR2	TAT AAG ATC T**AC TAG T** AGG TTT CGC TGC CGG C
phoF	TAT AAG ATC T**AC TAG T**CG GGC TGC TCA GGG CGA TAT
phoR	TAT A**CG GCC G**TT AGT GAT GGT GAT GGT GGT GTT TCA GCC CCA GAG CGG C
enprEF	TAT AAG ACT T **ACT AGT** GCC GCC GCC ACT GGA AGC
enprER	TAT A **CGG CCG** TTA GTG ATG GTG ATG GTG GTG CAA TCC AAC AGC ATT CCA GGC

Restriction sites are highlighted in bold letters.

Amino acid sequences of pre- and propeptides were investigated in this study

***S. hyicus lipA* prepeptide**

M K E T K H Q H T F S I R K S A Y G A A S V M V A S C I F V I G G G V A E A ↓N

***S. hyicus lipA* prepropeptide**

M K E T K H Q H T F S I R K S A Y G A A S V M V A S C I F V I G G G V A E A
N D S T T Q T T T P L E V A Q T S Q Q E T H T H Q T P V T S L H T A T P E H V D D S K
E A T P L P E K A E S P K T E V T V Q P S S H T Q E V P A L H K K T Q Q Q P A Y K D K
T V P E S T I A S K S V E S N K A T E N E M S P V E H H A S N V E K R E D R L E T N E
T T P P S V D R E F S H K I I N N T H V N P K T D G Q T N V N V D T K T I D T V S P K D
D R I D T A Q P K Q V D V P K E N T T A Q N K F T S Q A S D K K P T

***B. subtilis nprE* prepeptide**

MGLGKKLSVAVAASFMSLSISLPGVQA↓A

***B. subtilis nprE* prepropeptide**

MGLGKKLSVAVAASFMSLSISLPGVQA
AEGHQLKENQTNFLSKNAIAQSELSAPNDKAVKQFLKKNSNIF
KGDPSKRLKLVESTTDALGYKHFRYAPVVNGVPIKDSQVIVHVDKSDNVYAVNGELHN
QSAAKTDNSQKV
SSEKALALAFKAIGKSPDAVSNGAAKNSNKAELKAIETKDGSYRLAYDVTIRYVEPEPA
NWEVLVDAETG
SILKQQNKVEH

***B. megaterium nprM* prepeptide**
MKKKKQALKVLLSVGILSSSFAFA↓H

***B. megaterium nprM* prepropeptide**
M K K K K Q A L K V L L S V G I L S S S F A F A
H T S S A A P N N V L S T E K Y N K E I K S P E F I S G K L S G P S S Q K A Q D V V F H
Y **M** N T N K D K Y K L G N E N A Q N S F K V T E V V K D P V E Q A T V V R L Q Q V
Y N N I P V W G S T Q L A H V A K D G T L K V V S G T V A P D L D K K E K L K G Q K
Q V D S K K A I Q A A E K D L G F K P T Y E K S P S S E L Y V Y Q N G S D T T Y A Y V
V N L N F L S P E P G N Y Y Y F V D A I S G K V L D K Y N T I D S V A G P K A D V K Q
A A K P A A K P

CHAPTER FIVE

ISOLATION OF HIGH CONTENT METHIONINE-CONTAINING BACTERIA AND CHARACTERIZATION OF METHIONINE PRODUCTION

ABSTRACT

Methionine is the first limiting amino acid in poultry feed. Currently methionine supplement is synthesized from a chemical process and it is anticipated that it will soon not be allowed for use by organic poultry industry Therefore, the objectives of this study were isolation of methionine producing bacteria from environmental samples and quantification of methionine production in these isolated bacteria. MCGC medium was selected as the isolation medium for methionine-producing bacteria by using *Corynebacterium glutamicum* ATCC13032 and *E. coli* ATCC23798 as the positive and negative controls, respectively. Thirty-nine bacterial strains were obtained from environmental samples. Only strains A121, A122, A151, and A181 were able to tolerate upto 0.1% (w/v) of ethionine or norleucine. These isolated strains were identified by sequencing small subunit rRNA genes. The results revealed that bacterial strains A121, A122, A151and A181 were *Klebsiella* species, *Acinetobacter baumannii*, *A. baumannii* and *Pseudomonas aeruginosa*, respectively. When methionine production in strains A121 and A181 was quantitated, under these conditions strains A121 and A181 excreted methionine up to 31.109 and 124.578 μg/ml, respectively.

KEY WORDS: bacteria, ethionine, methionine production, norleucine,

INTRODUCTION

Methionine is a nutritionally essential amino acid required in the diets of humans and livestock, including poultry. Approximately 90% of poultry feed is composed of corn and soybean both of which are insufficient for methionine requirement and it is therefore one of the first limiting amino acids in poultry feed (Baker, 2009). Methionine deficiency has been linked to development of various diseases and physiological conditions in animals. Currently, methionine supplements in animal feed are produced either by chemical synthesis or by hydrolyzing proteins. These processes are expensive and require hazardous chemicals (Fong et al., 1981). Synthetic methionine is currently allowed as an additive to organic poultry feed by the U.S. Department of Agriculture's National Organic Program (NOP) however NOP is only extending its use until October 1st 2012 (USDA, Agricultural Marketing Service, http://www.paorganic.org/wp-content/uploads/2011/10/USDA-Methionine.pdf). Therefore, there is an urgent need for organic sources of methionine for feed additives.

Many studies have attempted to isolate methionine producing bacteria from environments or generate genetically modified organisms by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Several studies have reported that methionine-analog resistance in bacterial strains correlated to higher methionine production due to an alteration in the regulation of L-methionine biosynthesis (Adelberg, 1958; Chattopadhyay et al., 1991; Yamada et al., 1982). Although the mutation was successful in producing methionine over-producing microorganisms, any genetically-modified organisms are considered unacceptable for use in organic food production (Electronic Code of Federal Regulations: U.S. National Organic Program). Therefore, wild type strains with methionine-producing ability are necessary for the organic poultry industry. The aims of this

study were to isolate methionine-producing bacteria and to quantify methionine production from these strains.

MATERIALS AND METHODS

Bacterial strains

Corynebacterium glutamicum ATCC13032 and *E. coli* ATCC23798 were obtained from American Type Culture Collection (ATCC, Manassas, VA). In this study, *C. glutamicum* ATCC13032 and *E. coli* ATCC23798, a methionine auxotroph, were used as positive and negative controls respectively, for selective medium of methionine production.

Selection of isolation medium for detection of methionine-containing bacteria

For selection of isolation medium, *C. glutamicum* was cultured for one day and *E. coli* was cultured overnight in Luria-Bertani (LB) broth at 37^{o}C in a waterbath shaker (NBS C76, New Brunswick Scientific Co., Inc., Edison, NJ). Ten ml of the cultures were centrifuged (10000 x g, 10 min, 4^{o}C), washed twice with 10 ml of 0.9% NaCl, and re-suspended in 10 ml of 0.9% NaCl. Both strains were re-streaked on LB agar, basal medium agar (Kase and Nakayama, 1974), and MCGC agar (von der Osten et al., 1989). The agar plates were incubated at 37^{o}C for at least one day. The medium which showed growth of *C. glutamicum* while inhibiting the growth of *E. coli* was used in further studies to isolate methionine producing bacteria.

Isolation of methionine-containing bacteria

The sample sources included nine chicken intestinal contents, six soil samples, one water sample from Scull Creek, Fayetteville, AR, one from fresh horse manure, one from compost, and one from bovine rumen fluid. The nine chicken intestinal contents were obtained from 5 – week-old broilers (conventional straight-run/off-sex) maintained on conventional corn/soy feed. Each sample, except the water and bovine rumen fluid samples, was mixed with 25-ml normal saline

solution and a 0.5-ml suspension was inoculated into 4.5-ml MCGC broth incubated under both aerobic and anaerobic incubation conditions. Water and bovine rumen fluid samples were added directly into 4.5-ml MCGC broth. Samples were incubated at 37^{o}C in a waterbath shaker or in an anaerobic jar with a commercial anaerobic culture system (AnaeroPack, Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan), respectively. For eight consecutive days, the 1/100 volume of inocula was transferred into fresh MCGC broth. The mixed cultures were 10-fold diluted or streaked onto MCGC agar plates. Different morphological colonies were picked from each sample and transferred into MCGC broth.

Methionine feedback effect assay

The bacterial strains which grew on MCGC agar plates were subsequently cultured on MCGC agar plates containing 0.1% (w/v) norleucine, a methionine analog. Strains that grew well in the presence of 0.1% (w/v) norleucine were retained for further evaluations. *C. glutamicum* ATCC13032 was used as the positive control. *C. glutamicum* ATCC13032 and eight isolated strains were cultured on MCGC broth supplied with either ethionine or norleucine at 0.05 and 0.1% (w/v) on a 96-well plate. The 96-well plates were incubated in a Tecan Infinite M200 (San Jose, CA) at 37 ^{o}C for 24 hours and the optical density was measured every hour at 600 nm.

Identification of potential methionine-containing microorganisms

The methionine over-producing bacterial strains which grew in the presence of 0.1% (w/v) norleucine were identified by sequencing the small subunit rRNA (SSU rRNA). The SSU rRNA sequences of methionine over-producing bacteria were amplified by using primers 27F and 1492R as described in Schuurman et al. (2004). The thermal cycling profile was as follows:

2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 50°C, 2 min at 72°C, followed by 10 min at 72°C.

The PCR products were sequenced by the DNA Resource Center, University of Arkansas, Fayetteville, AR. The resulting consensus sequence, created from the double-stranded part of the alignment, was used for comparison with sequences stored in GenBank, by using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990) and the similarity matrix tool from the Ribosomal Database Project II (RDP-II; http://rdp.cme.msu.edu/html/analyses.html) (Cole et al., 2003). For both BLAST and RDP-II analyses, the highest-scoring species was reported.

Measurement of growth

The potential methionine-overproducing strains were cultured in MCGC medium at 37°C, 200 rpm for 12 hours. A 1/100 volume of cells was transferred into 50-ml fresh medium in three-baffled 500-ml flasks and cells were maintained under the same conditions until late log phase. The turbidity of the culture was read at 600 nm.

Methionine quantification by microbial assay

Bacterial strains A121 and A181 were cultured on MCGC medium until late log phase. Cells were collected by centrifugation at 14,000 x g, 4°C for 10 min and were washed three times with the same volume of 0.9% NaCl solution. For cell digestion, 1 ml of 0.1 N HCl was added into each sample and the samples stood at 110 °C for 1 hour. These samples were kept at 4 °C.

Methionine quantification was measured by using *E. coli* K12 with a partial *metC* deletion. To construct a methionine auxotrophic strain, the target *metC* region was amplified by PCR with primers UF2 and LR2. The 708 bp out of 1188 bp-*metC* gene in *E. coli* K12 was deleted by following the manufacture protocol (Quick and Easy *E. coli* Gene Deletion Kit, Gene

Bridges, Heidelberg, Germany). The *E. coli* K12 derivative with *metC* partially deleted was designated as strain K12C. A correct partial *metC* deletion on the chromosome of *E. coli* K12C was verified by PCR with primers CF6 and CR6. The thermal cycling profile was as follows: 2 min at 95 ^{o}C, followed by 30 cycles of 30 s at 95 ^{o}C, 30 s at 55 ^{o}C, 4 min at 72 ^{o}C, followed finally by 10 min at 72 ^{o}C.

E. coli K12C was cultured in Luria-Bertani medium at 37 ^{o}C, 200 rpm for 5 hours and then was harvested by centrifugation at 5,000 x g, 4 ^{o}C for 5 min and washed three times by MCGC medium (von der Osten et al., 1989). The cells were diluted 1/100 with fresh MCGC medium and methionine concentrations between 0 to 500 μg/ml were used as the standard. *E. coli* cells with various methionine concentrations and samples were added into the 96-well plates incubated in Tecan Infinite M200 (San Jose, CA) at 37 ^{o}C for 24 hours and the optical density was measured every hour at 600 nm.

Statistical analyses

Where standard deviations are presented in the respective figures, these values represent the average of at least triplicate measurements. The statistical tests for treatment effects were performed using an analysis of variance (ANOVA) procedure of Statistixs 9.1 Analytical Software (SAS Institute, Cary, NC). Means were further separated using least-significant difference multiple comparisons.

RESULTS

Selection of isolation medium for detection of methionine- containing bacteria

C. glutamicum ATCC13032 and *E. coli* ATCC23798 were cultured on basal medium and LB medium as the positive control medium. However, both bacteria were unable to grow on

basal medium agar. *C. glutamicum* ATCC13032 grew well on MCGC and LB agars while *E. coli* ATCC23798 grew only on LB agar. Therefore, MCGC agar was used in future steps.

Isolation and identification of methionine over-producing bacteria

As shown in Table 5.2, 38 bacterial strains were isolated from environmental samples. All strains in this study were able to grow under both aerobic and anaerobic conditions. All strains including *C. glutamicum* ATCC13032 were cultured on MCGC agar containing 0.1% norleucine (w/v). Only *C. glutamicum* ATCC13032, and strains A101, A111, A121, A122, A131, A151, A171, and A181 grew well on MCGC agar supplied with norleucine at 0.1% (w/v).

The strains that could grow in the presence of norleucine at 0.1% (w/v) on MCGC agar were further assayed on MCGC broth containing either ethionine or norleucine at 0.05 or 0.1% (w/v). The results showed that the types and concentrations of methionine analogs significantly affected the growth of these bacterial isolates (Fig. 5.1 to 5.9). Based on the results from strains ATCC13032 and A171 cultured in MCGC broth supplied with two methionine analogs, ethionine had significantly ($P < 0.05$) higher inhibitory ability to the growth than norleucine (Fig. 5.1 and 5.8). However, the growth of strains A122. , A151, and A181 with ethionine supplement was significantly ($P < 0.05$) higher than the growth without methionine analog addition (Fig. 5.5, 5.7, and 5.9). Overall, from 39 isolated bacterial strains and *C. glutamicum* ATCC13032, only bacterial strains *C. glutamicum* ATCC13032, and isolates A101, A111, A121, A122, A131, A151, A171, and A181 could tolerate norleucine at 0.1% (w/v) in MCGC agar (Table 5.1). By culturing 9 bacterial strains in MCGC broth containing either ethionine or norleucine at 0.05 and 0.1% (w/v), only strains A121, A122, A151, and A181 were able to grow well under these conditions.

By using the primers 27F and 1492R, a universal primer set for the domain *Bacteria*, approximately 1500 bp DNA fragments were obtained. Based on the comparison from BLAST and RDP-II, both databases unanimously confirmed these organisms at least at the genus level. The SSU rRNA sequences of strain A121, A122, A151, A171 and A181 were matched with *Klebsiella* species, *Acinetobacter baumannii*, *A. baunnii*, *Pantoea* species, and *Pseudomonas aeruginosa*, respectively.

Methionine quantification

The characterization of strains A121 and A181 was continued to evaluate the correlation between methionine-analog resistance and methionine biosynthesis. The growth of both strains were cultured on MCGC medium at 37 ^{o}C, 200 rpm (Fig 5.10) and the samples were collected to measure methionine biosynthesis at different time intervals (Table 5.2). The methionine in cell samples slightly increased between 4 to 12 hour incubation (Table 5.2). However, total methionine samples from both were much higher than the cell methionine samples. While methionine in cell samples of strain A181 was increased from 6.122 μg/ml at 4 hour incubation to 31.109 μg/ml at 12 hour incubation, the total methionine was increased from 63.200 to 124.578 μg/ml during that the same time period.

DISCUSSION

Methionine is the first limiting amino acid in poultry feed. Methionine supplementation in poultry production enhances feed efficiency, increases protein synthesis, and improves immune systems (Bunchasak, 2009). Dietary supplementation of methionine at 5.4 g/kg has been shown to improve color, water-holding capacity, and oxidative stability of male offspring meat (Wang et al., 2009). For long-term benefits, adequate methionine is considered necessary

for sustaining normal immunocompetence and achieving maximum egg production in laying hens in subtropical climates (Poosuwan et al., 2010).

Methionine production has been manufactured synthetically for more than 50 years (Leuchtenberger et al., 2005); however, synthetic methionine soon will no longer be allowed in organic poultry feed production (USDA, Agricultural Marketing Service, http://www.paorganic.org/wp-content/uploads/2011/10/USDA-Methionine.pdf). However, methionine biosynthesis from wild-type microorganisms could serve as an alternative way to support the organic poultry industry. In this study, bacterial strains were isolated from environmental samples and selected methionine over-production by using two methionine analogs.

Isolation of methionine-containing microorganisms

Minimal media without methionine were used to culture *C. glutamicum* ATCC13032 and *E. coli* ATCC23798 as the positive and negative controls in this study. Only the methionine-producing bacteria *C. glutamicum* was able to grow on MCGC medium; thus the MCGC medium was used as the selective medium for isolating methionine-producing bacteria. The inability of *C. glutamicum* ATCC13032 to grow on the basal medium may be due to the buffer capacity of the medium (Lawrence et al. 1968). Unlike the buffer capacity of basal medium, the buffer capacity of MGCG medium increases during fermentation as the pH of the culture decreases (von der Osten et al., 1989).

Identification of potential methionine-overcontaining microorganisms

In this study, 38 isolated strains and *C. glutamicum* ATCC13032 were cultured with methionine analogs for methionine over-production. Finally, 5 bacteria were identified by sequencing SSU rRNA genes. However, all 5 strains were potential plant and human pathogens

which are impractical for methionine production. The SSU rRNA gene of strain A121 isolated from a water sample was matched with *Klebsiella* species, this genus has been recovered from aquatic environments (Grimont and Grimont, 2001) while the SSU rRNA genes of strains A122 and strain A151 isolated from a water sample and a compost sample, respectively, were a match with *A. baumannii*, a bacterium that occurs naturally in soil and water (Juni, 2001). Strain A171 originating from a soybean field at the University of Arkansas belongs to the genus *Pantoea*, the genus generally associated with plants (Delétoile et al., 2009), while strain A181 isolated from bovine rumen fluid was identified as *P. aeruginosa*, a ubiquitous bacterium found in the ovine rumen (Duncan et al., 1999).

Methionine production from isolated microorganisms

Methionine detection methods have been developed by using chemical and biological means. For example, *Pediococcus acidilactici* ATCC8042 was employed as a biological approach to methionine detection (Steele et al., 1949). However, the methionine assay medium for *P. acidilactici* ATCC8042 is commercially unavailable and medium preparation is laborious. In addition, trace amounts of methionine can cause an error of results. Compared to the method based on *P. acidilactici* ATCC8042, the method based on *E. coli* K12C in this study was simple and the lowest detectable concentration was 5 μg/ml. The information available on *E. coli* ATCC23798 in The Coli Genetic Stock Center at Yale University cited only *met-59* and does not show which gene in methionine biosynthesis pathway was deleted. The *metC* was chosen for deletion because no bypass occurs at this step (Lee and Hwang, 2003). In addition, due to well known genetics, simple media requirement and rapid growth, *E. coli* auxotroph is a good candidate for quantification of amino acid bioavailability (Froelich and Ricke, 2005; Chalova et

al., 2010). By When comparing *E. coli* as a biosensor bioassay with an animal bioassay, lysine bioavailability of feed samples were not significantly different (Chalova et al., 2007).

By using two methionine analogs in this study, strain A181 which was more tolerant to these analogs produced more methionine. In addition, both strains excreted methionine into the surrounding media. In general, most methionine-producing bacteria secrete methionine externally. Ghosh and Banerjee (1986) reported *Serratia marcescens* under some conditions secreted methionine at approximately 100 μg/ml.

In this study, all identified strains were pathogens. For safety reasons, it might be better to isolate methionine-producing bacteria from specific food samples such as yoghurt and starter culture producers may represent a better approach. For example, Cebeci and Gurakan (2008) studied methionine biosynthesis in *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. In addition, molecular approaches might be useful for methionine biosynthesis detection. Since methionine biosynthesis pathways are variable in different organisms (Lee and Hwang, 2003), it is difficult to design specific primers for detecting methionie biosynthesis. However, Cebeci and Gurakan (2008) were successful to design primers for detecting methionine genes in *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* due to 95% similarity of methionine biosynthesis gene in both organisms.

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Table 5.1. Primers used in this study.

Primer	Sequence
UF2	TGCAGCCAAACACTAAAATCGTGTTTCTGGAATCGCCAGGCTCCATCAC C*AATTACCCTCACTAAAGGGCG*[a]
LR2	GTTGAAAAACTGGAAGCGGCGGTTTGGCAGACCGGAAATTCCCGCCATG G*TAATACGACTCACTATAGGGCTC*
CF6	ACAATACCTGCGCTGCAACATCAC
CR6	CGATGTGCGTTTGTTGTATGGCGA

[a]Italics show sequences for amplification of the FRT-PGK-gb2-neo-FRT cassette

Table 5.2. The bacterial isolates were grown in either aerobic or anaerobic condition and with norleucine at 0.1 % (w/v).

Sample source	Strains isolated under a aerobic condition		Strains isolated under an anaerobic condition		
	strain	Grow with norleucine [a]	strain	Grow with norleucine in a aerobic condition	Grow with norleucine in an anaerobic condition
	C. glutamicum ATCC13032[b, c]	3+			
chicken intestinal contents	A11[d]	-	AA11[e]	-	-
chicken intestinal contents	A21	-	AA21	-	-
chicken intestinal contents	A31	-	AA31	-	-
chicken intestinal contents	A41	-	AA41	-	-
chicken intestinal contents	A42	-			
chicken intestinal contents			AA51	-	-
chicken intestinal contents	A61	1+	AA61	-	-
chicken intestinal contents	A62	-			
chicken intestinal contents	A71	-	AA71	-	-
chicken intestinal contents	A81	1+	AA81	-	-
chicken intestinal contents	A91	-	AA91	-	-
Soil sample[f]	A101	3+	AA101	1+	-

Table 5.2. (continued).

Sample source	Strains isolated under a aerobic condition		Strains isolated under an anaerobic condition		
	strain	Grow with norleucine [a]	strain	Grow with norleucine in a aerobic condition	Grow with norleucine in an anaerobic condition
Soil sample[g]	A111	3+	AA111	1+	1+
Water sample[h]	A121	3+	AA121	-	-
	A122	2+			
Soil sample[i]	A131	3+	AA131	1+	-
Horse manure[j]	A141	-	AA141	1+	-
Compost	A151	-	AA151	-	-
			AA152	1+	-
Soil sample[k]	A161	-	AA161	1+	1+
Soil sample[l]	A171	3+			
Bovine rumen fluid[m]	A181	3+	AA181	1+	-

[a] Growth with norleucine was monitored on agar plate under a aerobic condition. The best, medium, and poor levels of growth are 3+, 2+, and 1+, respectively. No growth on agar plates was (-).

[b] *C. glutamicum* ATCC13032 as the positive control.

[c] only *C. glutamicum* ATCC13032 was unable to grow on both LB agar (enriched medium) and MCGC agar (minimal medium) under an anaerobic condition.

[d] A11 was isolated under a aerobic condition from the first sample and the first type strain.

[e] AA11 was isolated under an anaerobic condition from the first sample and the first type strain.

[f] Soil sample was collected from the bamboo root.

[g] Soil sample was collected from lawn at Chestnut apartment, Fayetteville, AR

[h] Water sample was collected from Scull Creek, Fayetteville, AR.

[i] Soil sample was collected from Scull Creek, Fayetteville, AR.

[j] The manure was collected from an American Quarter horse grazing mixed grasses.

[k] Soil sample was collected from roadside at Morning Side Rd., Fayetteville, AR.

[l] Soil sample was collected from soybean field at University of Arkansas, Fayetteville, AR.

[m] Rumen fluid was collected from Gelbveih Angus Cross grazing Bermuda grass.

Table 5.3. Methionine production in bacterial samples (μg/ml).

Samples	Incubation time (hours)				
	4	6	8	10	12
Cells methionine in strain A121	7.86 ± 1.62[a]	3.34 ± 2.61	4.56 ± 1.29	ND[b]	ND
Cells methionine in A181	6.12 ± 0.90	10.24 ± 1.98	17.91 ± 0.63	13.94 ± 4.58	31.11 ± 13.26
Total methionine in strain A121	44.13 ± 2.17	64.22 ± 3.87	78.93 ± 16.22	ND	ND
Total methionine in A181	63.20 ± 9.91	90.44 ± 7.63	102.62 ± 30.05	73.33 ± 35.47	124.58 ± 33.20

[a]The mean of methionine production (μg/ml) and standard deviations.

[b]ND, not determined.

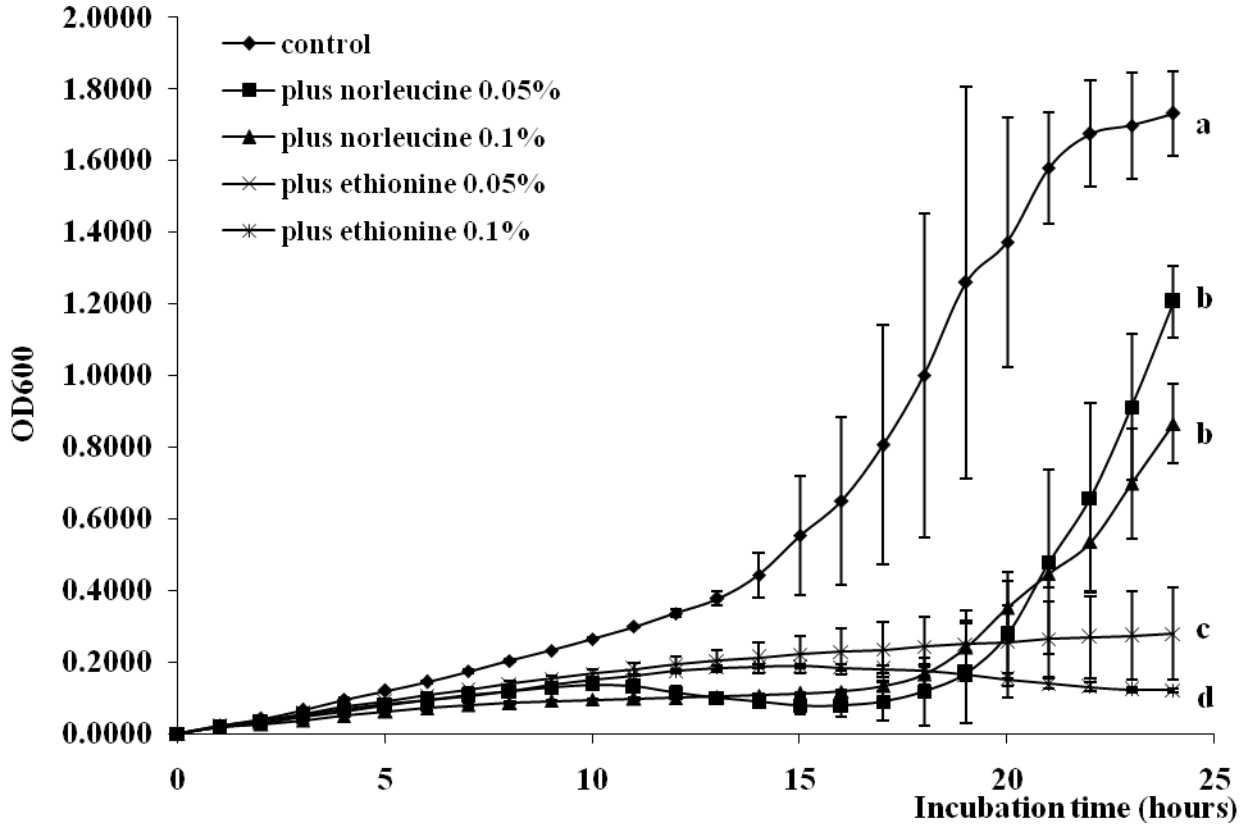


Fig. 5.1 The growth of bacterial strain ATCC13032. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

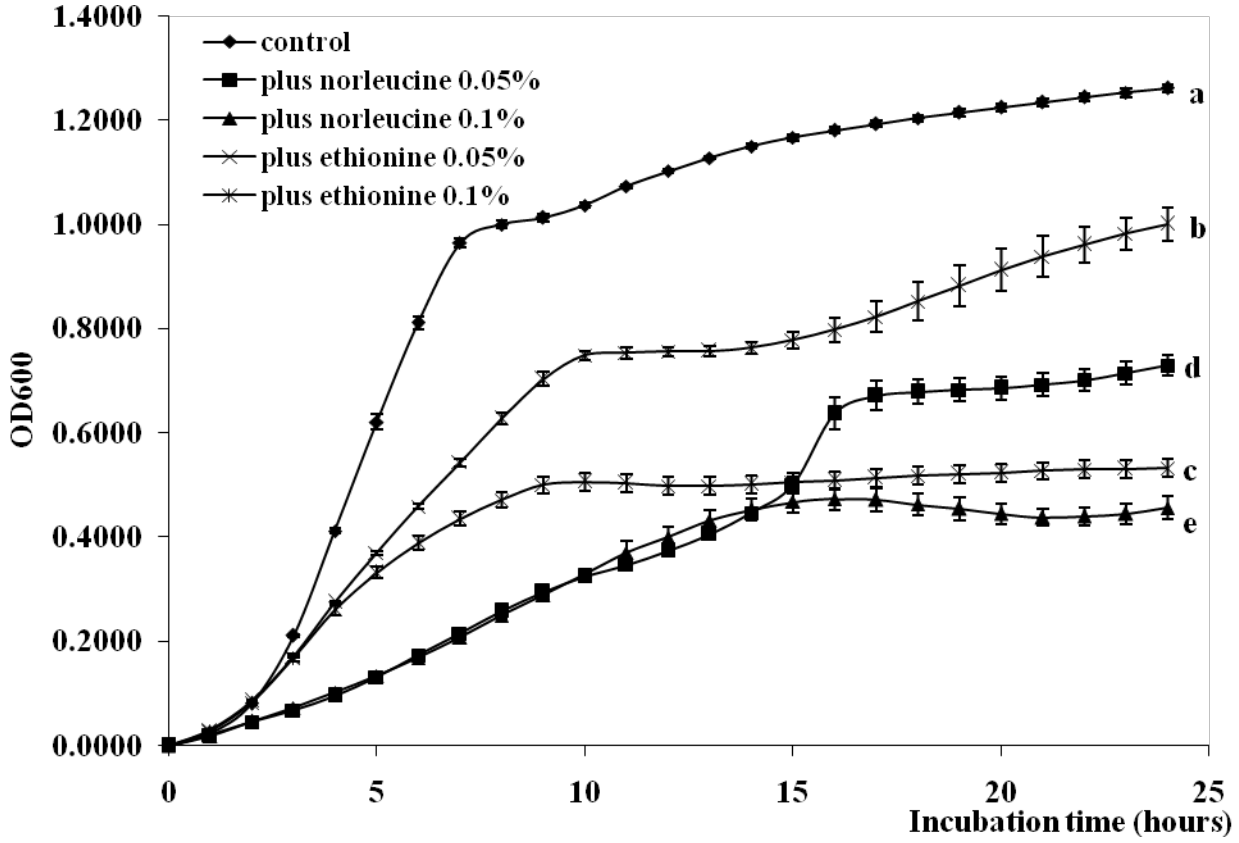


Fig. 5.2 The growth of bacterial strain A101. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

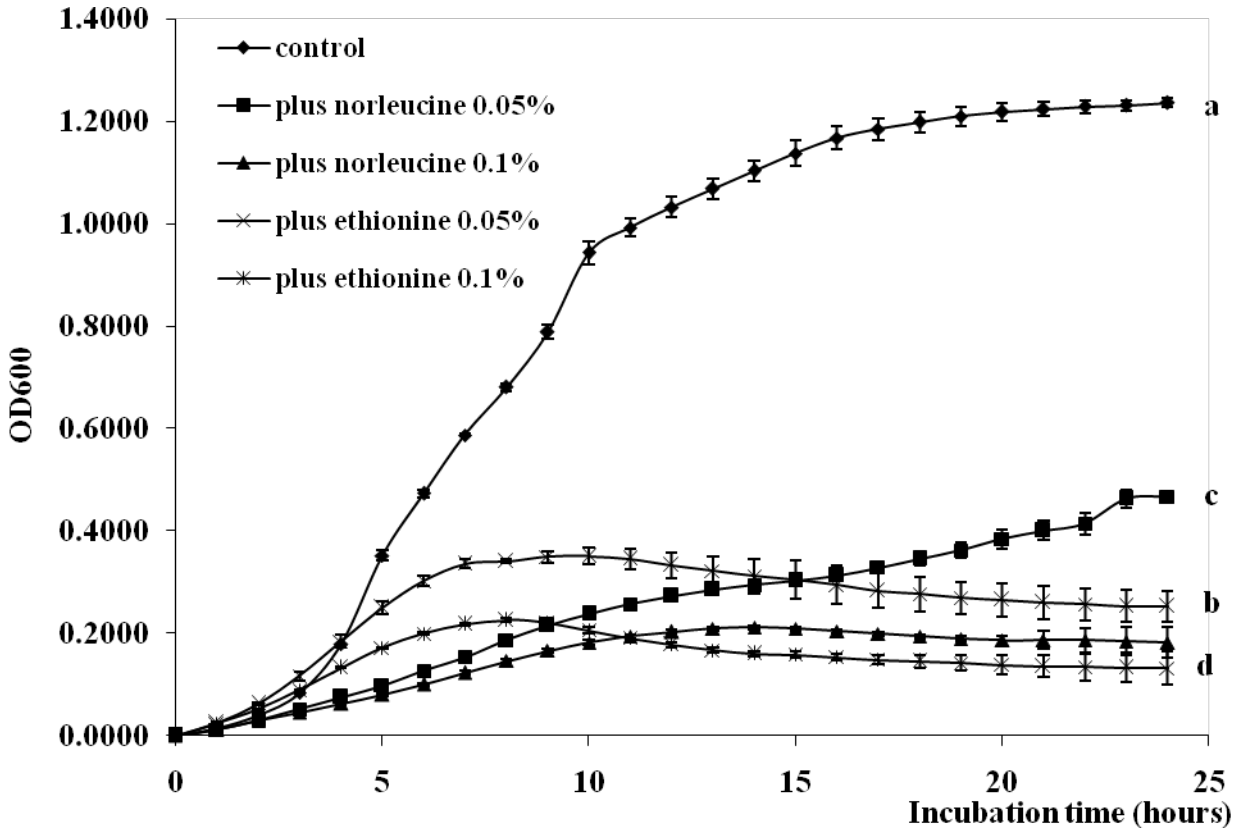


Fig. 5.3 The growth of bacterial strain A111. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

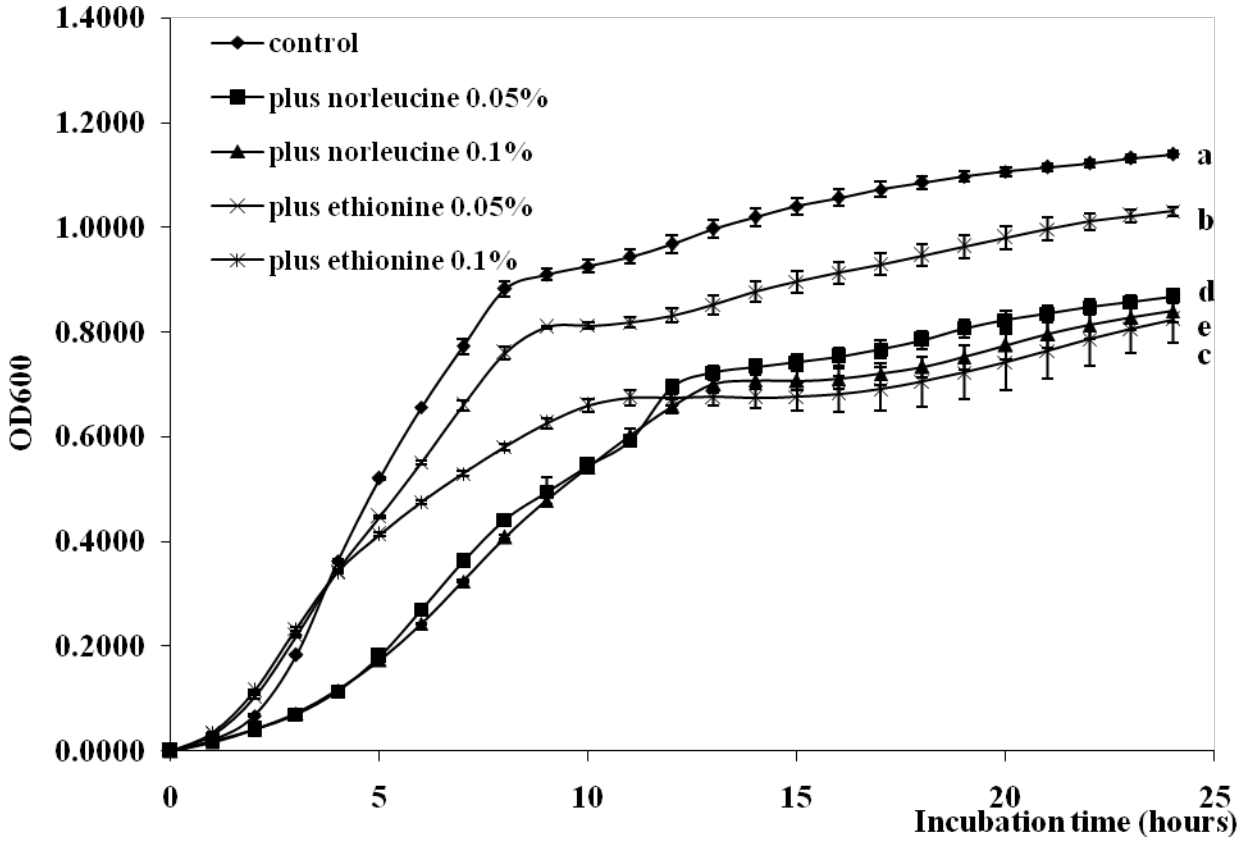


Fig. 5.4 The growth of bacterial strain A121. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

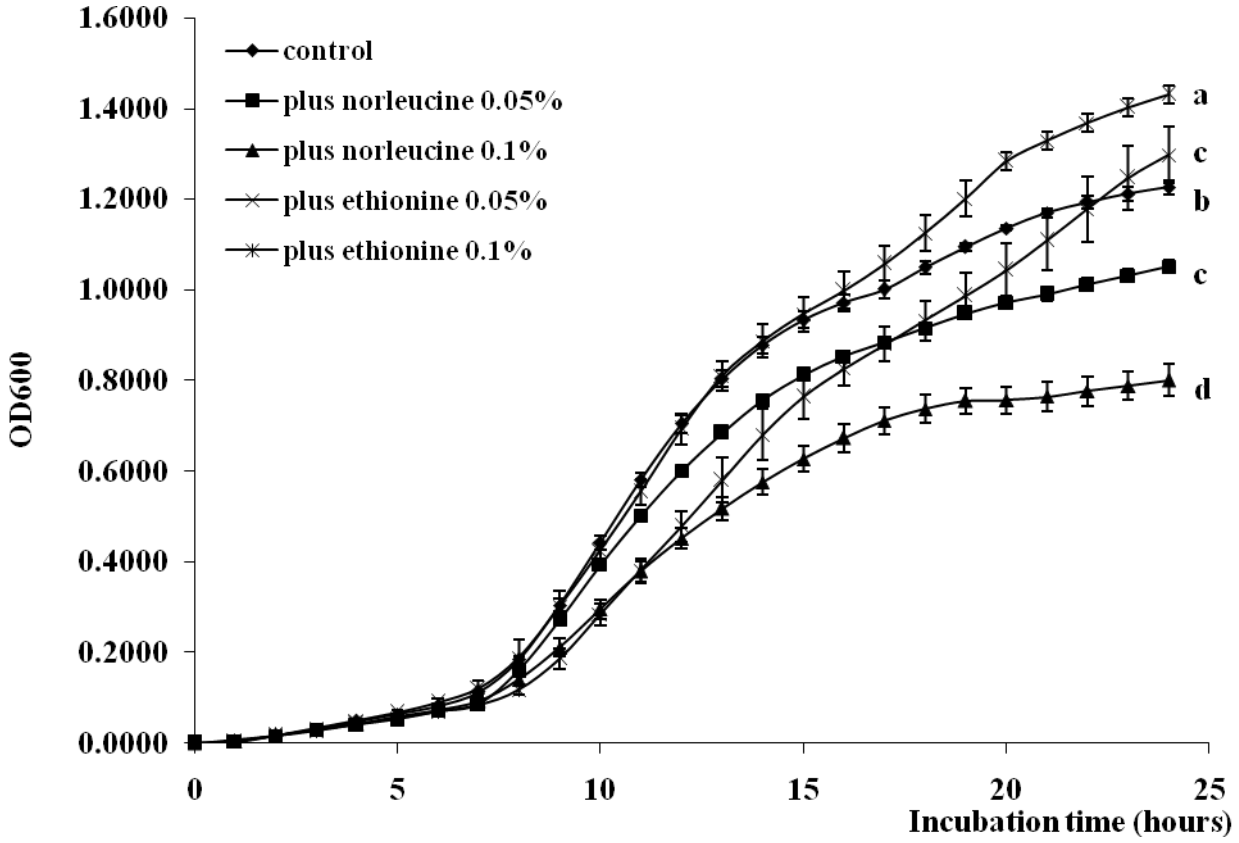


Fig. 5.5 The growth of bacterial strain A122. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

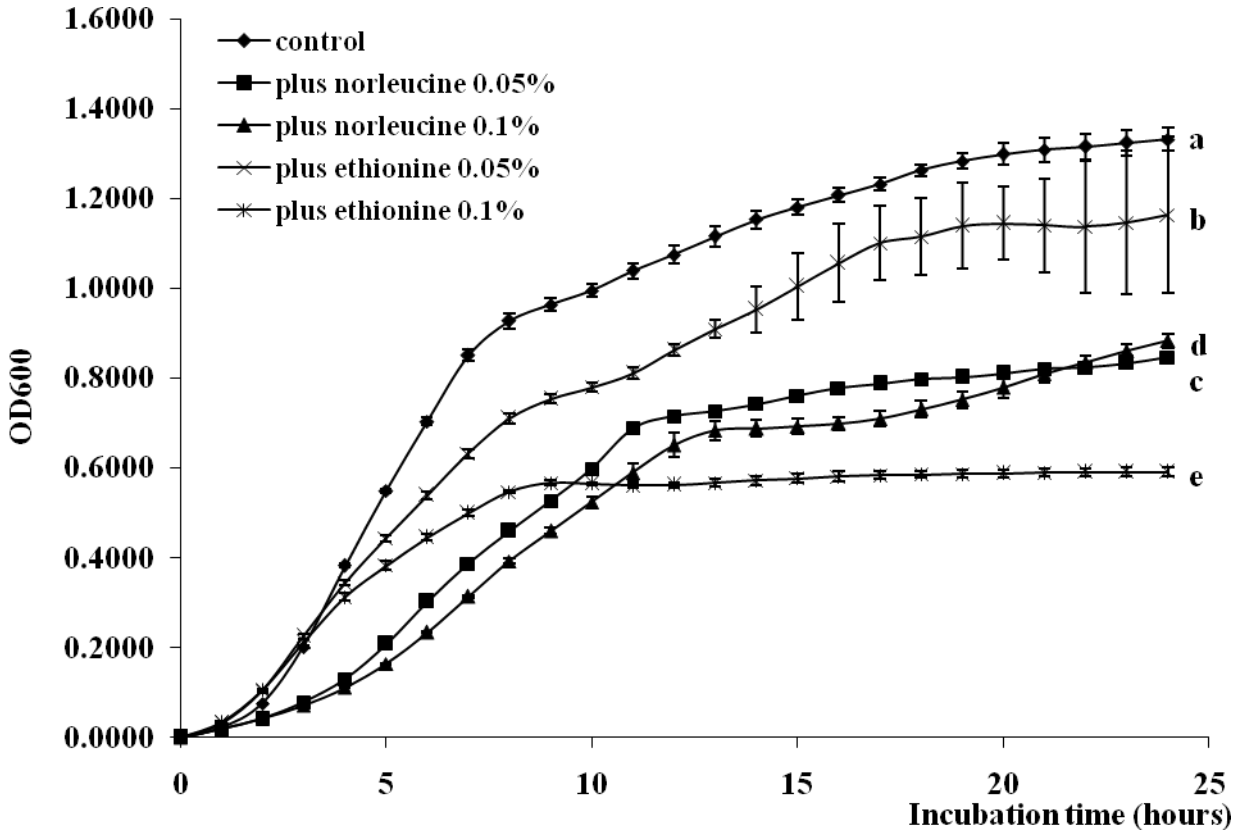


Fig. 5.6 The growth of bacterial strain A131. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

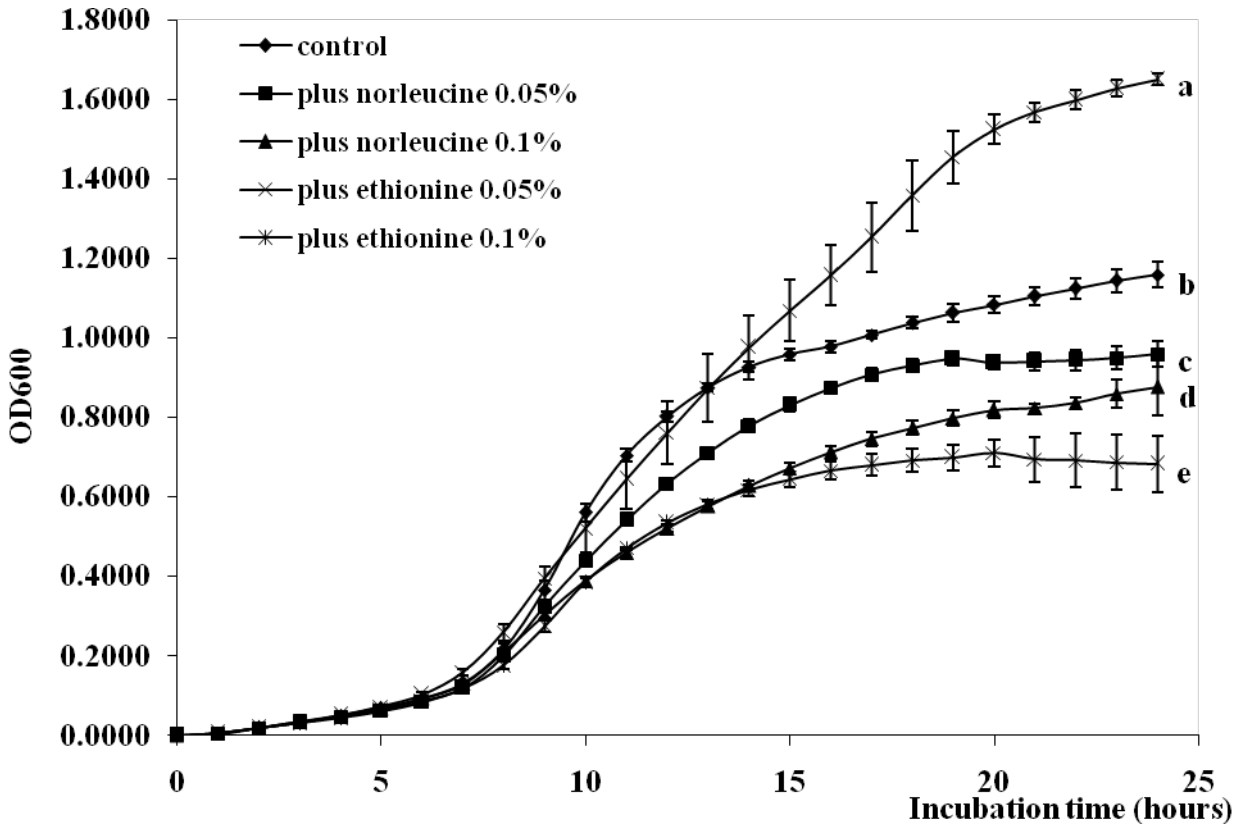


Fig. 5.7 The growth of bacterial strain A151. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

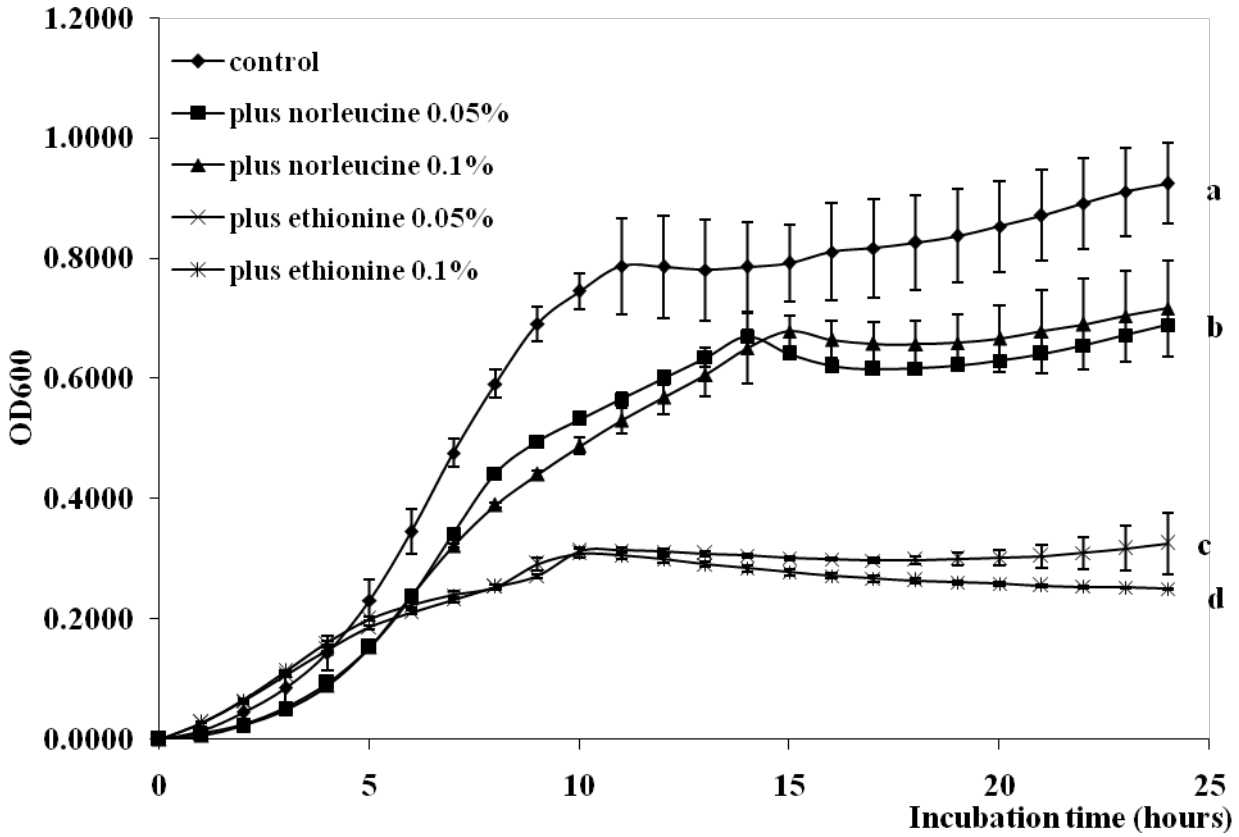


Fig. 5.8 The growth of bacterial strain A171. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

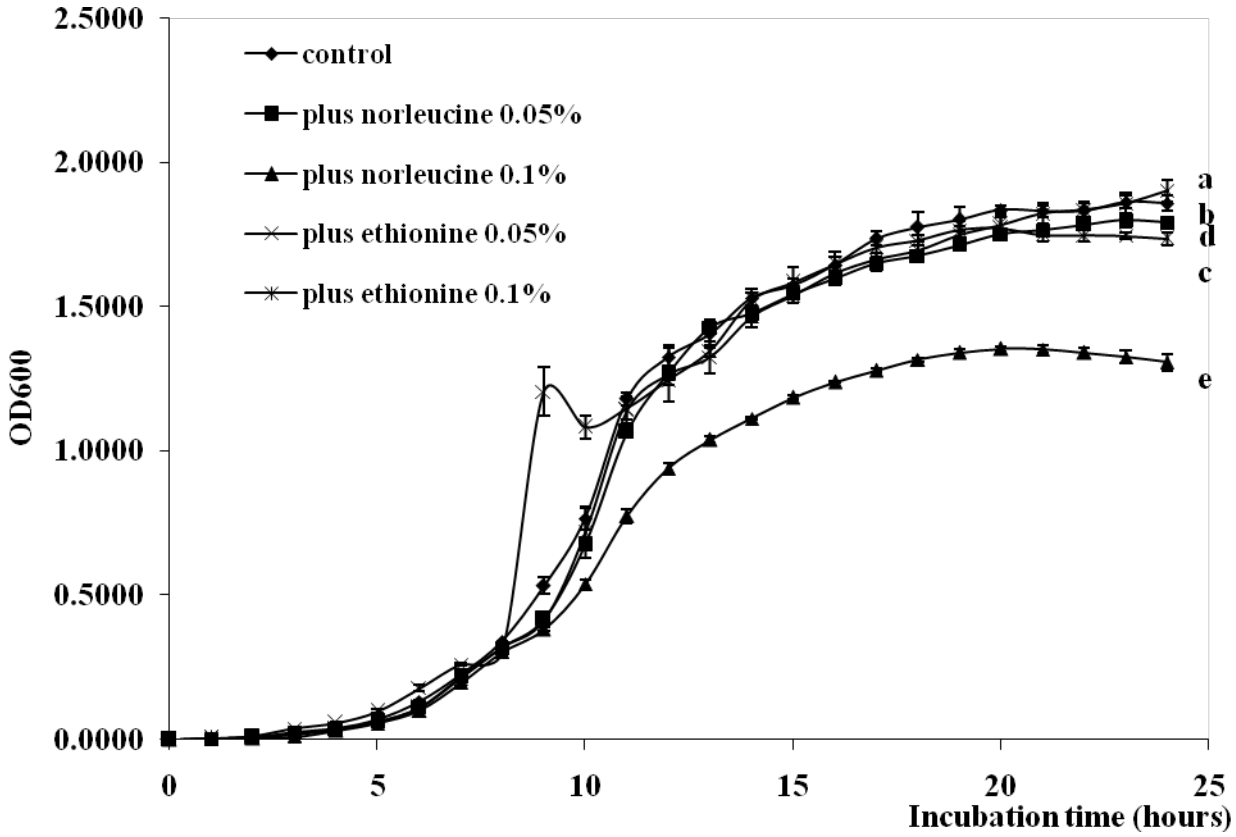


Fig. 5.9 The growth of bacterial strain A181. The strain was culture on MCGC medium containing either ethionine or norleucine at 0.05 and 0.1% (w/v). [a.b,c,d,e] Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

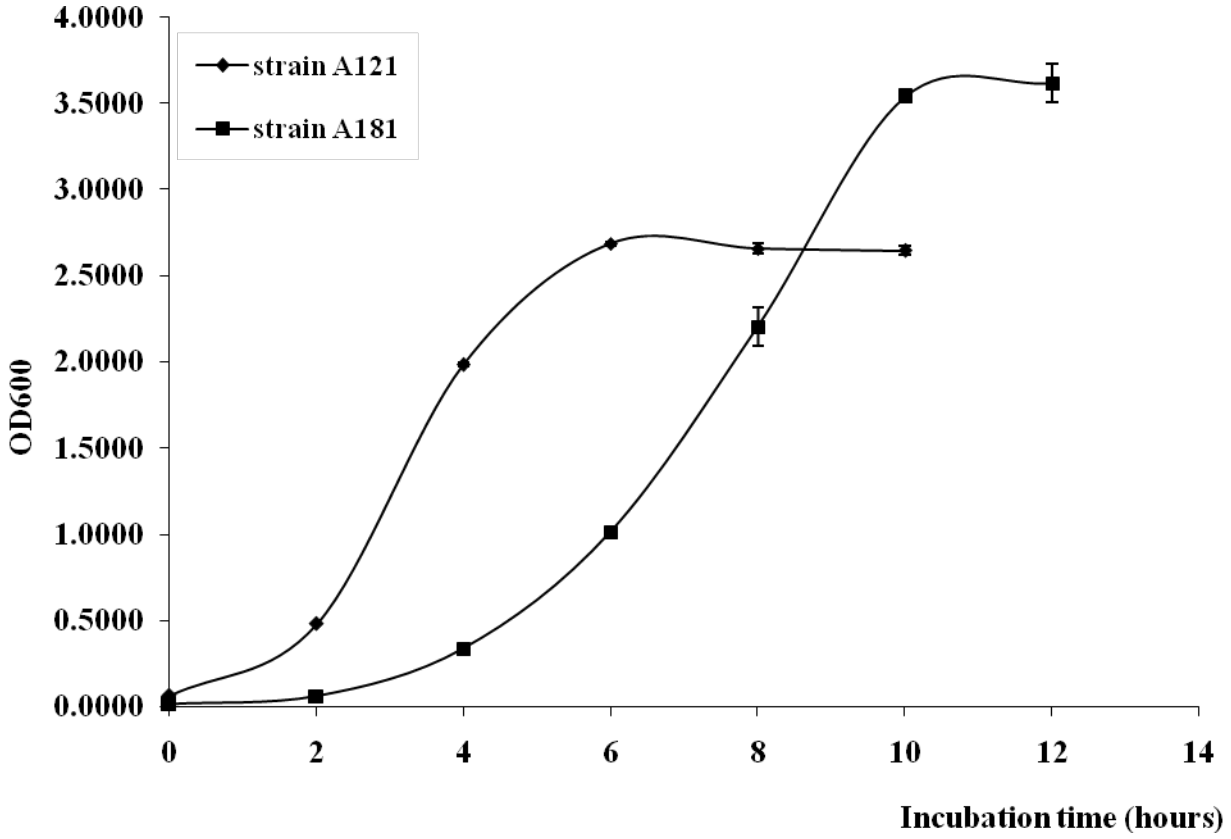


Fig.5.10. The growth of bacterial strains A121 and A181cultured on MCGC medium at 37 ^{o}C, 200 rpm. The growth measurements were measured at 600 nm and were performed with at least three independent cultures. Error bars indicate standard deviations between growth measurements.

CHAPTER SIX

ISOLATION OF HIGH METHIONINE-CONTAINING YEAST UTILIZING METHIONINE ANALOGS

ABSTRACT

Methionine is one of the first limiting amino acids in poultry nutrition and typically in most diets this amino acid has to be supplemented into the poultry feed mixture. It is anticipated that in the near future the organic poultry industry will not be allowed to add synthetic methionine generated from chemical synthesis to diets of birds. Therefore, the objectives of this study were identification of high content methionine containing yeasts and quantification of methionine content in yeasts. Minimal medium was used as the selection medium and the isolation medium of methionine-producing yeasts from yeast collection and environmental samples, respectively. Two out of the eleven yeasts previous collected and six additional strains were isolated from Caucasian kefir grains, air-trapped, cantaloupe, and three soil samples that could grow on minimal medium. Only two of the newly isolated strains K1 and C1 grew better than others in minimal medium supplied with either ethionine or norleucine at 0.5% (w/v). Based on large subunit rRNA sequences, these isolated strains were identified as *Pichia kudriavzevii/Issatchenkia orientalis*. *P. kudriavzevii/I. orentalis* is recognized as a ″generally recognized as safe″ (GRAS) organism. In addition, methionine produced by K1 and C1 yeast hydrolysate could be measured by microbial assay and yielded 19.30 mg per gram dry cell. Yeast strain K1 may be suitable as a source of methionine for dietary supplements in organic poultry feed.

KEY WORDS: ethionine, methionine production, norleucine, yeast

INTRODUCTION

Methionine is an essential amino acid for proper growth and development of chickens. Chickens are unable to produce methionine and therefore must obtain it externally as a dietary supplement. However, in the U.S., approximately 90% of poultry feed is composed of corn and soybean but both are insufficient to meet the birds' methionine requirement and it is therefore one of the first limiting amino acids in poultry feed (Baker, 2009). The organic poultry industry faces an even greater challenge regarding methionine supplementation in organic poultry feed. Currently available organic poultry feed contains insufficient amounts of methionine resulting in reduced broiler growth rates (Rodenburg et al., 2008) as well as lower egg weights for laying hens (Elwinger et al., 2008).

Current methods of methionine production include either *de novo* chemical synthesis or by hydrolysis of proteins. Both methods are expensive and require the use of hazardous compounds (Kumar and Gomes, 2005). Synthetic methionine is currently allowed as an additive to organic poultry feed by the U.S. Department of Agriculture's National Organic Program (NOP) however NOP is only extending its use until October 1st 2012 (USDA, Agricultural Marketing Service, http://www.paorganic.org/wp-content/uploads/2011/10/USDA-Methionine.pdf). Therefore, there is an urgent need for organic sources of methionine for feed additives.

Many attempts have been made to utilize yeast cells as protein sources in livestock and aquaculture industries. For example, *Candida utilis* is widely used for animal feed as a protein source because it is able to grow on inexpensive substrates (Demain et al., 1998). Gilter et al. (1958) reported that torula yeasts could replace fish meal up to 25% without influencing the rate of growth in chickens. Recently, Garcia-Hernandez et al. (2012.

doi:101.1016/j.rvsc.2011.09.005) isolated yeasts from the excreta of broilers and found that some strains have potential as probiotic supplements. However, yeast hydrolysate used as a protein source in animal feed has been shown to be insufficient as a source of methionine (Komatsu et al., 1974; Rastogi and Murti, 1964). To improve methionine content, mutagenesis by UV irradiation or administering *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine have been used to select methionine-analog resistant mutants that are characterized by greater methionine biosynthesis (Brigidi et al., 1988; Dunyak and Cook, 1985; Komatsu et al., 1974; Morzycka et al., 1976; Musilkova and Fencl, 1964; Tani et al., 1988). However, based on USDA regulations, the organic poultry industry will not be allowed to use methionine supplements from chemical production systems after October 1st, 2012. The objective of this study was to identify high content methionine containing yeast for potential use in the organic poultry industry; utilizing methionine analogs to screen methionine overproduction in wild-type yeasts.

MATERIALS AND METHODS

Isolation of methionine producing yeast

In this study, eleven yeast strains were provided by Professor Ines Pinto, Department of Biological Sciences, University of Arkansas, Fayetteville, AR (Table 6.1). An additional six yeast strains were isolated from a variety of environmental samples. Air-trapped yeast cells were prepared by mixing all purpose flour and water. The air-trapped yeast sample and cantaloupe were allowed to stand in an open-environment for 10 hours. All environmental samples were inoculated into minimal medium (Table 6.2) (Morzycka et al., 1976). The cultures were maintained at 30^{o}C, 200 rpm in a shaker (incubator model G-25, New Brunswick Scientific, New Brunswick, NJ) and were transferred to fresh media for eight consecutive days.

Methionine feedback effect assay

For screening of methionine-overproducing yeast, each strain was assayed on minimal medium containing either ethioine or norleucine at 0.5% (w/v). The 96-well plates were incubated in a Tecan Infinite M200 (San Jose, CA) at 30^{o}C for 24 hours and the optical density was measured every hour at 580 nm.

Measurement of yeast growth

The potential methionine-overproducing yeast strain was cultured in both minimal medium and lactose medium (Table 6.2) (Lukondeh et al., 2005) at 30^{o}C, 200 rpm overnight. A 1/100 volume of yeast cells was transferred into 50-ml fresh medium in three-baffled 500-ml flasks and these cells were maintained under the same conditions until reaching stationary phase. The turbidity of the culture was read at 580 nm (Tecan Infinite M200, San Jose, CA).

Methionine quantification

Yeast strain K1 and C1 were cultured in minimal and lactose media until late log phase. Yeast cells were collected by centrifugation at 14,000 x g, 4^{o}C for 10 min and were washed three times with the same volume of 0.9% NaCl solution. In this study, yeast samples were first hydrolyzed by acid and were subsequently detected by microbiological assay similar to the other studies (Kitamoto and Nakahara, 1974; Komatsu et al., 1974). For cell digestion, 1 ml of 6 N HCl was added into each sample and the samples allowed to stand at 110^{o}C for 1 hour. These samples were kept at 4 ^{o}C and the samples were diluted 10 fold by 0.1 N HCl before being quantified.

E. coli K12 was obtained from the *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, CT. A part of *metC* gene was deleted by using Quick and Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, Germany) and designated as *E. coli* K12C. *E.*

coli K12C was cultured in Luria-Bertani medium at 37 ^{o}C, 200 rpm for 5 hours and was subsequently harvested by centrifugation at 5,000 x g, 4 ^{o}C for 5 min and washed three times by MCGC medium (von der Osten et al., 1989). The cells were diluted 1/100 with fresh MCGC medium and methionine concentrations between 0 to 500 μg/ml were used as the standard. *E. coli* cells with various methionine concentrations and samples were added into the 96-well plates and incubated in a Tecan Infinite M200 (San Jose, CA) at 37 ^{o}C for 24 hours and the optical density was measured every hour at 600 nm.

Identification of potential methionine-producing yeasts

The large subunit rRNA (LSU rRNA) genes of methionine over-producing yeast strains were amplified by using primers NL-1 and NL-4 as described in Kurtzman and Robnett (1998). The thermal cycling profile was as follows: 2 min at 95^{o}C, followed by 30 cycles of 30 s at 95^{o}C, 30 s at 52^{o}C, 1 min at 72^{o}C, followed by 10 min at 72^{o}C.

The PCR products were sequenced by the DNA Resource Center, University of Arkansas, Fayetteville, AR. The partial LSU rRNA sequence of K1 was used to query GenBank. To place K1 within a phylogeny of representative yeasts, sequences from GenBank were included in the analysis. The alignment was generated and a neighbor joining tree was constructed using CLC genomics workbench 4.0.3 employing the default settings for nucleotide data. The tree was subjected to 1,000 replicates of bootstrapping, and the percentages of replicates supporting a given node are indicated in Fig. 6.9.

Statistical analyses

Where standard deviations are presented in the respective tables and figures, these values represent the average of at least triplicate measurements. The statistical tests for treatment effects were performed using an analysis of variance (ANOVA) procedure of Statistixs 9.1 Analytical

Software (SAS Institute, Cary, NC). Means were further separated using least-significant ($P < 0.05$) difference multiple comparisons.

RESULTS

Only *Sacchaeomyces(Sac.) cerevisiae* ATCC4126 and *C. kefyr* ATCC2512 out of eleven strains from a yeast collection at the University of Arkansas were able to grow in minimal medium. Six strains were isolated from Caucasian Kefir grains, air-trapped, cantaloupe, and three soil samples. *Sac. cerevisiae* ATCC4126, *C. kefyr* ATCC2512 and five strains isolated from environmental samples were further tested in yeast minimal medium containing 0.5% (w/v) either ethionine or norleucine. For safety reasons, since strain Y1, isolated from air-trapped sample was identified as *C. parasilosis* and is considered a human pathogen, it was discarded from further evaluations.

To further select the high content methionine containing yeasts, seven strains were cultured in minimal medium containing either ethionine or norleucine at 0.5% (w/v) (Fig. 6.1 to 6.7). Compared to cultures without methionine-analogs and norleucine supplement, ethionine addition affected the growth of all five strains. Methionine analogs significantly ($P < 0.05$) inhibited the growth of most strains, except strain S2. The growth of all strains supplied with ethionine was significantly lower than the growth with norleucine supplement. Surprisingly, norleucine enhanced ($P < 0.05$) the growth in strain S2 but ethionine completely inhibited the growth (Fig. 6.6). Strains K1 and C1 were further evaluated since both had greater ability to tolerate norleucine and ethionine than the other isolates (Fig. 6.1 and 6.4).

The biomass from both strains K1 and C1 showed that these organisms preferred to grow in minimal media rather than lactose media (Fig. 6.8 and Table 6.3). While dry cell weight cultured on minimal medium was approximately 1.4 mg per ml culture, biomass from lactose

medium was approximately 0.2 mg per ml (Table 6.3). Methionine biosynthesis from C1 cultured in lactose medium was 15.3 mg per g dry cell weight, but methionine concentrations from cultures in lactose were not detectable (Table 6.3). Methionine concentrations when strain K1 was cultured in lactose medium and minimal medium were 9.3 and 19.3 mg per g dry cell weight, respectively. Overall, minimal medium supported more growth and methionine production in both strains than lactose medium. Based on the phylogenetic analysis, the LSU rRNA sequences of strains K1 and C1 were determined to be *Pichia kudriavzeii/Issatchenkia orientalis* (Fig.6.9).

DISCUSSION

Methionine is an important amino acid for the poultry industry. Methionine supplementation prevented broiler chicks from developing neurological symptoms when raw grass pea seeds (*Lathyrus sativus*) were used as protein and energy sources in the diet (Fikre et al., 2010). In addition, methionine in diets enhanced the oxidative stability, increased color stability, and decreased drip loss in chicken meat (Wang et al., 2009). In addition, sufficient methionine levels in the diet have been demonstrated to be necessary for sustaining normal immunocompetence and achieving maximum egg production in laying hens in subtropical conditions (Poosuwan et al., 2010). Bunchasak and Silapasorn (2005) reported that laying hens under tropical conditions fed a low-protein diet (14 % crude protein) supplemented with 0.44% methionine improved egg production and egg weight. In the same study bird mortality was reduced and egg shell thickness was improved when these hens were supported with methionine in feed.

Poultry feed contains insufficient amounts of methionine which can lead to physiological and production problems in birds. Currently methionine is produced from chemical processes or

protein hydrolysis and used as a supplement in poultry feed. Synthetic methionine is expensive and soon will no longer be allowed in organic poultry feed production (Kalbande et al., 2009). Therefore, a natural alternative needs to be found for methionine supplementation in poultry feed. Methionine over-producing microorganisms may be an inexpensive source of natural methionine for the poultry industry.

Isolaton of high content methionine containing yeasts

C. utilis is widely used as a single cell protein for animal feed (Demain et al., 1998); however, in this study this organism was unable to grow in minimal medium. Only *Sac. cerevisiae* ATCC4126 and *C. kefyr* ATCC2512 grew under these conditions and most yeast strains in collection were unable to produce detectable methionine.

Yeasts are heterotrophs and the presence of yeasts depends on their available substrates in habitats. In nature, yeasts are ubiquitous in aquatic, terrestrial, and airborne environments (Yarrow, 1998). Agricultural soils may have 40,000 viable yeasts per gram (Walker, 1998). Three strains originated from soil samples and the results showed that yeasts in agricultural soils were abundant. However, no more than one yeast strain was isolated from each soil sample. In a particular habitat, the number of yeast species was found to be between one to four (Phaff and Starmer, 1980). By screening yeasts with ethionine and norleucine, strains K1 and C1 may be capable of over-producing methionine since both showed more tolerance to ethionine and norleucine than others.

Methionine quantification

Methionine detection methods have been developed by using chemical and biological means. For example, *Pediococcus acidilactici* ATCC8042 was employed as a biological approach to methionine detection (Steele et al., 1949). However, the methionine assay medium

for *P. acidilactici* ATCC8042 is commercially unavailable and medium preparation is laborious. In addition, trace amounts of methionine can cause an error results. When a chemically generated *E. coli* methionine auxotroph (ATCC 23798) was used, Froelich et al. (2002) observed no differences based on substrate affinities of an *E. coli* methionine auxotroph to methionine and methionine hydroxy analog, respectively. Estimated maximum growth rate of the *E. coli* auxotroph when grown on both substrates was also found to be similar. To avoid problems as mentioned above, in this study partial *metC* deletion was constructed from wild-type *E. coli* K12. Without methionine supplement at 1 μg/ml, this mutant was unable to grow. The method based on partial *metC* deletion in *E. coli* K12 in this study was simple and based on this assay the lowest detectable concentration was 5 μg/ml.

Methionine content in yeasts

Since the yeast does not usually excrete amino acids into the culture medium (Tani et al., 1988), only intracellular methionine amounts were determined. Compared to lactose medium, minimal medium enhanced the growth of both strains. It is possible that glucose as a sugar substrate on minimal medium may support the growth better than lactose on lactose medium. By culturing kefir grains with various sugar substrates, Harta et al. (2004) reported glucose as the substrate that yielded greater biomass compared to cultures grown on lactose.

Yeast biomass in this study was between 0.16 to 1.46 g/L. It is possible that strains K1 and C1 produced sufficient ethanol at levels to be somewhat inhibitory and thus cause a decrease in biomass syhthesis compared to previous studies. Gallardo et al. (2011) and Shin et al. (2002) detected ethanol during *I. orientalis* fermentation. The biomass in yeasts was varied from 0.3 up to 10.4 g/L (Dunyak and Cook, 1985; Kitamoto and Nakahara, 1994; Tani et al., 1988). However, under optimal conditions, Shin et al. (2002) produced 34.5 g/L of *I. orientalis.*

The amount of methionine produced by yeast strain K1 in this study is similar to the results reported in the previous investigations for some yeast strains (Table 6.3). However, methionine biosynthesis in yeasts has been reported to vary between 1.69 to 16.02 g/g dry wt. Also, the biomass yields in various yeast strains were reported from 0.27 to 38.5 mg dry cell wt/ml. The methionine biosynthesis and biomass production possibly depend on yeast strains and culture conditions. By culturing yeast *Candida boidinii* No. 2201, one study could achieve methionine biosynthesis up to 16.02 mg/g dry cell wt (Tani et al., 1988) while another study reported methionine production was between 5.86 to 10.16 m/g dry cell wt (Lim and Tani, 19888). In addition, yeast strains were able to tolerate methionine analogs at 0.5% (w/v). *Kluyveromyces lactis* that produced methionine at 14.15 mg/g dry cell wt, also grew under 2% (w/v) of ethionine. Lukondeh et al. (2003) concluded that methionine was one of the primary amino acids in *Kluyveromyces marxianus* and *Sac. cerevisiae* and the methionine concentrations were between 1 to 4 mg per g dry weight of cells.

NRC (1994) recommends methionine requirement at 0.5 and 0.38% in broiler starter feed and white-egg laying hen feed. Based on broiler starter feed (NRC, 1994) and white-egg laying hen feed (Narvaez-Solarte et al., 2005), methionine supplement requires 170 and 140 mg per 100 g feed, respectively. Therefore, yeast dry wt would have to be added 8.81 and 7.25 g per 100 g of broiler start feed and white-egg laying hen feed, respectively, to meet the methionine requirement.

I. orientalis and *P. kudriavezevii* have been isolated from raw milk (Chen et al., 2010 a and b). *I. orientalis* usually found in fermented dairy products including kefir (Bai et al., 2010; Latorre-García et al., 2007; Ongol and Asano, 2009). Bai et al. (2010) reported that *I. orentalis* population was between 4 to 11% of total isolated yeast from cow, yak, goat, and camel milk.

I. orientalis and *P. kudriavzevii* are recognized as a "generally recognized as safe" (GRAS) organism and exhibits probiotic abilities (Chen et al., 2010b; Yao et al., 2010). In addition, previous studies have demonstrated the benefits of *I. orientalis* and *P. kudriavzevii* as probiotics. By inoculating the mixture of *I. orientalis*, *Aspergillus* sp., and *Bacillus* sp. in black soybeans, antioxidant and free radical scavenging activities in the fermented product increased (Yao et al., 2010). In addition, *I. orientalis* has been used as commercial product for animal feed (Koh and Suh, 2009). Broiler chicks fed with 0.25% rice bran fermented with *Bacillus subtilis* and *I. orientalis* exhibited higher immunity to against a virulent strain of Newcastle disease virus than broilers supported with Avilamycin (Koh and Suh, 2009).

Chen et al. (2010b) proposed that *P. kudriavzevii* might be a potential probiotic in human intestine since it can assimilate cholesterol and in chickens (Garcia-Hernandex et al. 2012. doi:10.1016/j.rvsc.2011.09.005). This organism was isolated from broiler excreta and exhibited the ability to agglutinate *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, and *Enterococcus faecalis* which could be a mechanism to support animals to resist pathogen establishment.

For several decades yeast cells have been used as single cell proteins because these organisms grow rapidly and the protein contents are generally high (7 to 12 % nitrogen on a dry wt basis) (Kihlberg, 1972). In addition to being a protein source in animal feed, yeast cells are also rich sources of B vitamins (Kihlberg, 1972). Yeast hydrolysate has been used as the sole source of nitrogen in the diet in chicken and rat experiments (Gitler et al., 1958; Rastogi and Murti, 1961). Yeast supplied diets have been used as chicken feed; however, methionine had to be added (Gitler et al., 1958).

From an economic aspect, it is possible to culture strain K1 from various agro-industrial wastes. Harta et al. (2004) proposed producing kefir grains wastes to add value to wastes and reduce pollution. Choi et al. (2002) used waste Chinese cabbage (*Brassica campestris*) as a substrate for yeast biomass production. The advantage of kefir cultured in bioreactor is the ability to form granules (Koutinas et al. 2005). When harvesting kefir biomass, only supernatant removal without a centrifugation step would be required. In addition, the production cost for making wet kefir was 1.4 Euro per kg (Koutinas et al., 2010). From a technology aspect and economical feasibility, strain K1 has potential for use as a supplement or probiotic in the poultry industry.

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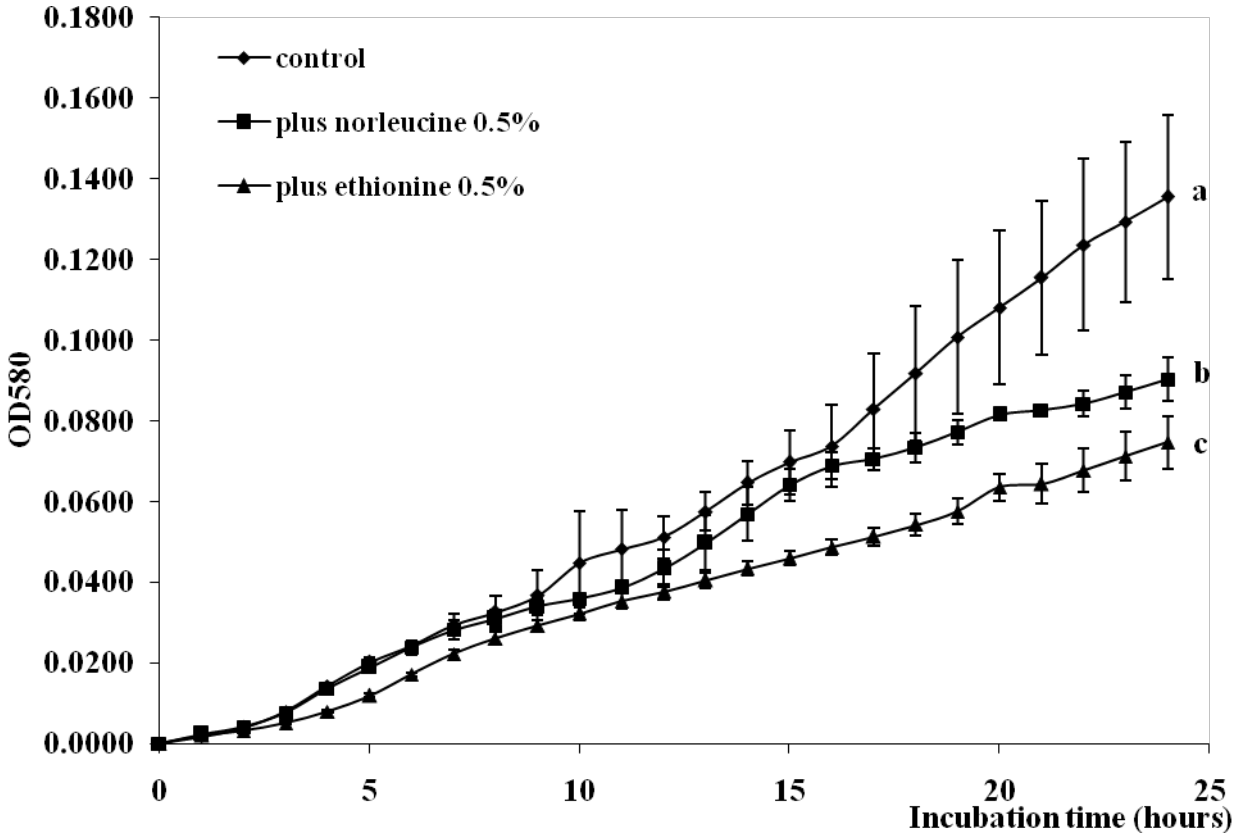


Fig.6.1 The growth of yeast strain K1. This strain was cultured on minimal medium containing either ethionine or norleucine at 0.5% (w/v). Strain K1 isolated from Caucasian kefir grains. [a.b,c]Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

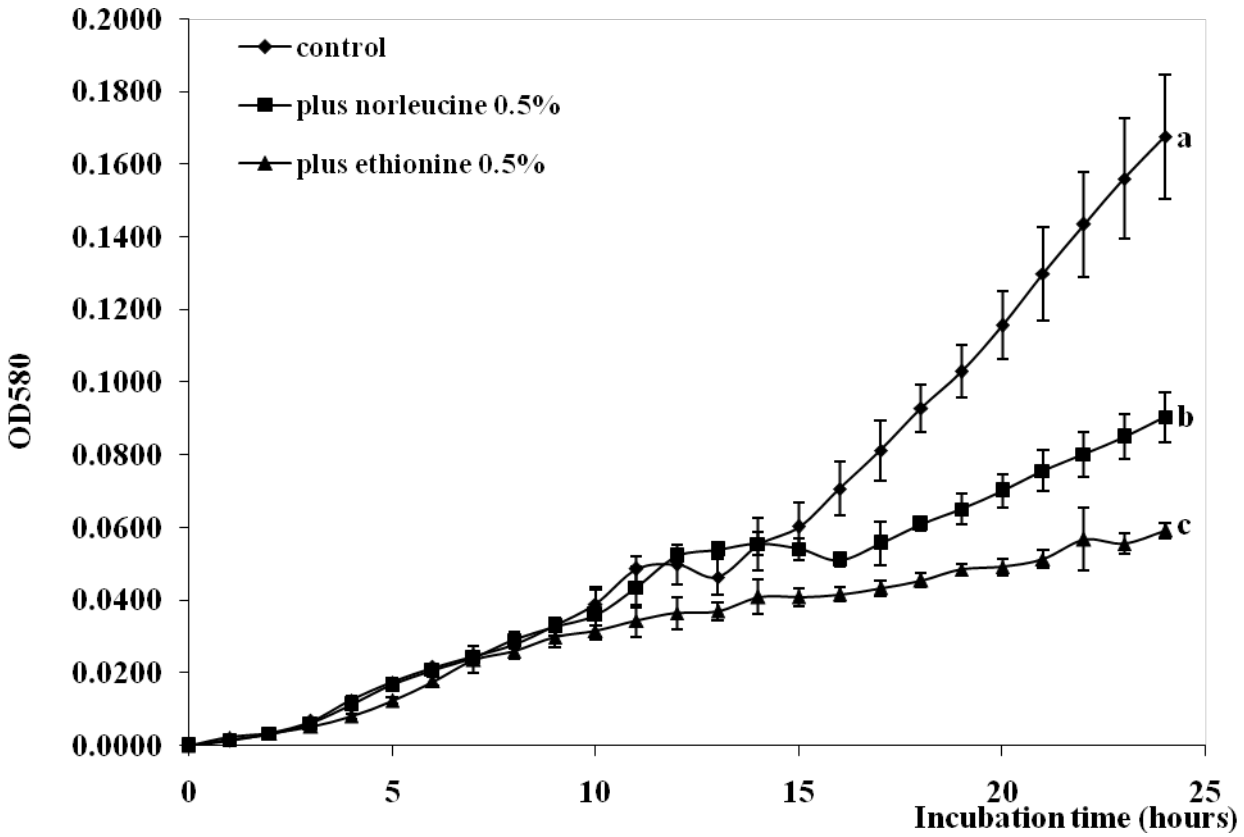


Fig.6.2 The growth of yeast strain *Sac. cerevisiae* ATCC4126. This strain was cultured on minimal medium containing either ethionine or norleucine at 0.5% (w/v). [a.b,c]Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

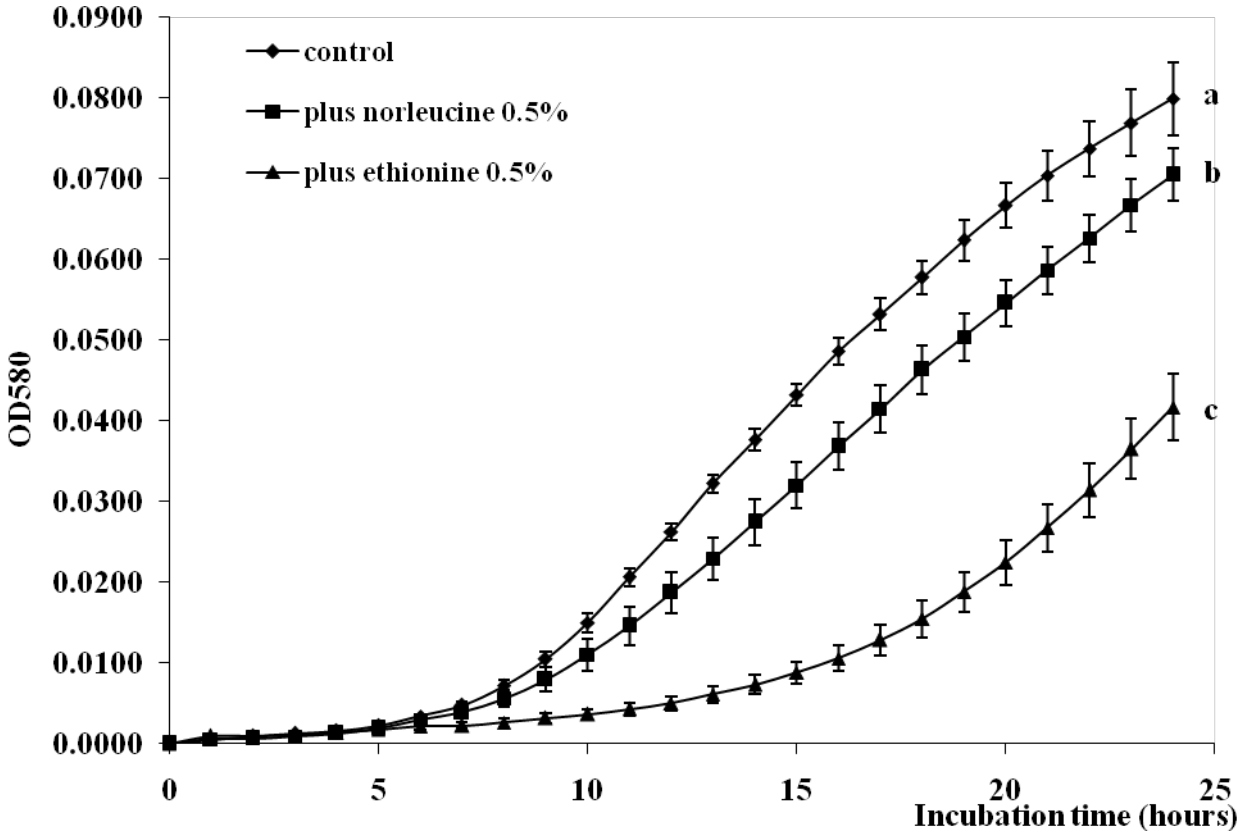


Fig.6.3 The growth of yeast *C. kefyr* ATCC2512. This strain was cultured on minimal medium containing either ethionine or norleucine at 0.5% (w/v). [a.b,c]Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

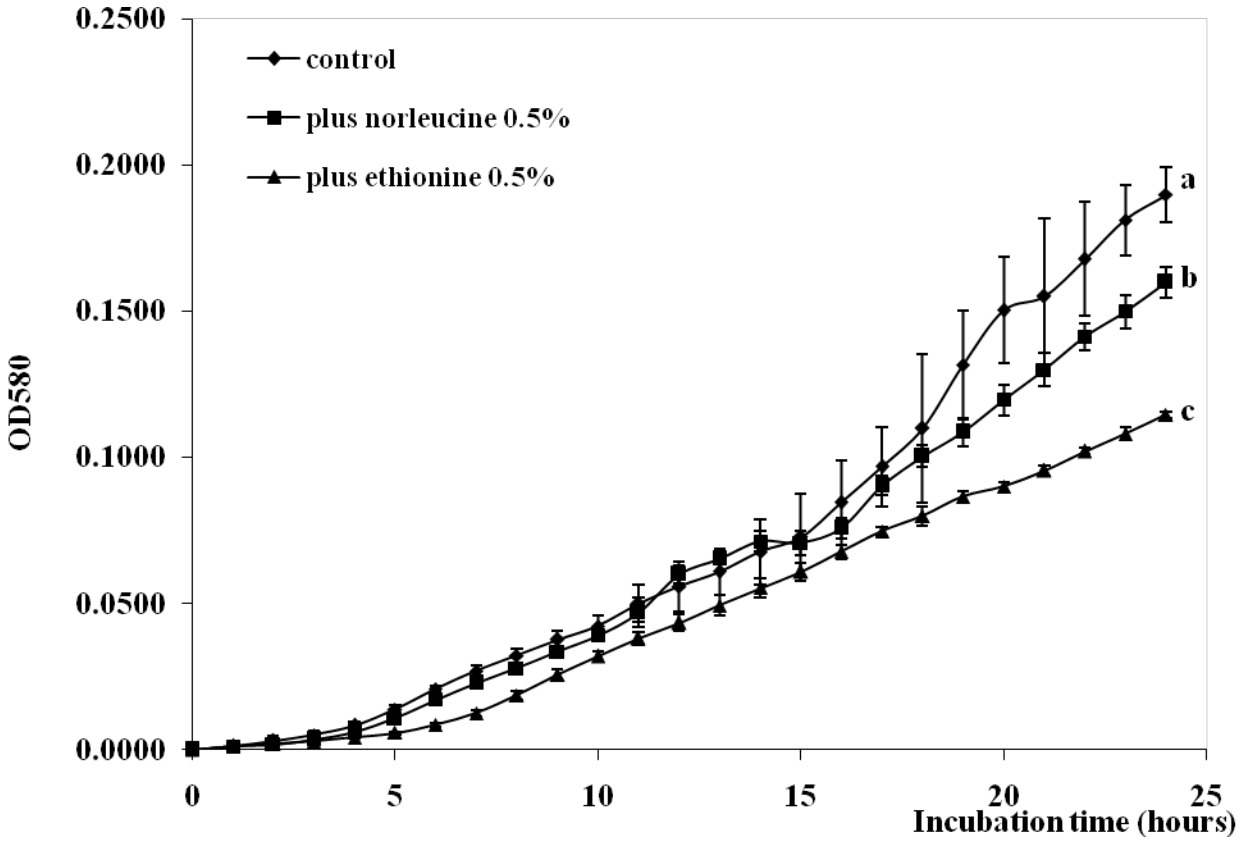


Fig.6.4 The growth of yeast strain C1 isolated from a cantaloupe sample. This strain was cultured on minimal medium containing either ethionine or norleucine at 0.5% (w/v). [a.b,c]Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

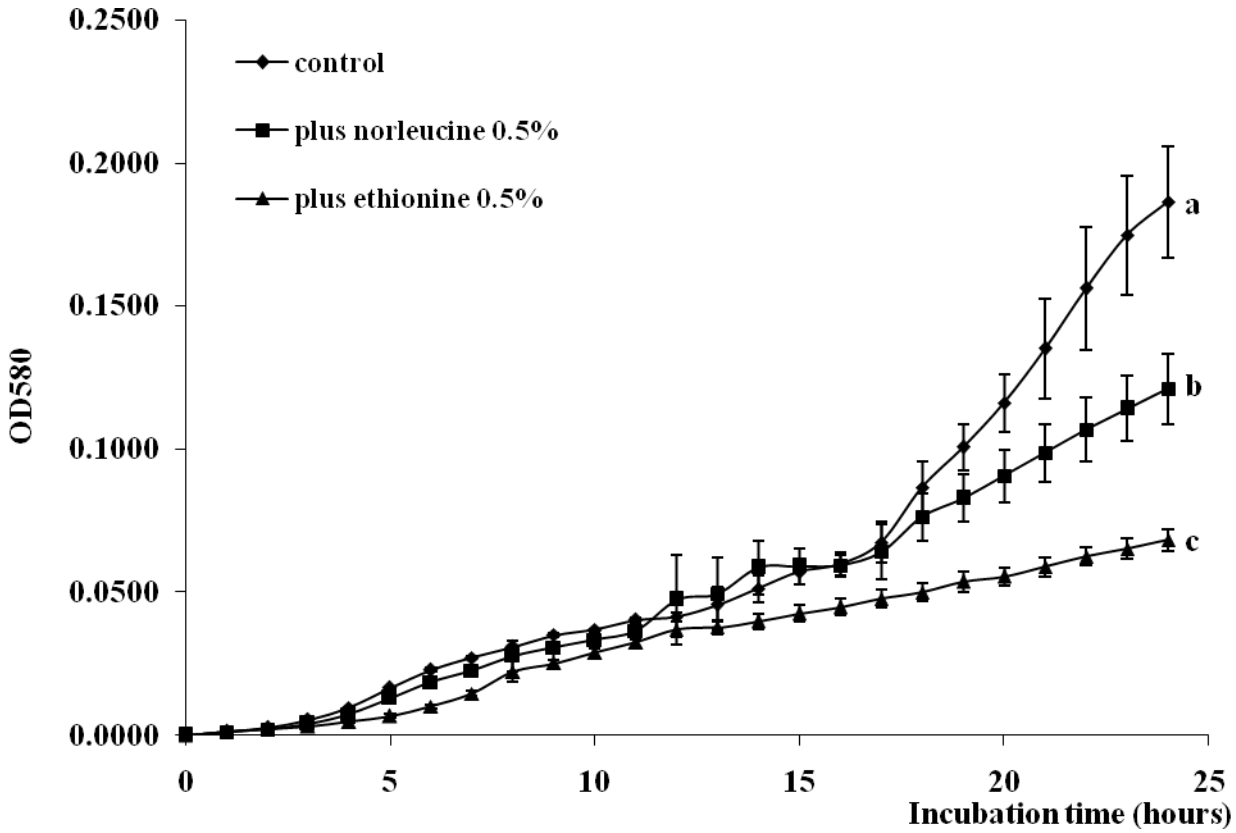


Fig.6.5 The growth of yeast strain S1 isolated from a soil sample. This strain was cultured on minimal medium containing either ethionine or norleucine at 0.5% (w/v). [a.b,c]Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

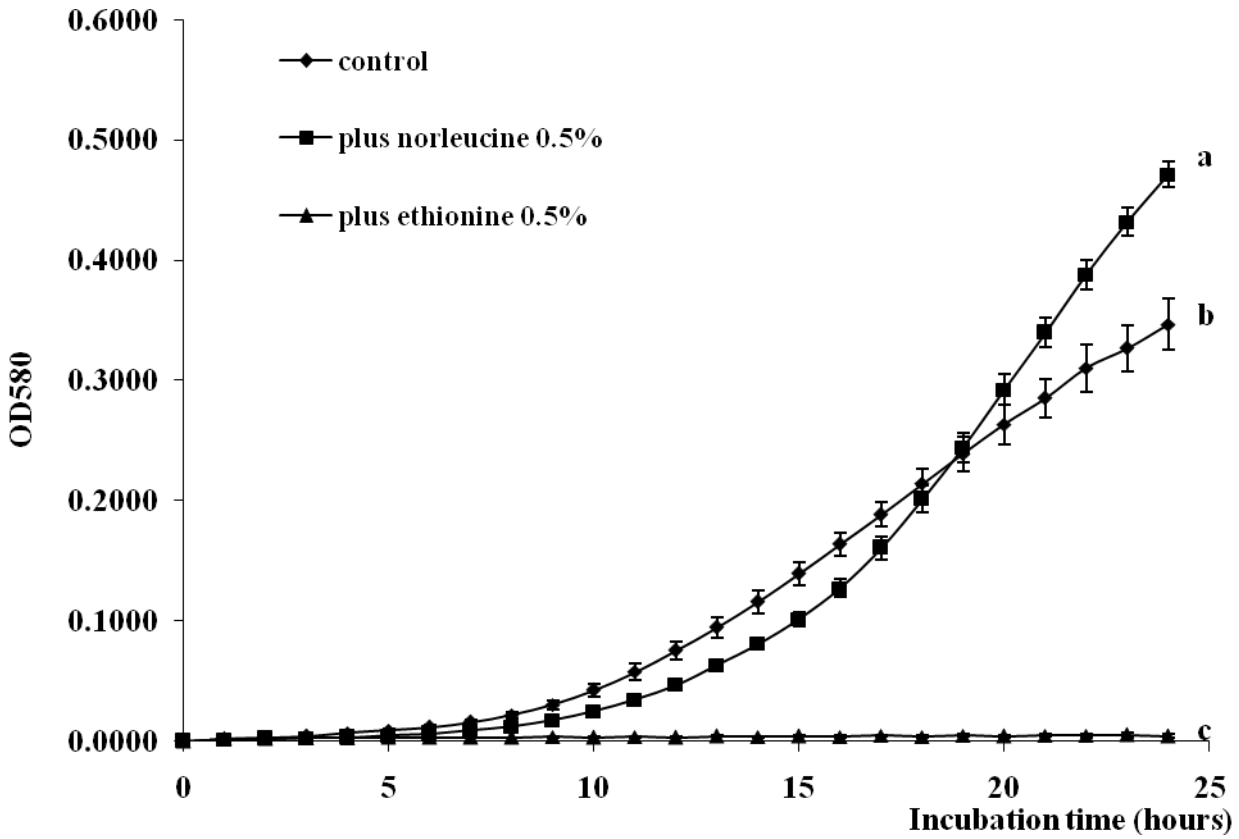
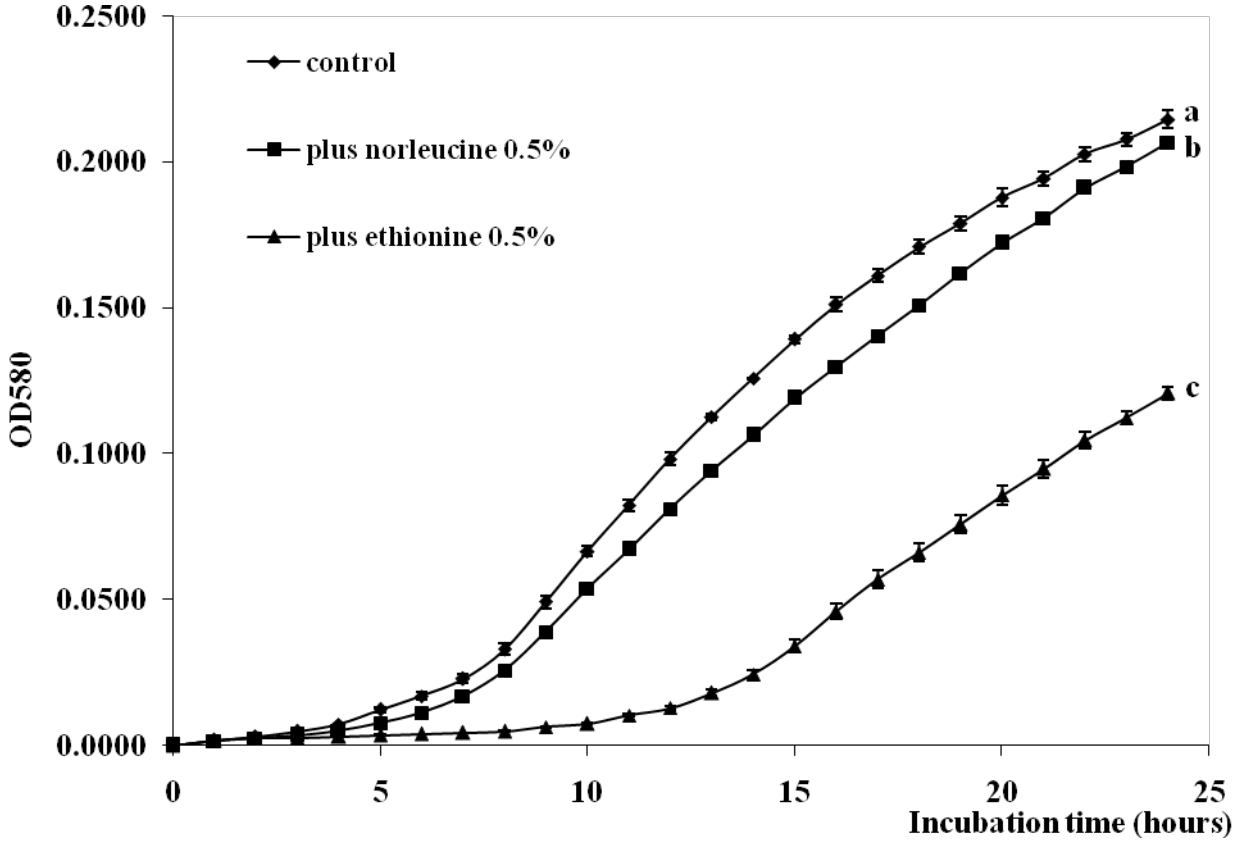


Fig.6.6 The growth of yeast strain S2 isolated from a soil sample. This strain was cultured on minimal medium containing either ethionine or norleucine at 0.5% (w/v). [a.b,c]Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.



(g)

Fig.6.7 The growth of yeast strain S3 isolated from a soil sample. This strain was cultured on minimal medium containing either ethionine or norleucine at 0.5% (w/v). [a.b,c]Means within the same growth measurements with unlike superscripts ($P < 0.05$). Error bars indicate standard deviations between growth measurements.

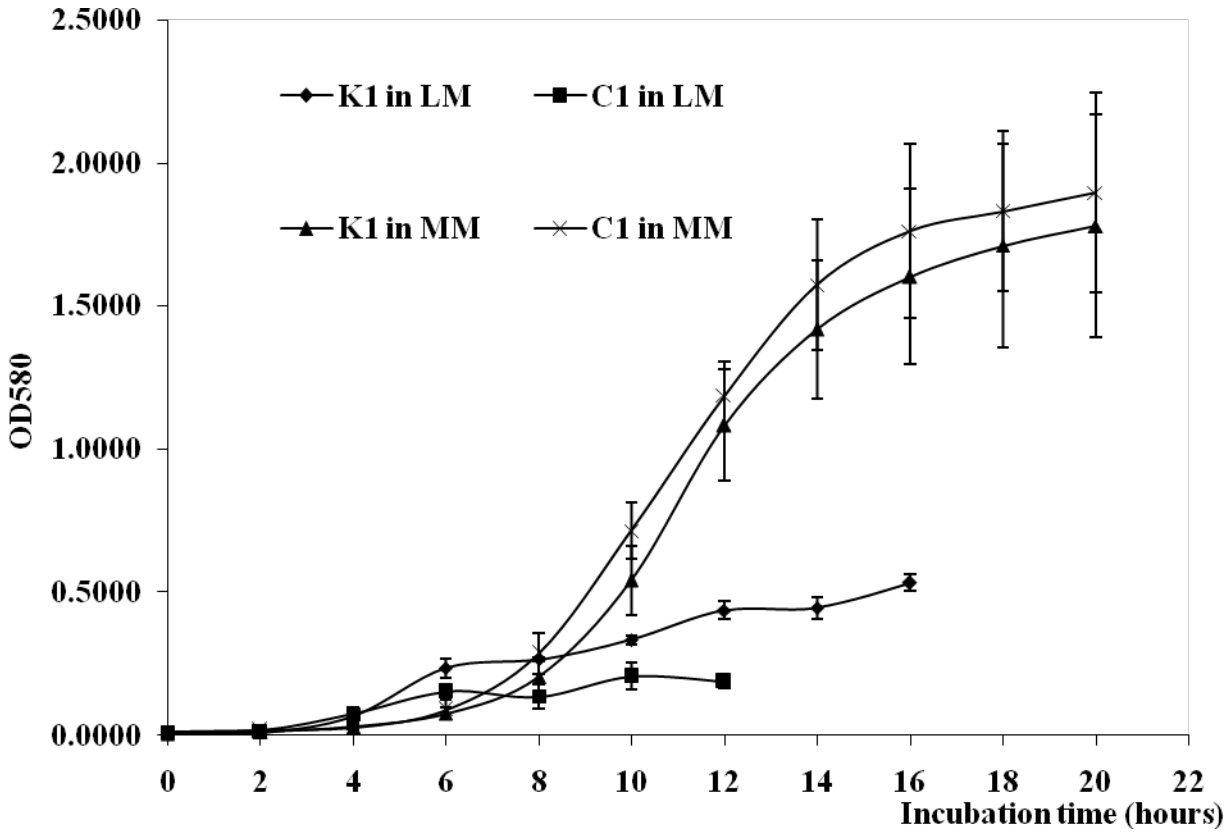


Fig. 6.8. The growth of yeast strain K1 and C1 cultured on lactose medium (LM) and minimal medium (MM) at 30 ^{o}C, 200 rpm. The growth measurements were measured at 580 nm and were performed with at least three independent cultures. Error bars indicate standard deviations between growth measurements.

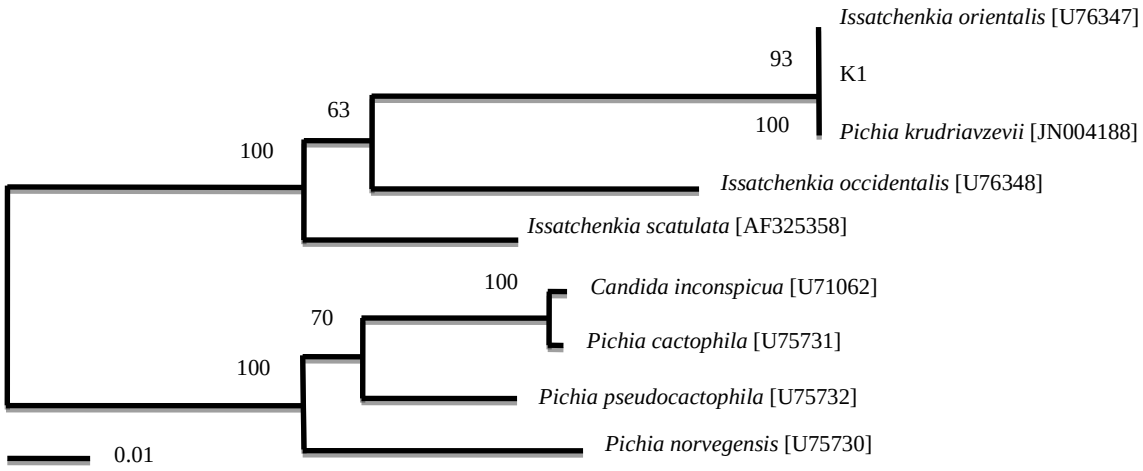


Fig. 6.9. Phylogeny of partial LSU rRNA sequences from the K1 isolate and several closely related yeast species identified by BLAST search. Sequences harvested from GenBank are followed by accession numbers in brackets. The scale represents the nucleotide substitution rate. This Neighbor Joining tree is midpoint rooted.

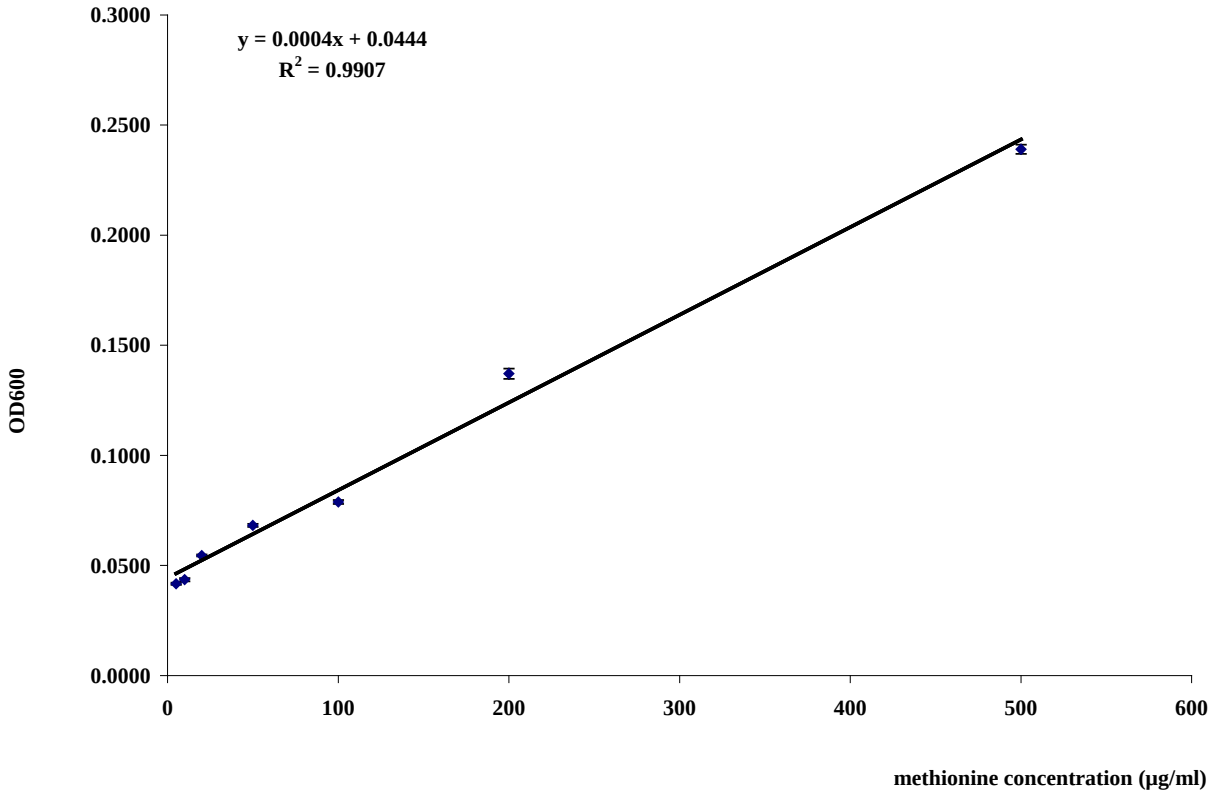


Fig. 6.10. Standard curve generated by *E. coli* K12C cultured on MCGC media supplemented by a dilution series of L-methionine. The mean values and standard deviation of OD600 were performed from triplicates within the same run.

Table 6.1. Yeast strains used in this study.

Yeast species/strain	Source
Schizosaccharomyces pombe ATCC26192	Professor Ines Pinto, University of Arkansas
Saccharomyces (*Sac*.) *pastorianus*	Professor Ines Pinto, University of Arkansas
Sac. cerevisiae HAO	Professor Ines Pinto, University of Arkansas
Sac. cerevisiae ATCC 90607	Professor Ines Pinto, University of Arkansas
Sac. cerevisiae ATCC 4126	Professor Ines Pinto, University of Arkansas
Candida kefyr ATCC2512	Professor Ines Pinto, University of Arkansas
Kluyveromyces marxianus ATCC16045	Professor Ines Pinto, University of Arkansas
Schizosacharomyces (*Sch*.) *octosporus*	Professor Ines Pinto, University of Arkansas
Sch. e*llip*	Professor Ines Pinto, University of Arkansas
C. utilis	Professor Ines Pinto, University of Arkansas
Torulaspora delbrueckii ATCC20100	Professor Ines Pinto, University of Arkansas
K1	Caucasian kefir grains
Y1	Air-trapped yeast sample
C1	Cantaloupe sample
S1	Soil sample
S2	Soil sample
S3	Soil sample

Table 6.2. Media used in this study.

	Minimal medium[a] (per L)	Lactose medium[b] (per L)	MCGC medium[c] (per L)
Glucose	20 g		10 g
Lactose		20 g	
KH_2PO_4	0.5 g	4 g	3 g
Na_2HPO_4			6 g
NaCl			1 g
$MgSO_4.7H_2O$	0.5 g	2 g	200 mg
$(NH_4)_2SO_4$	1 g	5 g	4 g
$CaCl_2.2H_2O$	0.3 g		56 mg
Yeast extract		2 g	
$ZnCl_2$	70 ug		
H_3BO_3	10 ug		
$MnCl_2$	10 ug		
$MnSO_4.2H_2O$			2 mg
$CuSO_4$	10 ug		
$CuCl_2.2H_2O$			0.2 mg
$FeSO_4$	10 ug		20 mg
$FeCl_3$			2 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	10 ug		
$ZnSO_4.7H_2O$			0.5 mg
D-biotin	0.1 ug		1 mg
Thaimin HCl			10 mg

[a] Minimal medium (Morzycka et al., 1976)

[b] Lactose medium (Lukondeh et al., 2003)

[c] MCGC medium (von der Osten et al., 1989)

Table 6.3. Biomass and methionine concentrations in yeast samples

	Biomass (mg dry cell weight/ml) [a]	Methionine concentration (mg/g dry cell weight) [b]	Reference
K1 in lactose medium	0.2142 ± 0.0321	9.2562 ± 0.7307[f]	This study
C1 in lactose medium	0.1567 ± 0.0705	ND[c]	This study
K1 in minimal medium	1.4658 ± 0.1874	19.3037 ± 0.1975[d]	This study
C1 in minimal medium	1.3763 ± 0.0831	15.2984 ± 0.0837[e]	This study
Sac. uvarum	NR[g]	6.2	Brigidi et al. (1988)
Candida petrophilum	NR	12.9	Komatsu et al. (1974)
Kluyveromyces lactis	8.5 to 9.5	14.15	Kitamoto and Nakahara (1994)
Candida utilis	0.27	3.7 to 4.5	Dunyak and Cook (1985)
Candida boidinii	1.7 to 3.6	16.02	Tani et al. (1988)
Sac. lipolytica	NR	1.7	Morzycka et al. (1976)
Candida boidinii	38.5[h]	5.86 to 10.16	Lim and Tani (1988)

[a] The mean of dry cell weight mg per ml culture and standard deviations

[b] The mean of methionine concentration mg per g dry cell weight and standard deviations.

[c] ND, not detected

[d,e,f] Means within the same columns with unlike superscripts differs ($P < 0.05$)

[g] not reported

[h] under optimal conditions

CHAPTER SEVEN

CONCLUSION AND FUTURE RESEARCH

Bifidobacterium linoleic acid isomerase LAI genes were successfully cloned into *E. coli* BL21 (DE3); however, the proteins formed inclusion bodies and no activities were detectable. Possibly, enzyme was an inactive form or activity was very low. Fusion between *Priopionibacterium acnes* LAI with prepropeptide *Staphylococcus hyicus* lipase was successful when this structure was expressed in *Bacillus megaterium* YYBm1. Unfortunately, the propeptide still attached with the secreted *P. acnes* LAI and could impede enzymatic activity. Conversely, prepropeptides of *B. subtilis nprE, B. subtilis amyE, B. megaterium nprM* did not protect *P. acnes* LAI from proteolytic degradation. Therefore, other hosts deleted intracellular proteases may need to be considered

Many bacteria isolated from natural environments appeared to overproduce methionine; however, all of them were subsequently identified as pathogens. In addition, bacteria excreted methionine into the surrounding medium and it is impractical to recover methionine. Thus, it might be better to concentrate on isolating only specific bacterial groups such as those occurring in yoghurt and starter culture producers. Conversely, isolated yeast strain K1 exhibited high methionine content, compared to other yeasts. Based on large subunit rRNA sequencing, these isolated strains were identified as *Pichia kudriavzevii/Issatchenkia orientalis*. *P. kudriavzevii/I. orentalis* is recognized as a ″generally recognized as safe″ (GRAS) organism. Yeast strain K1 may be suitable as a source of methionine for dietary supplements in organic poultry feed.