BIOTECHNOLOGICAL APPROACHES TO THE PHOSPHORUS CYCLE: RECOMBINANT PHYTASE PRODUCTION IN BACILLUS SUBTILIS

by

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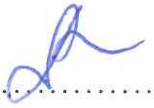
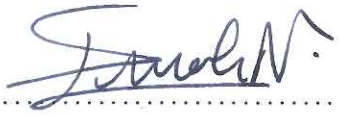
Assoc. Prof. Dr. Elif Damla Arısan ..... 

Assist. Prof. Dr. Emrah Nikerel ..... 

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ABSTRACT

BIOTECHNOLOGICAL APPROACHES TO THE PHOSPHORUS CYCLE: RECOMBINANT PHYTASE PRODUCTION IN BACILLUS SUBTILIS

Phosphorus (P) is classified as one of the most essential macronutrients for all living organisms. It plays a key role in many vital processes such as growth, protein synthesis, repair and energy utilization. Phosphate (P_2O_5), the common inorganic form of P, is mostly found as phytic acid or phytate form (myo-inositol 1,2,3,4,5,6-hexakisphosphate), which cannot be consumed by plants and most of the animals. In order to prevent P deficiency, inorganic phosphate fertilizers are excessively used in agriculture. This strategy is unsustainable since these fertilizers are mostly dependent on phosphate rocks. Besides, the use of fertilizers causes eutrophication in aquatic environments, which results in impairment of the ecosystem. Phytase (3.1.3.8) is an enzyme from the phosphatase family, which catalyzes the hydrolysis of phytic acid into myo-inositol and lower phosphate esters. Especially microbial phytase enzyme is used as a fertilizer or sometimes as a food/feed additive to overcome P deficiency. Therefore, studies have been focused on high yield enzyme production, which also has contribution to both phosphorus cycle and relevant industries.

In this study, *Bacillus subtilis* was chosen as the host for recombinant phytase production due to its ability of extracellular protein secretion. Phytase gene was cloned from *B. subtilis* 168 strain and transferred to *B. subtilis* RIK1285, a protease deficient strain. Considering the importance of the signal peptide for the secretion, as an integral part of the cloning strategy, signal peptides have been screened from the transformants with varying phytase activity. In total, twenty-nine *B. subtilis* transformants were obtained with a phytase activity range of 0.03-0.3 U/mL. Sequencing of the selected recombinant plasmids revealed that the colonies contained the signal peptides, lipB or dltD. Also, comparison of different growth media showed that the presence of rice bran increased the efficiency of enzyme production. This study underlines the importance of not only the modifications on a molecular level but also the choice of growth medium for increasing the efficiency of recombinant enzyme production.

ÖZET

FOSFOR DÖNGÜSÜNE BİYOTEKNOLOJİK YAKLAŞIMLAR: BACILLUS SUBTILIS'DE REKOMBİNANT FİTAZ ÜRETİMİ

Fosfor (P) bütün canlılar için gerekli bir makro besin olarak sınıflandırılmaktadır. Büyüme, protein sentezi, onarım ve enerji kullanımı gibi birçok yaşamsal süreçte kilit rol oynamaktadır. Fosfat (P_2O_5), P'nin en yaygın inorganik formu, çoğunlukla fitik asit ya da fitat (myo-inositol 1,2,3,4,5,6-hexakisphosphate) formunda bulunmaktadır, fakat bitkiler ve çoğu hayvan tarafından tüketilememektedir. Tarımsal alanlarda, P eksikliğini önlemek için yüksek seviyede inorganik fosfor gübresi kullanılmaktadır. Bu gübrelerin üretimi çoğunlukla fosfat kayalarına bağımlı olduğundan sürdürülemez bir stratejidir. Ayrıca, gübre kullanımı akuatik ortamlarda ötrofikasyona yol açmaktadır, bunun sonucunda ekosistem zarar görmektedir. Fitaz (3.1.3.8), fosfataz ailesinden bir enzim olup fitik asitin myo-inositol ya da daha düşük fosfat esterlerine hidrolize olmasını sağlamaktadır. P eksikliğinin giderilmesinde, özellikle mikrobiyal fitaz enzimi, gübre veya gıda/yem maddesi katkısı olarak kullanılmaktadır. Bu nedenle, çalışmalar hem fosfor döngüsüne hem de ilgili sektörlere katkıda bulunan yüksek verimli enzim üretimine odaklanmıştır.

Bu çalışmada, hücre dışı protein salgılama özelliği nedeniyle *Bacillus subtilis*, recombinant fitaz üretimi için konakçı hücre olarak seçilmiştir. *B. subtilis* 168 suşundan klonlanan fitaz geni, proteaz yoksunu olan *B. subtilis* RIK1285 suşuna aktarılmıştır. Rekombinant proteinin hücre dışına lokalizasyonunda sinyal peptidlerinin önemi dikkate alınarak, farklı sinyal peptidleri ile hücre dışı en yüksek fitaz aktivitesi veren transformantlar elde edilmeğe çalışılmıştır. Fitaz aktiviteleri 0.03-0.3 U/mL arası değişkenlik gösteren toplamda yirmi dokuz tane *B. subtilis* transformantı elde edilmiştir. Seçilen rekombinant plazmidlerin sekanslama sonuçları, kolonilerin lipB ya da dltD sinyal peptidi içerdiğini ortaya çıkarmıştır. Ayrıca farklı büyüme ortamlarının karşılaştırması, besiyerinde bulunan pirinç kepeğinin enzim üretim verimini arttırdığını göstermiştir. Bu çalışma, rekombinant enzim üretim verimini arttırmak için sadece moleküler düzeydeki modifikasyonların değil, büyüme ortamı seçiminin de önemini vurgulamaktadır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

A. ficuum	*Aspergillus ficuum*
A. fumigatus	*Aspergillus fumigates*
A. niger	*Aspergillus niger*
A. thaliana	*Arabidopsis thaliana*
B. megaterium	*Bacillus megaterium*
B. mucilaginosus	*Bacillus mucilaginosus*
B. subtilis	*Bacillus subtilis*
E. coli	*Escherichia coli*
P. lycii	*Peniophora lycii*
P. pastoris	*Pichia pastoris*
S. cerevisiae	*Saccharomyces cerevisiae*
Taq	*Thermus aquaticus*
α	Alpha
β	Beta
C	Celcius
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
C	Carbon
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
Df	Dilution factor
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (2-aminoethylether)-*N,N,N,N'*-tetraacetic acid

Fe	Iron
$FeSO_4 \cdot 7H_2O$	Iron (II) sulfate heptahydrate
g	Earth's gravitational force
gDNA	Genomic DNA
GRAS	Generally Recognized as Safe
$H_2PO_4^-$	Dihydrogen phosphate
HAP	Histidine acid phosphatases
HPO_4^{2-}	Hydrogen phosphate
IP6 sodium salt	Phytic acid sodium salt
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IUPAC-IUB	International Union of Biochemistry
K	Potassium
K_2HPO_4	Potassium phosphate dibasic
K_2O	Potassium oxide
K_2SO_4	Potassium sulfate
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
L	liter
LB	Luria-Bertani
LPS	Lipopolysaccharides
MCS	Multiple cloning site
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
min	minute
ml	milliliter
mM	millimolar
mmol	millimole
$MnSO_4 \cdot 5H_2O$	Manganese sulfate pentahydrate
N	Nitrogen
$(NH_4)_2SO_4$	Ammonium sulfate
$Na_3citrate.2H_2O$	Sodium citrate dihydrate
NB	Nutrient Broth

NCBI	National Center for Biotechnology Information
OD	Optical density
P	Phosphorus
P_2O_5	Phosphorus pentoxide
PA	Phytate
PAP	Purple acid phosphatase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGPR	Plant Growth Promoting Rhizobacteria
pH	Potential of hydrogen
phy	Phytase
Pi	Inorganic phosphate
PSM	Phytase screening medium
rpm	revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sec	second
SOC	Super Optimal Broth with Catabolic Repressor
SP mix	Signal peptide mix
SP	Signal peptide
Std	Standard
TCA	Trichloroacetic acid
Tm	Melting temperature
tRNA	Transfer ribonucleic acid
U	unit
U.S.	United States
UV	Ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume
X-gal	5-Bromo-4-Chloro-3-Indolyl-D-Galactopyranoside
Zn	Zinc
µg	microgram

µl	microliter
µmole	micromole

1. INTRODUCTION

Phosphorus (P) is classified as one of the most essential macronutrient for all living organisms. It is used in many processes such as growth, protein synthesis, repair, energy production and pH regulation. In nature, phosphate (P_2O_5) is mostly found in the form of phytic acid. Especially the largest phosphorus source in the soil, phytate (salt form of phytic acid), binds to free metals such as Calcium (Ca), Zinc (Zn) and Iron (Fe), and inhibits the uptake of these metals by other organisms. Plants use their roots for obtaining the phosphorus needed for their growth. Even though phytic acid is the main storage form of phosphorus in plants, they can only take up the inorganic phosphorus (Pi) and cannot make use of the phytate. In order to prevent P deficiency, inorganic phosphorus fertilizers are excessively used in agriculture. This strategy is unsustainable for high crop yield production since these fertilizers are mostly dependent on phosphate rocks. These sources are not only non-renewable but also economically unstable which point outs a shortage of inorganic phosphorus in the near future.

Although phytic acid is the storage form of the phosphorus in seeds, plants cannot directly utilize the phytic acid found in soil. They use an intracellularly produced phytase to break down the phytic acid, which is needed for seed germination. The high amount of phytic acid located in seeds, grains and seed dependent products that are used as feed does not provide animals the sufficient amount of phosphorus since their gastrointestinal track is not suitable for the digestion of phytic acid (PA). In order to solve these nutritional problems, a common solution is to provide inorganic phosphorus supplements for animals and fertilizers for plants. Besides the nutritional problems, the unutilized phosphorus found in the animal waste and the applied fertilizers cause soil pollution leading to eutrophication. In order to protect the environment from the undigested phytic acid found in the animal waste, research studies have focused on a favorable and an economical source of inorganic phosphorus.

Phytase (3.1.3.8) is an enzyme from the phosphatase family, which catalyzes the hydrolysis of phytic acid into myo-inositol and lower phosphate esters. It has been considered as an artificial fertilizer and sometimes as a food/feed additive to overcome the aforementioned problems. Recombinant DNA technology, which is one of the most

commonly addressed molecular tool, has been used for high scale extracellular phytase production. Heterologous protein expression systems differ accordingly with the desired product and its specifications. An efficient protein expression system considers three main properties: 1) Gene expression system, 2) Secretion system, and 3) Fermentation conditions. Most of the recombinant enzymes are produced via utilization of *Escherichia coli* as a host due to its easy culture, fast growth and developed expression tools. For each recombinant protein production, a new set up should be designed considering the types of constraints and the aim of the investigation. One of the disadvantages of recombinant protein production is the low efficiency at larger scales. To obtain higher efficiency through recombinant protein production, new expression systems that are capable of high level production and purification have been developed. The main requirements of these expression systems are easy control, fast growth rate and high level protein production.

In this study, *Bacillus subtilis* was chosen as the host for recombinant phytase production due to its ability of extracellular protein secretion. Phytase gene was cloned from *Bacillus subtilis* 168 strain and transferred to *Bacillus subtilis* RIK1285, a protease deficient strain. Considering the importance of the signal peptide (SP) for extracellular localization of the recombinant protein, different signal peptides were experimented for the highest extracellular phytase activity. In total, twenty-nine *B. subtilis* transformants were obtained with an activity range of 0.03-0.3 U/mL. Sequencing of the selected recombinant plasmids revealed that the colonies contained the signal peptides, lipB or dltD. Also, comparison of different growth media showed that the presence of rice bran increased the efficiency of enzyme production. This study underlines the importance of not only the modifications on a molecular level but also the choice of growth medium for increasing the efficiency of recombinant enzyme production. The outcome of the study targets optimizing the expression system further for a more approachable and efficient phytase enzyme production and emphasizes the importance of SP engineering.

2. LITERATURE REVIEW

2.1. PHOSPHORUS / PHYTIC ACID / PHYTATE

All living systems require circulation of various elements such as Carbon (C), Nitrogen (N), Potassium (K) and some other minerals to survive and grow. One of the most essential elements, Phosphorus (P), acts as a key constituent by its presence in both DNA and RNA. It is also used in many cellular processes such as growth, protein synthesis, repair, energy production and even pH regulation [1].

Phosphate (P_2O_5) is basically an inorganic compound that consists of phosphorus elements bound with Oxygen (O). The most abundant form of phosphates is myo-Inositol-1,2,3,4,5,6-haxakisphosphate, which is also known as phytic acid (PA) [2]. This dominant form of organic phosphate makes up almost 70 per cent of the soil phosphorus [3]. It consists of an inositol ring with six phosphate ester bonds (Figure 2.1).

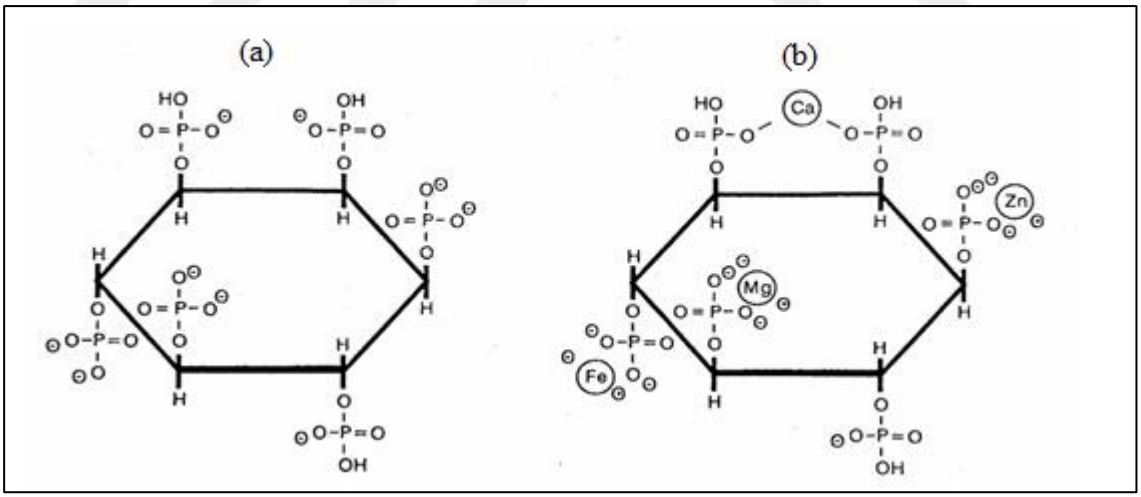

Figure 2.1. Chemical structures of phytic acid (a) Phytic acid myo-inositol hexakisphosphate, and (b) Phytic acid in a chelate form (phytate) [2].

When phytic acid is examined in a complete dissociation state, it is observed that PA has the capability of binding to other minerals in a wide range of pH. This property of phytic acid creates a high affinity for chelating with other minerals and salts such as Magnesium

(Mg), Iron (Fe), Zinc (Zn), Calcium (Ca) and even carbohydrates and some proteins (Figure 2.1). Phytic acid is generally found in its salt form called phytate, or phytin if it is a calcium or magnesium salt. It is recognized with its anti-nutritional behavioral by limiting the bioavailability of important minerals [4]. Though there is abundant amount of phytate in soil, this P source cannot be used directly by most of the animals and plants. P fertilizers and supplementations are used for regular growth in agriculture and animal husbandry.

Plants supply their phosphorus need by up-taking inorganic forms such as dihydrogen phosphate ($H_2PO_4^-$) and hydrogen phosphate (HPO_4^{2-}) via their roots. Rhizosphere organisms located near the roots breakdown the phosphorus into primary and secondary ortho-phosphate ions and make it available for plants. If the plant cannot find any phosphorus source or the rhizobacteria cannot efficiently utilize phosphorus, a biomass decrease is observed.

Looking into the phosphorus storage mechanisms in plants, it is observed that phosphorus is mainly stored in the form of phytic acid or its salt form, phytate. Even though the amount of inorganic phosphorus (Pi) shows diversity among different plants and plant parts, it can be up to 50 to 80 percent of the total phosphate in plant seeds, and 90 percent in cereals, legumes and seeds [6]. Phytate is formed during the maturation of the plant seed and in dormant seeds it represents 60 to 90 percent of the total phosphate [7]. The minerals bound to phytate also serve as a mineral deposition for the plants especially in the seeds, with priority being used for the energy metabolism at the germination phase [8]. In the latter phases, the phosphorus is supplied to the plant from the soil.

Due to the poor intake of the available form of P, inorganic phosphorus fertilizers and manure are applied for compensating the inorganic phosphorus need of the plant. Unfortunately, a high amount of the applied fertilizer is rapidly fixed in the soil and becomes unavailable for plants. Therefore, while crops utilize 10 to 20 percent, the rest of the phosphorus is transformed into its organic forms creating a fruitless cycle (Figure 2.2.).

The predicament is that animals and humans use the plants as nutrient source, but phytic acid, the major form of organic phosphorus in plants, is not accessible for them. Though ruminants incorporate the enzyme that breaks down phytic acid, called phytase, other non-ruminants and monogastric animals lack the endogenous enzymes for phytic acid digestion.

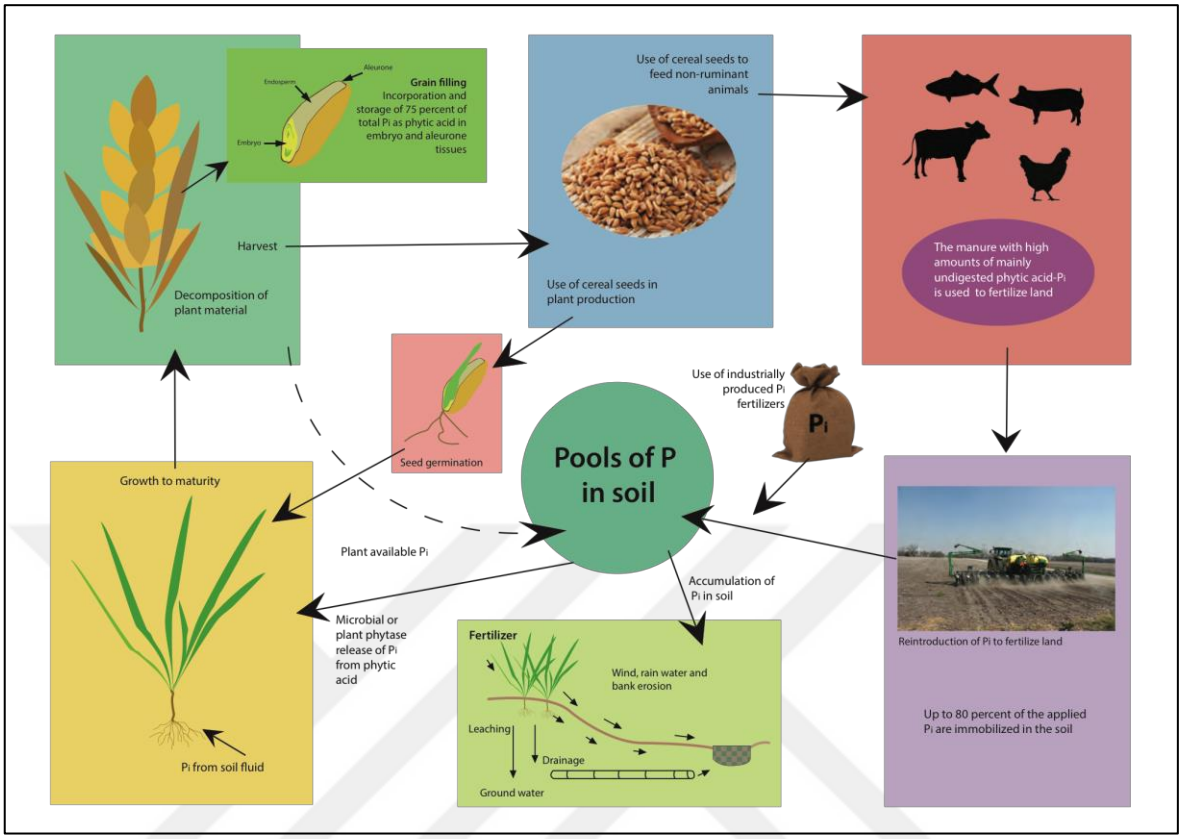

Figure 2.2. Phosphorus cycle in real life [5] (modified from Brinch-Pedersen et. al., 2002).

When animals cannot get enough phosphorus, their growth is affected. Therefore, supplementation with extra phosphorus additives or exogenous enzymes such as phytase enhances animal feed performance for increasing feed conversion rate (proportion of growth relative to the amount of feed consumed) [9].

2.1.1. World Phosphorus Demand

Total amount of phytate content stored in fruits and crop seeds are around 51 million tons. In nature, phosphorus can be found in many different forms and availability but compared with its demand, the usable phosphorus (i.e. inorganic phosphorus) is not sufficient. The inorganic phosphate demand was declared to be 45 million of tones in 2016 with an increasing number [10,11].

Mostly, phosphate rock reserves are used to meet the inorganic phosphorus requirement of the world, which is non-renewable and costly. Taking into consideration of the locations of

the phosphate mines, they are predominantly controlled by five countries which results in a compelling inorganic phosphorus market since it is a necessity. The depletion of these mines is a growing concern. Since it is a fossil source, the formation of these mines is a very slow process [12]. Phosphorus is an essential component of commercial fertilizers and the world inorganic phosphorus demand is increasing (Figure 2.3). Phytase is a promising alternative to rock phosphate fertilizer application by its ability to release inorganic phosphors from the phytic acid or its salts [13].

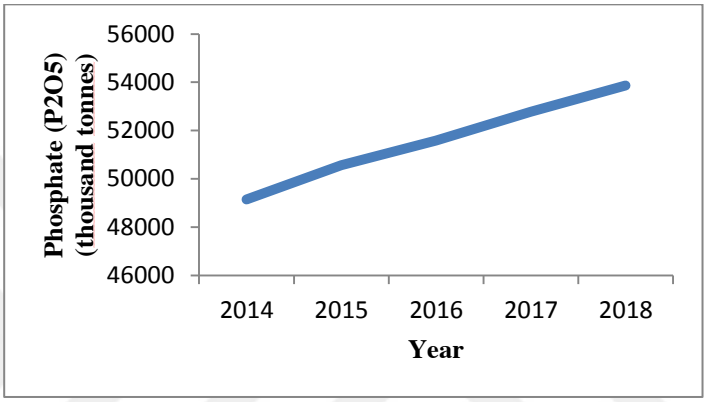

Figure 2.3. Total world demand for phosphorus (P_2O_5) [10].

2.2. PHYTASE

Phytase (EC 3.1.3.8) is an enzyme belonging to phosphatases or phosphohydrolase group that is recognized by its ability to release inorganic phosphorus from phytic acid or its salt (Figure 2.4). Phosphohydrolase plays role in initiating the removal of at least one phosphate group from phytate, hydrolyzing to less phosphorylated myo-inositol intermediates with at least one free inorganic phosphorus [14].

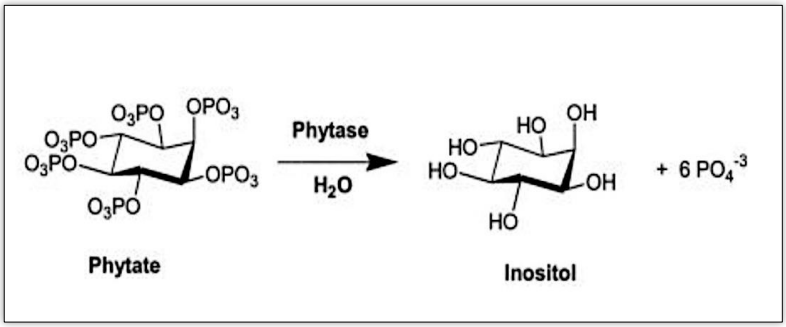

Figure 2.4. Phytate chair conformation and its conversion by phytase [15].

Phosphorus is mainly stored as phytic acid/phytate form in plants, mainly in the seeds. The phytate stored in the seeds is used as an energy source for the primary initiation of growth as well as mineral storage. To access this phosphorus source in the seed, plants use their own intracellular phytase when all the conditions are optimized for the germination such as the temperature and pH. It is stated that the intracellular phytase of plants is specific for their own conditions and not effective as microbial phytases. The main obstacle for plants is that they cannot use their enzymes for utilizing the soil phosphorus. Therefore, they take help from microorganisms that live in the soil which are capable of producing extracellular phytase.

Although ruminants and some fish can digest phytic acid with the help of phytase producing living microorganisms in their stomach or intestines, monogastric animals and humans lack such microorganisms [16]. As a strategy, either exogenous phytase can utilize the phytic acid, or inorganic phosphorus is added to the feedstuff. Generally, microbial phytase is assigned for this mission although the thermo stability can be a problem.

Phytase is therefore referred as an environmental friendly product because it can degrade the phytic acid and decrease phosphorus contamination in nature. Thus, phytase can be an alternative to costly and non-renewable inorganic phosphorus [17].

2.2.1. Sources and Classification of Phytase

Phytase can be found in different sources in nature ranging from microorganisms to animals and plants, but the activity and its optimized conditions change respectively [18]. For almost all commercial phytases, microbial sources are preferred due to the lack of need for post-translational modification. Phytase found in animals show almost zero activity compared to phytase found in plants and microbes [19].

Phytase has been found in many different microbial organisms with each of them exhibiting different properties. Among 2000 phytate hydrolyzing microbial isolates from the soil, the majority produce intracellular phytase [20]. All phytases have different sizes with a range between 12.8 to 700 kDa. Bacterial phytase is smaller when compared to fungal phytase. The phytase produced in *B. subtilis* is known to be 41 kDa, in *E. coli* as 43 kDa and *Aspergillus niger* as 49 kDa. The molecular mass changes according to the origin

of phytase because of the differences in the glycosylation pattern of different organisms [21]. In the cases where the bacterial phytase was expressed in yeast cells, the product was observed to have a bigger molecular mass due to glycosylation mechanisms with a higher thermostability [19].

In literature, there are different ways of classifying phytase into families or classes according to their catalytic mechanism or position of the first dephosphorylation characteristics. Most common type of classification is according to their biochemical properties, similarity in their genomic sequence and catalytic mechanisms. There are three known types, Histidine Acid Phosphatases (HAP), β-propeller phytase and purple acid phosphatase (PAP) [21–23].

Histidine acid phosphatases are the most frequent phytase family with an acidic pH range that is usually produced by fungus or bacteria. They are characterized with their low pH profile (pH 1.3-5.5) and activation by ethylenediaminetetraacetic acid (EDTA) [24]. β-propeller phytase, which is named after its chemical structure that resembles a propeller with 6 blades, is considered unique due to its neutral pH and calcium dependency [25]. Phytase isolated from *Bacillus* genus is classified under this category. A disadvantage of this phytase is inhibition by ethylene glycol tetraacetic acid (EGTA) or EDTA. Purple acid phosphatase (PAP) and protein tyrosine phosphatase-like family are less popular among others due to their low catalytic activity [22,25].

Another nomenclature stated by International Union of Biochemistry (IUPAC-IUB) is according to the position of the first dephosphorylation on the inositol cycle, i.e. 3-phytase (EC 3.1.3.8), 6-phytase (EC 3.1.3.26) or 5-phytase (EC 3.1.3.72). Generally, 3-phytase and 6-phytase are found in microorganisms whereas 5-phytase associates with the plant phytase [2]. Although they all have the ability to dephosphorylate a phytic acid molecule, 6-phytase also has the capability to completely destruct phytic acid. The most common 3-phytase producer is *A. niger* where as 6-phytase producers are *E. coli* and *Peniophora lycii*.

2.2.2. Industrial and Agricultural Applications of Phytase

Phytase has many industrial applications, mostly concentrated in feed enzyme market. The commercial enzyme is in use since 1991 for both agriculture and animal feed. First

commercial phytase was isolated from *A. niger* belonging to the histidine acid phosphatases family. Phytase covers almost 60 percent of the total feed enzyme market among other enzymes such as β-glucanases, xylanases and galactosidases [11].

Phytase is used as a feed supplement in diets for swine and poultry and for some fish species [26]. Using phytase enzyme not only eliminates the need of inorganic phosphorus but also releases other nutrients that are bound to phytate. Studies show that the inorganic phosphorus load has become dangerously high in water supplies near animal husbandries [8]. As an alternative for expensive inorganic phosphate additives, phytase can minimize the phosphorus waste and balance the phosphorus cycle in a long-term usage.

Plant material generally contains high phytin amount. In areas where these materials are essential, they must be dephytinized in order to be safe. Degradation of phytic acid with phytase does not produce any carcinogenic or toxic byproducts; hence it's seen as an environmental friendly option for pulp and paper industry [27]. Another possible usage of phytase is improvement of the nutritional values in human food.

2.3. RECOMBINANT DNA TECHNOLOGY

Proteins are important macromolecules not only for metabolic activities of a cell but also for industrial purposes. The downside of natural protein translation is low expression rate, which is an issue when industrial production is the focus. Recombinant DNA technologies are used for solving the yield problem and altering the products for specific needs. With the recent improvements in genetic engineering, these productions have become easier and cheaper. Different types of organisms such as bacteria, fungi, plants and mammalian cells became the biological factories for these targeted proteins used in industrial, medical, agricultural and research intended proteins [28,29].

Recombinant DNA technology brings together some of the molecular tools used for manipulation of genes. Mainly, a desired gene sequence is cloned and ligated into a piece of DNA, known as the vector, with restriction endonucleases. This vector, consisting of the desired gene, promoter and the required elements is then transferred to the host for production. By the use of this technology, specific genes belonging to an organism can be

isolated for amplification, functional investigation and characterization, or production in another organism.

Recombinant DNA technology is a useful tool for producing desired proteins or other products in specifically planned conditions. According to the need, different parameters can be manipulated and optimized for the highest yield. For example, to facilitate the isolation and purification step and to overcome the extraction step, the product can be expressed exogenously with a specific tag, or a specific promoter can be selected for expression. As a simple, yet delicate technology, the affecting factors should be analyzed all together for a collaborative system to yield efficient production.

In some cases, the produced amount cannot reach the expected large-scale production. To be able reach higher yields through this technology, expression systems have been developed. Some of the important criteria include easy culture, fast growth, and high amounts of production. Since for each protein a different number of criteria are involved to reach optimized production, it's important to find the right system. Therefore, basically, the molecular and physiological properties of the protein and the scale of production guide should be tailor made to choose the expression system since no system can be described as universally optimal [30].

2.3.1. Recombinant Protein Expression Systems

2.3.1.1. Bacterial Expression Systems

Bacteria are commonly preferred for recombinant production due to their easily cultured nature and short doubling time. Low cost culture media and easy adaptation of these conditions to large-scale production make bacterial systems more preferable [31].

Generally, *E. coli* is one of first host that comes to mind when bacterial systems are the subject. The availability of its whole genome sequence and its relatively easier genetic manipulation have created a large area of study combining both physiological and genomic investigations. Also, *E. coli* cells can grow in high densities, which make it preferable in high scale fermentation productions. On the other hand, *E. coli*, being a gram-negative bacterium, produces endotoxins also known as lipopolysaccharides (LPS), which

contaminate the protein solutions during production [32]. Unfortunately, these endotoxins are highly dangerous for mammalians, in particular for humans, and there is no 100 per cent safe method for purification [33]. Also, due to its prokaryotic nature, *E. coli* is not capable of post-translational modifications such as glycosylation, acetylation, ubiquitination and phosphorylation, which can be underlined as a huge obstacle when it comes to the production of eukaryotic proteins. Despite of these downsides, *E. coli* holds its place for the host choice for 30 years for producing more than 50 per cent of the total recombinant protein. As a result of its popularity, a large array of molecular tools and engineered strains have been developed [32].

Bacillus subtilis is a well-known gram-positive bacterium that is targeted for development in recombinant protein production. Some well-known industrial enzymes such as amylase and cellulase are produced in recombinant *B. subtilis* [4]. On contrary to *E. coli*, *B. subtilis*, being a gram-positive bacterium, does not produce endotoxins. Thus it is free of the risks and dangers that were related with the lipopolysaccharides. U.S. Food and Drug Administration (FDA) announced *Bacillus* species as a Generally Regarded As Safe (GRAS) organism, which highlighted its potential [34]. Besides the absence of lipopolysaccharides, *Bacillus subtilis* has a mechanism for secretion of proteins into the medium, which leads to easy downstream processes. The growth medium can be prepared with low-cost substrates but it generally requires optimization for high yield expressions.

The lack of significant bias in codon usage and ease of genetic manipulation are other important properties that make *Bacillus* a remarkable choice for recombinant protein production [4]. A drawback of this system is that while producing the targeted protein extracellularly, proteases, which have the capacity to degrade heterologous proteins, are produced and secreted into the medium as well. To prevent the degradation of the targeted enzyme by proteases, strain modifications have focused on producing protease deficient strains such as RIK168 [35]. Nevertheless, there is a large gap in the development of expression plasmids and modified strains when *Bacillus* is compared to *E. coli*. Overall, *Bacillus subtilis* has a long way ahead to become a popular industrial production with the ability of extracellular protein secretion [36–38].

2.3.1.2. Yeast Recombinant Systems

Another popular host for recombinant protein production is the eukaryotic organism model, *Saccharomyces cerevisiae* whose whole genome sequence has been available since 1996 [39,40]. Even though these yeast cells share some advantages with bacteria such as short doubling time and ease of genetic manipulation, they lag behind in the downstream processes since most of the yeast cannot secrete the produced recombinant protein. Also, the requirement of complex culture conditions for optimal growth and production should never be ignored along with difficulty in selection after transformation step. Even though low plasmid stability is a concern, it can be overcome by the integration into genome. Nevertheless, the ability to carry out post-translational modifications such as protein folding, glycosylation, proteolytic processing and acylation makes this yeast more attractable [41].

Among yeast and bacterial systems, *S. cerevisiae* used to be the primary choice for heterologous protein production. However, lately due to the finalization of its whole genome sequencing, another yeast cell, *Pichia pastoris*, is considered as a promising organism for production of both industrial and biopharmaceutical proteins [42]. One of the most outstanding features is that *P. pastoris* grows in high cell densities, thus is capable of high expression levels. As single cell methylotrophic yeast, *P. pastoris* uses methanol as a carbon source, which enables the control of protein production by using methanol induced promoters. Also, the post-translational modifications, excluding the over glycosylation situations, renders the system attractable. One of the drawbacks of this system, especially for large scale fermentations, is the usage of methanol which can be harmful to the work space and the product [43].

The expression plasmids and engineered strains are limited for yeasts as these systems are not as common as bacterial ones. Also, for both yeast systems, the competent cells require fresh preparation and the transformed cells may vary between the colonies [44].

2.3.1.3. Plant Expression Systems

For production of high scale industrial and pharmaceutical proteins generally microbial systems are preferred. As an alternative, plants can be used as expression systems since they meet the requirements of a pathogen free production factory [45]. The usage of plants for recombinant protein production, also referred as molecular farming, ensures low-cost, high quality, safe and practical production peculiarly for pharmaceutical proteins [46]. Due to their naturally existing transcription and translational machinery, they gain an advantage over microbial systems by performing more accurate/native folding and other post-translational modifications. Strategies for molecular farming can be divided as plant cell cultures and creating transgenic plants. Both approaches offer high quality products, but transgenic plants fall behind when the time scales are compared [47]. Another factor to be considered is to find an optimized purification method with low cost. In most cases, transgenic seeds are produced for high yields in small amounts. Also, it should be noted that plant cells grow by forming clusters in contrast to microbial and mammalian cells which are observed as single cells [48–51].

2.3.2. Factors Affecting the Recombinant Protein Expression

2.3.2.1. Codon Optimization

Recombinant DNA technology is based on the successful cloning of a gene sequence, which is followed by the expression of this sequence in another organism preferably with higher amounts. One of the points, which should be considered thoroughly at this step, is that each prokaryotic and eukaryotic system favors the usage of some nucleotides and encodes their codons by following these patterns [52]. This phenomenon, also known as codon bias, influences the speed of the translation relatively with the frequency of the used codons. So, the same protein can be found with different genomic sequences when analyzed even among the same species. It can be said that the codons used in an organism reflects the genomic tRNA pool present and when a coding sequence is transferred to a non-native organism, the transcription and translation rate may decrease. If the protein is overexpressed, the tRNA pool of the host cell can fall into a depletion [53]. The codon

frequency serves as a tool for guidance to synthesis of the proteins. The sequence should be optimized according to the host in order to prevent the low expression if the gene to be expressed is in a different organism. Considering the emerging developments in *de novo* gene synthesis, the easiest way for codon optimization is generally to synthesize the gene from scratch.

2.3.2.2. Promoter Optimization

Promoter is a region of DNA, which is generally found at upstream of transcription unit of a gene. When a gene is transcribed, RNA polymerase recognizes the promoter and starts the transcription [36,54]. The promoter sequence can vary greatly and influence the amount and the time of production. Besides determining the frequency of the gene, they are the regions at which activators or repressors bind for gene regulation. Distinct types of promoters have been isolated and inducible ones are mostly used in recombinant protein production. In the process of optimizing an expression scaffold, the selection of the promoter sequence is one of the essential factors. The promoter also should be compatible with the terminator sequence found at the end of the gene sequence.

Even though the main focus in recombinant protein production is to increase the yield of the protein of interest, an excess amount of protein can be harmful to the host cell. In order to prevent such situations and reduce the metabolic burden, promoters induced by heat, chemicals, cellular cycles or even stress are preferred [4].

2.3.2.3. Signal Peptide Optimization

Signal peptides (SP), found in both eukaryotic and prokaryotic systems, are short sequences usually composed of 16-30 amino acids that are placed at the N-terminals of the proteins. The mission of these peptides is to guide the protein to its target site, to make sure that the protein is correctly delivered to the subcellular compartment. Once the protein reaches the destination, the signal peptide is removed by the signal peptidases. Using the right signal peptide, proteins can be secreted extracellularly.

In recombinant protein production, one of the aims is to decrease the cost and to create easy to handle routes for achieving the protein. By adding the appropriate signal peptide sequence, the proteins can be secreted directly into the growth media with higher purity. Previous studies state that the selection of the suitable signal peptide has a direct effect on the efficiency of secretion [55,56]. At the moment, the best strategy to find the optimum SP is to create a SP library and screen for the most fitting one for each target protein. Even though there are some online tools available for predicting the signal peptide, they are not very efficient when it comes to heterologous protein production [56–59].

2.3.2.4. Selection of the Host Organism

In general, according to the target protein, the host organism for the synthesis is chosen and the other contributing factors such as the vector, the promoter, the gene sequence and the fermentation conditions are optimized [31]. One of the important factors to achieve high yield is to choose not only the suitable host organism but also the correct strain. As the recombinant technology and biotechnology developed, many different strains, mostly belonging to the popular hosts, have been engineered for various circumstances. These genetically altered strains mainly aim to improve the secretion of a protein and/or declining the degradation of the produced proteins. Some common strains chosen for protein production are BL21 for *E. coli* and RIK168 for *B. subtilis*, which are protease deficient in order to prevent degradation of foreign proteins [35].

2.3.2.5. Fermentation Conditions

The culture conditions are considered as a main part of the production since it directly affects the growth of the host cells. In some cases, the growth medium can be used as an inducer and create a stressful environment for an increase in the expression [60]. By making adjustments in the medium composition such as forced starvation, the cell can be compelled to produce the desired protein. It is important to note that a recombinant bacterium carries a metabolic burden, especially, when a foreign gene is overexpressed. Forcing the cell to express and synthesize an extra molecule diverts the host cells' energy and resources and it may even interfere with some of the vital cellular processes, leading to

growth arrest. For preventing such cases, besides promoter optimization, adjustments in the medium composition, inoculation volume, inducer specifications and even the aeration should be considered for higher yield. It has been stated that changing the carbon source, addition of supplementary amino acids and finding the optimum pH all have positive effects on the growth [4,61].

Aside from helping the efficient growth of the host cells, adjustments such as the addition of other chemicals and the growth temperature can affect the solubility of the produced protein. In the process of expression of a protein, chaperones assist in the folding which directly manages the solubility of the protein.

2.3.3. Recent Studies on Improvement of Phytase Production

2.3.3.1. Microbial Phytase Production

A high interest in phytase and phytase producing organisms has emerged recently. A different type of phytase is needed with specific characteristics according to the commercial and environmental application purpose. Researches are either focused on screening for a novel enzyme or improve the current enzyme. Generally, the wild types have low production rates, thus isolation of the natural phytase is quite difficult. Therefore, there are two approaches; finding new phytases or optimizing the available ones by means of genetic and protein engineering to produce highly active enzymes. The large scale production of recombinant enzymes is commonly applied to organisms varying from yeast to bacteria (Figure 2.5).

Within recombinant production, versatile host vector combinations, strong promoters, signal sequences for extracellular secretion and multiple gene copies are studied. These parameters are important for reaching a high production yield with low production costs and the system should also be easy to scale up. Phytase researches focus on an enzyme with high catalytic efficiency, improved stability under different temperature and pH conditions, and resistance to protease since it will be used as feed additive. Hence it should be able to function in the digestive track of the animals.

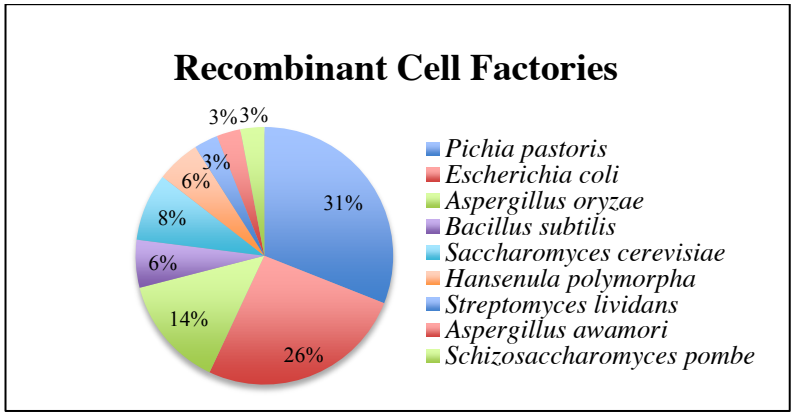

Figure 2.5. Recombinant cell factories used for the production of commercial phytases. The usages are expressed in percentages [61].

Generally, most of the phytases used commercially are derived from *A. niger* or *E. coli*, but recently other bacterial phytases such as *Bacillus* phytase has become a focal point despite their low yield. *Bacillus subtilis* is classified as Generally Recognized As Safe (GRAS), which allows direct usage as food ingredients of recombinant produced proteins. Also, their extracellular secretion mechanism creates an easy downstream of protein purification just by collecting supernatant [62]. Recombinant enzyme production of xylanases, carbohydrases and proteases in *Bacillus subtilis* is already well established and phytase production has a great potential for applications.

2.3.3.2. Transgenic Plants Expressing Phytase

Since 1950's, the alternative methods against phosphorus deficiency have been focused more on the problems faced in the animal breeding. The existing phosphorus cycle in the ecosystem (Figure 2.3.) enforce agriculture systems to use phosphate fertilizers due to the lack of inorganic phosphorus in the soils. Self-sufficient plants without the need of inorganic phosphorus fertilizers could be a promising back up for the current or future phosphorus shortage. Recently, following this approach, generation of microbial phytase expressing transgenic plants has become a goal for utilization of the soil phytate [63–66].

A few studies have been conducted on creating transgenic plants that have the ability to express phytase extracellularly. Some studies have focused on cloning *Aspergillus niger* phytase into *Arabidopsis thaliana*, *A. ficuum* phytase into tobacco, alfalfa and potato to be

expressed in the roots with successful results showing that the plant was able to synthesize and use the phytase from bacterial source successfully [11,45]. It was also shown that specific promoter usage such as *Arabidopsis* phosphate transporter initiates secretion of *A. niger* phytase only in the roots of phosphate deprived plants [67]. Other biofarming examples have been demonstrated by cloning *A. ficuum* phytase to tobacco [68].

Besides transgenic plants, transgenic phytase expressing seeds has been a strategy as well [69]. This approach is mostly applied on plant seeds that are used as feedstuff such as maize, corn and wheat [70].

Recently, low phytate containing plant seeds have been popular as an alternative [71]. Some studies include generation of mutants with low phytic acid content for seed biofortification but due to the fact that the phytic acid plays different roles in cellular mechanism ranging from energy metabolism to signaling pathways, the genes related to the biosynthesis should be isolated and well recognized [72]. It has been observed that in most cases a random mutation results in a manner in which the plants life cycle is affected even if the target was only the seed [73]. It should always be a primary objective to follow the impacts on the total plant growth while creating a low phytate mutant for a sustainable solution. Transgenic seeds expressing phytase instead of feed additive is cheaper and preferred in some conditions.

2.3.3.3. Phytase Producing Bacteria as a Plant Growth Promoting Rhizobacteria Strategy

The contribution of soil microorganisms is well established for the utilization of the P in soil. Microorganisms living in the soil, beneficial for plants' growth and health, are referred as Plant Growth Promoting Rhizobacteria (PGPR). A strategy for the improvement of plants' inorganic phosphorus uptake is to generate compatible and more efficient Plant Growth Promoting Rhizobacteria strains that produce extracellular phytase for degradation of phytate in soil. Studies have shown that *Bacillus* species can act as Plant Growth Promoting Rhizobacteria due to its antimicrobial activities, and growth promotion [74,75]. It was shown that different *Bacillus* strains have the ability of extracellular secretion; *B. muciloginosus* and *B. megaterium* promote growth and increase the

phosphorus content in the plant by degrading the phytate in the soil [74]. Recombinant *Bacillus subtilis* expressing phytase gene extracellularly was tested with maize development and the results showed higher development rate and biomass since the plant had more access to inorganic phosphorus due to the presence of bacteria [74,76,77].

2.4. THE OBJECTIVE OF THE STUDY

The objective of this study is to produce *Bacillus subtilis* strain(s) yielding high capacity extracellular production of the recombinant phytase enzyme by cloning the phytase gene from *B*. *subtilis* 168 strain to *B*. *subtilis* RIK1285, a protease deficient strain. The effect of different parameters such as varying signal peptides and growth media is analyzed for the optimal production.

3. MATERIALS

3.1. CHEMICALS

Agarose (Sigma, USA), Ammonium hepta molybdate (Sigma, USA), Ammonium Molybdate (Merck, USA), Ammonium Sulfate (Aldrich, USA), Ampicillin (Sigma, USA), Bacto Yeast Extract (BD, USA), Bovine Serum Albumin (Gibco, UK), Calcium Chloride (Sigma, USA), Calcium Chloride Dihydrate (Sigma, USA), Dextrose (Sigma, USA), Dipotassium Phosphate (Merck, USA), dNTP mix (Thermo Fisher, USA), Dulbecco's Phosphate Buffered Saline (Gibco, United Kingdom), Eco52I (Thermo Fisher, USA), Xho I restriction enzyme (Thermo Fisher, USA), Ethylene Glycol-bis (β-aminoethyl Ether) (Sigma, USA), Ethidium bromide (Applichem, Germany), Ethanol (J. T. Beaker, USA), Ferrous Sulfate (Merck, USA), GeneRuler DNA Ladder Mix (Thermo Fisher, USA), Glucose (Carlo Erba, France),), Glutamine (Merck, USA), Glycerol (Sigma, USA), IP6 sodium salt (Sigma, USA), Isopropyl β-D-1-thiogalactopyranoside (Thermo Fisher, USA), Isopropanol (Merck, USA), Kanamycin (Thermo Fisher, USA), LB Agar (Sigma, USA), LB Broth (Sigma, USA), Loading Dye (Thermo Fisher, USA), Lysine (Santa Cruz, USA), Lysozyme (Thermo Fisher, USA), Magnesium Chloride (Aldrich, USA), Magnesium Sulfate (Merck, USA), Magnesium Sulfate Heptahydrate (Merck, USA), Manganese Sulfate Penthydrate (Merck, USA), Mlu I (Thermo Fisher, USA), Monopotassium Phosphate (Santa Cruz, USA), NB Broth (Sigma, USA), Peptone (Thermo Fisher, USA), Phytic Acid (Sigma, USA), Potassium Chloride (AppliChem, Germany), Potassium dihydrogen diphosphate (Merck, USA), Potassium dihydrogen monophosphate (Merck, USA), Potassium Dihydrogen Phosphate (Merck, USA), Potassium Sulfate (AppliChem, Germany), Proteinase K (Thermo Fisher, USA), Rice Bran (Altınateş), SOC Medium (Sigma,USA), Sodium Citrate Dehydrate (AppliChem, Germany), Sodium Phytate (Merck, USA), Sodium Phytate Salt (Sigma, USA), Sulfuric Acid (AppliChem, Germany), Taq Buffer (Thermo Fisher, USA), Taq DNA polymerase (Thermo Fisher, USA), Tryptophan (Sigma, USA),X-gal (Sigma, USA), Yeast Extract (BD, USA)

3.2. KITS

B. subtilis Secretory Protein Expression System (Takara Bio Inc., Japan), In-Fusion® HD Cloning (Clontech, USA), NucleoSpin® Gel and PCR Clean-up Kit (Macherey Nagel, Germany), NucleoSpin® Plasmid Purification Kit (Macherey Nagel, Germany), Roche High Pure PCR Template Preparation Kit (Roche, Switzerland)

3.3. INSTRUMENTS

+4°C Refrigerator (Arçelik, Turkey), -80°C Freezer (Thermo Scientific, USA), -20°C Freezer (Arçelik, Turkey), Agarose Gel Electrophoresis System (Bio-Rad, USA), Autoclave (Hirayama, Japan), Microbalance (Kwipped, USA), Centrifuge (Eppendorf, Germany), Gel Electrophoresis Tank (Cleaver Scientific, UK), Heating block (Thermo Fisher, USA), Incubator (Thermo Scientific, USA), Inverted Phase Contrast Light Microscope (Nikon Eclipse TS-100, USA), Laminar Flow Cabinet (TEZ-SAN Class II Biohazard Safety Cabinet Type 2A), Liquid Nitrogen Storage (Taylor-Wharton, USA), Microplate Spectrophotometer (Thermo Scientific, USA), Microwave MD809 (Arçelik, Turkey), Nanodrop2000 Spectrophotometer (Thermofisher, USA), pH meter (Metler-Toledo Seven Compact, USA), Power Supply (Cleaver Scientific, UK), Shaking Incubator (Inova, USA), Sonicator (Emerson, USA), Thermal Cycler (Bio-Rad, USA), UV Transilluminator (Fisher Scientific, USA), Vortex (Scilogex, USA), Water Bath (Memmert, Germany)

3.4. BACTERIAL STRAINS AND PLASMIDS

Bacillus subtilis 168, *B. subtilis* RIK1285, Stellar Competent Cells, *E. coli*, DH5α, pUC19, pBS-DNA (Clontech, TAKARA, Japan)

4. METHODS

4.1. CLONING OF THE TARGET GENE: PHYTASE

4.1.1. Cultivation of *Bacillus subtilis*

Bacillus subtilis stocks from -80 °C were thawed and plated on LB agar plates for single colony formation. After 14-16 hours incubation at 37 °C, single colonies were picked and inoculated in 50 ml NB in 250 mL Erlenmeyer flasks. At the end of 18 hours of incubation at 37°C and 150 rpm, the culture was transferred to a falcon tube when the OD_{600} was close to 1.0. The absorbance value was analyzed via spectrophotometer. When the culture OD_{600} reached 1.0, the bacterial culture was used for DNA isolation.

4.1.2. Genomic DNA Isolation from *Bacillus subtilis* 168

Genomic DNA of *Bacillus subtilis* was isolated with Roche High Pure PCR Template Preparation Kit and the procedures were conducted according to the manufacturer's instructions.

Bacillus subtilis culture was centrifuged at 10,000 x g for 5 minutes and the pellet was resuspended in 1 mL NB. 200 µl of this cell suspension was added to a 1.5 ml centrifuge tube and centrifuged at 3000 x g for 5 minutes. After discarding the supernatant, the pellet was resuspended in 200 µl of PBS. 5 µl of lysozyme was added and the solution was incubated for 20 minutes at 37 °C. 200µl binding buffer with the addition of 40µl proteinase K was incubated at 70 °C for 10 minutes. 100 µl of isopropanol was added and mixed well before centrifugation at 8,000 x g for 1 minute. The flow through was discarded and the filter was placed in a new collection tube. 500 µl of Inhibitor Removal Buffer was added and then it was centrifuged at 8,000 x g for 1 minute. Again, the filter was transferred into a new collection tube and 500 µl of Wash Buffer was added. The filter inside the collection tube was centrifuged for 1 min at 8,000 x g. After discarding the flow through, the washing step was repeated by addition of 500 µl Wash Buffer and

centrifugation for 1 min at 8,000 x g. After discarding the flow through, the tube was spinned at full speed (~20,000 x g) for 10 seconds. The filter was then removed into 1.5 ml micro centrifuge tube and 200 µl of pre-warmed elution buffer was added. After centrifugation for 1 minute at 8,000 x g, the isolated genomic DNA was analyzed for concentration and purity (A260/280) via Nanodrop2000.

4.1.3. Primer Design for Cloning of the Phytase Gene

The phytase gene sequence belonging to *Bacillus subtilis* 168 was obtained from NCBI database (NC_000964.3) and phytase gene-specific primers with 15 bp extensions homologous to vector ends were designed for further In-fusion cloning reactions. *XhoI* restriction enzyme was chosen to linearize the vector, pBE-s DNA. *XhoI* has restriction enzyme recognition site in MCS of pBE-s DNA vector and doesn't cut the target gene. Forward and reverse primers, FW1 and RV1, were designed by using Clontech online primer design tool (Table 4.1.).

Table 4.1. The primers used in this study.

FW1Primer	5' GCTCGGTACCCTCGAGATGAAGGTTCCAAAAACAATG 3'
RV1Primer	5' ATTCGGATCCCTCGAGGCCGTCAGAACGGTCTTT 3'
SP_phy_F	5' GTCTCTACGGAAATAGCGAGAGATG 3'

4.1.4. Polymerase Chain Reaction Amplification of Phytase Gene

Genomic DNA isolated from *Bacillus subtilis* 168 was used as a template for cloning of the *phytase* gene. 100 ng/µl of the genomic DNA was used as a template and a gradient PCR was applied for optimization of the melting temperature (T_m). The gradient PCR program for amplification of phytase gene was as follows; 5 minutes at 95 °C for initial denaturation, then 31 cycles of 95 °C for 60 sec, 50-60 °C for 60 sec, and 72 °C for 90 sec, and the final extension at 72 °C for 10 minutes. The final concentrations were adjusted as 1 x Taq buffer, 2.5mM $MgCl_2$, 1mM $dNTP_{mix}$, 1mM of each primer and 0.5unit/µl Taq enzyme in a total reaction volume of 25µl.

4.1.5. Gel Extraction

The PCR products were analyzed by agarose gel electrophoresis on 1 per cent agarose gel. Ten µL from each PCR product was loaded with a final concentration of 1 X Loading dye. After running at 90 volts for 30 min, the gel was visualized via UV transilluminator. The bands at 1200 bp were cut from the agarose gel by a sterile scalpel and purified by using NucleoSpin Gel and PCR Clean-Up Kit. The weight of the gel slice was determined and transferred into to a sterile microcentrifuge tube. For each 100 mg of agarose gel, 2 per cent 200 µL Buffer NTI was added and the tubes were incubated for 5–10 min at 50 °C. The sample was vortexed during incubation for every 2–3 min until the gel slice was completely dissolved. A NucleoSpin® Gel and PCR Clean-up Column was placed into a Collection Tube (2 mL) and 700 µL sample was loaded. It was centrifuged for 30 sec at 11,000 x g. The flow-through was discarded and the column was placed back into the collection tube. After addition of 700 µL Buffer NT3 to the column, it was centrifuged for 30 sec at 11,000 x g. Flow-through was discarded and the column was placed back into the collection tube. It was centrifuged for 1 min at 11,000 x g for complete removal of Buffer NT3. At this step, the column was placed into a new sterile 1.5 mL microcentrifuge tube and 25 µL Buffer NE (elution buffer) was added and incubated at room temperature for 1 min. Then, the sample was centrifuge for 1 min at 11,000 x g and the isolated DNA was analyzed for concentration and purity (A260/280) via Nanodrop2000.

4.1.6. Preparation of Competent *Escherichia coli* Cells

E. coli cells from -80 °C were spread on a LB plate for single colony isolation and incubated at 37 °C for 12-16 hours. After the colony formation, a single colony was picked and transferred into 5 mL LB. The liquid culture was incubated for 12-14 hours at 37 °C in a shaking incubator and then 2 mL of the culture was transferred into 200 ml LB in a 500 ml Erlenmeyer flask, which was incubated for 1.5-2 hours at 37 °C and 220 rpm. The OD_{600} was measured until it reached between 0.35-0.45. The *E. coli* cells were then transferred to a sterile 50 mL falcon tube and cooled on ice for 5 minutes. The cells were centrifuged for 10 minutes at 2000 x g and 4 °C, and then the supernatant was discarded. The bacterial cell pellet was resuspended with 20 mL ice-cold improved buffer. Improved

buffer was prepared by mixing 15 per cent glycerol and 75 mmol/L $CaCl_2$ and autoclaved. The falcon tube was centrifuged at 1000 x g at 4 °C for 6 minutes after incubation on ice for 5 minutes. The supernatant was then discarded and again resuspended in 20 mL ice-cold improved buffer. After the third centrifugation at 1000 x g and 4 °C for 6 minutes, again the supernatant was discarded and the pellet was resuspended in 4 mL of chilled improved buffer. The prepared competent cells were distributed as 100 µL aliquots and then dipped in liquid nitrogen before storage at -80 °C.

4.1.7. Linearization of the Transformation Plasmid pBE-s DNA with *Xho*I

Thermo Scientific Fast Digest *XhoI* restriction enzyme was used for digestion of pBE-s DNA. Recommended amounts by the manufacturer were used for digestion (Table 4.2). Digested plasmid pBE-s DNA was run on 0.7 per cent agarose gel for confirmation of a successful digestion. (30 min, 90 volts).

Table 4.2. Components used at plasmid linearization of pBE-S DNA.

Component	**Amount (µL)**
Water (nuclease free)	15
10X fast digest buffer	2
DNA	2 (up to 1 µg)
Fast digest enzyme	1
Total volume	20

4.1.8. Transformation of Stellar Cells by Heat Shock

E. coli cells were taken from -80 °C and placed on ice until thawed while 2 µL of ligation reaction was added to a sterile 2 mL microcentrifuge tube that was kept on ice. The cells were mixed by gentle flicking while avoiding excessive pipetting. 50 µL of the cells were transferred into the tube with ligation reaction. The tube was gently flicked for

homogenized distribution and then placed on ice for 20 minutes. After this step, the tube was incubated at 42 °C for 45-50 seconds for the heat shock. The tube was immediately returned on ice for 2 minutes incubation. 950 µL of fresh SOC medium was added to the tube and incubated at 37 °C for 1.5 hours with 150 rpm shaking. Finally, the cells were centrifuged at 3,000 x g for 5 minutes and the supernatant (~700 µL) was discarded. The cells were resuspended and plated on LB agar plates with ampicillin (50 µg/mL). pUC19 plasmid was used as a positive control for the transformation process. The media for pUC19 transformation were prepared by addition of X-gal and IPTG. The plates were incubated at 37 °C for 24-36 hours until colony formation was observed.

4.1.9. Selection of Transformants: Colony PCR

Colony PCR was performed to confirm that phytase gene was successfully inserted into pBE-s DNA plasmids. Colonies from each cloning reaction plates contain pBE-s DNA + phytase and 2 colonies from negative control and 2 colonies from positive control plates were prepared to grow the colonies. Each colony was inoculated to 5 mL of LB amp+ broth medium and incubated overnight at 37 °C by shaking at 150 rpm.

The same PCR procedure that was used for amplifying the phytase gene was applied. The DNA templates were obtained from the individual colonies in petri dishes via touching by a sterile tip that was then streaked inside the PCR tube. PCR results were loaded on 1 per cent agarose gel and according to PCR results, the selected transformants were subjected to plasmid purification by NucleoSpin® Plasmid Purification Kit. Plasmid concentrations were measured by Nanodrop.

4.1.10. Plasmid Isolation from Stellar Competent Cells (pBE-phy plasmids)

The plasmids isolations were carried out according to the Nucleospin Plasmid (No-Lid) protocol for isolation of high copy plasmids. Transformed Stellar cells were grown in liquid LB culture and 5 mL was transferred into a 15 mL falcon tube. It was centrifuged at 11,000 x g for 30 seconds, and the supernatant was discarded. The cell pellet was resuspended with 250 µL Buffer A1 and incubated at room temperature for 5 minutes.

Then 300 µL of Buffer A3 was added and mixed gently by inverting the tube 6-8 times. The tube was centrifuged for 5 minutes at 11,000 x g and room temperature. This step was repeated if the supernatant was not clear as expected. 750 µL of the supernatant was transferred to a Nucleospin column placed in a collection tube. The column was centrifuged for 1 minute at 11,000 x g and the flow through was discarded. 600 µL of Buffer A4 was added and centrifuged for 1 minute at 11,000 x g. After discarding the flow through, the column was centrifuged empty at 11,000 x g for 2 minutes, and then the collection tube was replaced with a 1.5 mL micro centrifuge tube. 50 µL elution buffer was added and incubated at room temperature for 1 minute and then centrifuged at 11,000 x g for 1 minute. The concentration and purity (A260/280) of the obtained plasmid DNAs were measured via Nanodrop2000.

4.1.11. Plasmid Sequencing

The plasmids were sequenced via Sentegen company with the designed primer shown in Table 4.1. The sequencing results were analyzed with Jalview and EMBOSS Needle program for pairwise sequence alignment.

4.2. PLASMID LIBRARY PREPARATION WITH SIGNAL PEPTIDES

4.2.1. Linearization of the pBE-phy Plasmid with *Mlu*I & *Eco*52I

In order to obtain successfully linearized plasmids, plasmids containing phytase gene sequence were chosen as stated in section 4.1.10. The plasmids were digested simultaneously by *Mlu* I and *Eco52*I restriction enzymes. Thermo Fischer's double digestion calculator tool was used. Digestion reaction was performed simultaneously for both enzymes with the reagents stated in Table 4.3.

Table 4.3. Components used at simultaneous plasmid linearization of pBE-phy.

Components	**Amount (µL)**
Eco52 I	1
10X *Eco52* I Buffer	2
2-fold excess of MluI	2
0,1 per cent BSA	2
Substrate DNA	≤1 µg (3 µL)
Sterilized distilled water	10
Total	20

Reaction mixture was incubated at 37 °C for 3 hours. The digested plasmid was mixed with 4 µL of loading dye and loaded to a 0.7 per cent agarose gel for confirmation. Linear plasmids were isolated from agarose gel after visualization.

4.2.2. Cloning of Signal Peptides with In-fusion Cloning

In-fusion reaction was set up to insert SP DNA into the digested pBE-DNA plasmid containing phytase gene (pBE-phy). Reagents and amounts of In-fusion reaction for each plasmid were stated in Table 4.4. Reaction tubes were mixed gently and incubated for 15 min at 50 °C, and then placed on ice.

Table 4.4. Components used at cloning of signal peptides into pBE-phy.

Components	**Cloning Reaction**	**Positive Control Reaction**
Linearized expression plasmid	3.3 µl	1 µl pUC 19 control vector
SP DNA mix	1.37 µl	2 µl control insert
5X In-Fusion® HD Enzyme Premix	2 µl	2 µl
Sterilized distilled water	3.3 µl	5 µl
Total	**10 µl**	**10 µl**

4.3. TRANSFORMATION OF *BACILLUS SUBTILIS* WITH PBE-PHY-SP

4.3.1. Preparation of Competent *Bacillus subtilis* RIK1285 Cells

To prepare *B. subtilis* RIK1285 competent cells, the tip of loop was touched to the glycerol stock of *B. subtilis* RIK1285 cells from -80 °C and spread on LB plate and incubated at 37 °C overnight. Next day, 2 mL of LB medium was inoculated with a full loop of *B. subtilis* and incubated overnight on the LB plate. The culture was grown overnight at 28 °C and 170 rpm. Next day, 50 µL of the culture broth grown overnight was added to 5 mL of SPI medium, and incubated at 37 °C and 175 rpm. OD_{660} was measured every 30 minutes, just beginning 1 hour after the start of culturing, and culturing was stopped once the culture entered the plateau phase. 0.5 mL of the culture broth was added to 4.5 mL of SPII medium, and incubated at 37 °C and 95 rpm for 90 minutes. Lastly, 50 µL of 100 mM EGTA was added to the culture medium and incubated at 37° C and 95 rpm for 10 minutes.

4.3.2. Transformation of *Bacillus subtilis*

Freshly prepared competent *B. subtilis* cells were used for transformation. 300 µL of the culture was divided into each 1.5 mL and 2 mL microcentrifuge tubes and transformed immediately. 1 µg of plasmid library solution was added per 300 µL of the competent *Bacillus subtilis* cells. Tubes were incubated at 30 °C and 95 rpm for 90 minutes. 100 µL of the culture broth was spread on LB plates containing kanamycin (10 µg/mL). Plates were incubated overnight at 37 °C. At the same time, *B. subtilis* cells were transformed with pBE-S DNA plasmid and spread on LB plates containing kanamycin (10µg/mL) as controls. Also, a loop of *B. subtilis* competent cells was spread on LB plates to check the growth. After the overnight incubation at 37 °C, colonies were observed on plates and they were placed at +4 °C for further procedures.

4.4. PHYTASE ACTIVITY ANALYSES

4.4.1. PSM Agar Plate Assay

Phytase secretion was assayed using phytase screening medium (PSM) agar plates containing; 0.1 per cent (w/v) K_2HPO_4, 0.6 per cent K_2HPO_4, 0.1 per cent $Na_3citrate{\cdot}2H_2O$, 0.02 per cent $MgSO_4{\cdot}7H_2O$, 0.2 per cent K_2SO_4, 0.02 per cent glutamine, 0.5 per cent IP6 sodium salt (pH 6.5), 1.5 per cent glucose, 0.3 per cent $(NH_4)_2SO_4$, 0.05 per cent KCl, 0.01 per cent lysine, 0.005 per cent tryptophan, 0.2 per cent $CaCl_2{\cdot}2H_2O$, 0.05 per cent $MgSO_4{\cdot}7H_2O$, 0.015 per cent $MnSO_4{\cdot}5H_2O$ and 0.01 per cent $FeSO_4{\cdot}7H_2O$ [77]. *B. subtilis* colonies were spread and inoculated on prepared PSM agar plates. After 48 hours of incubation at 37 °C, plates were analyzed according to the clear zone around the colonies.

4.4.2. Phytase Enzyme Activity Assay

In order to check phytase activity of *B. subtilis* cells transformed with pBE-phy-SP plasmid, supernatant of the culture was used as crude enzyme solution. The activity assay was performed with a modified protocol based on measurement of the amount of Pi released by the phytase enzyme [78]. A substrate solution was prepared with sodium phytate salt, phytic acid that has bonded with salt, for the break down by the phytase. Another element of the buffer; $CaCl_2$, is used for the stabilization of the enzyme [76], while Tris-HCl with a pH of 7.0 is the main buffer of the solution (0.1 M Tris-HCl with pH 7.0 was supplemented with 2 mM sodium phytate and 2 mM $CaCl_2$). 600 µL of substrate buffer was mixed with 150 µL of enzyme solution in 15 mL falcon tubes. The mixture was incubated at 37 °C for 30 minutes. The enzymatic reaction was stopped by adding 750 µL of 10 per cent TCA. Color reagent was prepared by mixing 4 volumes of 1.5 per cent (w/v) ammonium molybdate solution supplemented with 5.5 per cent (v/v) sulfuric acid and 1 volume of 2.7 per cent (w/v) ferrous sulfate solution. 1.5 mL of color reagent was added. After this step, the amount of inorganic phosphate is analyzed by ammonium molybdate binding [22] with spectrophotometry at 700 nm wavelength.

Working standards were prepared with 0,02 mM, 0,04 mM, 0,06 mM, 0,088 mM, and 0,1mM potassium dihydrogen phosphate solution by dilution of stock phosphate dihydrogen phosphate solution (K_2HPO_4). Procedure was carried out as described above and the absorbance difference of the standard solutions against the corresponding exactly calculated amount of potassium dihydrogen phosphate was plotted. A standard curve was plotted with K_2HPO_4 for calculations of activity and the regression equation was found (Figure 4.1). The equation acquired from the standard curve (Figure 4.1) was used for calculating all of the phytase activity measurements in this study.

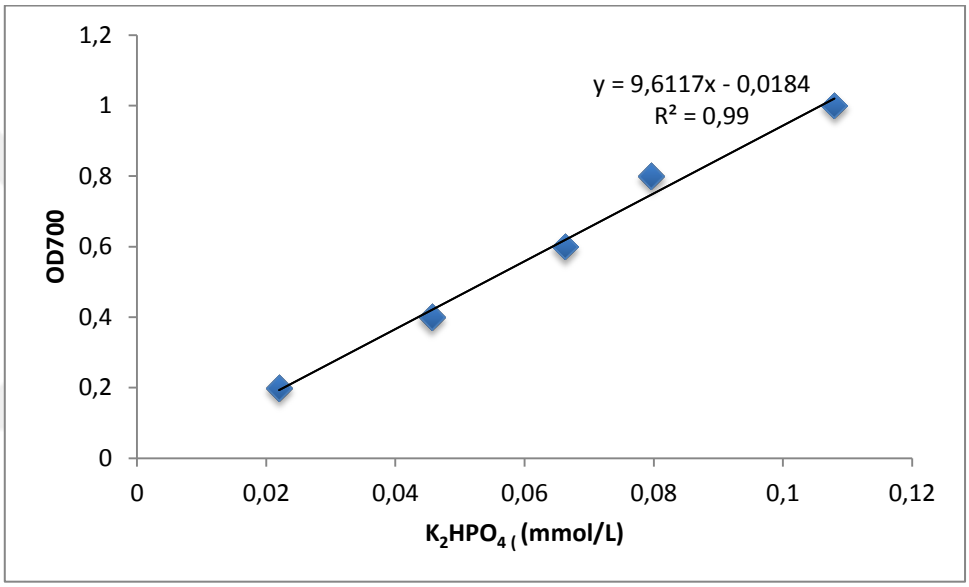

Figure 4.1. Standard curve for phytase activity determination.

Activity of sample (U) was calculated as follows:

$$units/_{mL}\ enzyme = \frac{\mu moles\ of\ phosphate\ released\ x\ dilution\ factor}{t\ x\ v} \quad (4.1)$$

df: Dilution factor

t: Time (30 minutes) of assay per the Unit Definition

v: volume (0.15 mL) of enzyme used

4.4.3. Preparation of Different Growth Media

Bacterial stocks of transformant *Bacillus subtilis* RIK1285 from -80 °C were initially cultured in 5 mL LB in 15 mL falcons, by using 2 per cent inoculum from stock. The culture was grown at 37 °C and 150 rpm overnight. Then, again using 2 per cent inoculum, the cultures were grown in different culture media for comparison. LB [10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract], YPD [10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose], IP6 Salt Medium [0.5 g/L dextrose, 10 g/L peptone, 5 g/L yeast extract, 1 g/L $MgSO_4$, 1 g/L $CaCl_2$, 1 g/L sodium phytate (pH 7.0)] [79], Modified LB Medium [10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 75 g/L rice bran] [80], Rice Bran Medium [15 g/L yeast extract, 15 g/L $(NH_4)_2SO_4$, 50 g/L rice bran, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 2.6 g/L $CaCl_2$] [77] were prepared and the bacterial cultures were grown at 37 °C and 150 rpm. Samples were collected at 24^{th}, 48^{th}, 72^{th}, 96^{th} hours for analysis.

4.4.4. Trichloroacetic acid (TCA) and Acetone Precipitation

TCA-acetone precipitation was done in order to concentrate enzyme solution samples before SDS-PAGE analysis. 180 µL of sample was mixed with 20 µL of 100 per cent TCA (prepared freshly) to achieve a final concentration of 10 per cent of TCA. The sample was vortexed and incubated on ice for 1 hour. After the incubation, the samples were centrifuged at 4 °C for 20 minutes at 12.000 x g. The supernatant was discarded and 800 µL of acetone chilled to -20 °C was added to the pellet. For resuspension, the pellet was vortexed thoroughly and then incubated overnight at -20 °C. Next day, the sample was centrifuged at 4° C for 10 minutes at 6,500 x g. The supernatant was discarded and 800 µL of acetone (20 °C) was added. After vortexing, it was incubated at -20 °C for 30 minutes. The supernatant was discarded and this step was repeated one more time. After the final centrifugation, the acetone was discarded and the pellet was air-dried. The pellet was resuspended in water.

4.4.5. SDS-PAGE Assay

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used for observing the produced protein. Firstly, 12 % resolving gel [(32.6% (v/v) dH_2O, 40% (v/v) 30% acrylamide, 25.3% (v/v) 1.5 M Tris (pH 8.8), 1.0% (v/v) 10 % SDS, 1.0% (v/v) 10% APS and 0.04% (v/v) TEMED)] was prepared by mixing all of the components in a 50 mL falcon tube and loaded into the chamber between the glass plate and white plate. After the polymerization of the resolving gel, 5% stacking gel [(68% (v/v) dH_2O, 16.6% (v/v) 30% acrylamide, 12.6% (v/v) 1.0 M Tris (pH 6.8), 1.0% (v/v) 10 % SDS, 1.0% (v/v) 10% APS and 0.04% (v/v) TEMED)] was prepared and poured on top. The gel was left for polymerization after insertion of the comb.

The samples were mixed with 1:1 Laemni buffer and incubated in water bath at 95 °C for 10 minutes and then waited on ice. The samples were loaded into the wells of the SDS gel and run at 300V for 1 hour. Afterwards, the gel was stained with Coomassie stain for 40 minutes on an orbital shaker. When the staining procedure ended, the gel was left in dH_2O overnight for the destaining procedure.

5. RESULTS

5.1. AMPLIFICATION OF THE *Bacillus subtilis* 168 PHYTASE GENE

The genomic DNA of *B. subtilis* 168 was isolated via Roche High Pure PCR Template Preparation Kit with a concentration of 47 ng/µL and purity of 1.86 at A260/280, where the expected yield of nucleic acid, 1-3 µg, was achieved.

For the following step, phytase specific primers FW1Primer and RV1Primer (Table 4.1) were designed via Clontech online primer design tool to amplify phytase gene with 15 bp extensions homologous to pBE-S DNA vector ends. The primers designed with *XhoI* site at either ends provided necessary conditions such as GC content between 40-60 per cent, a melting temperature (Tm) between 58-65°C, and a total length of 18-25 bp long. *XhoI* restriction enzyme was chosen from the vector's multiple cloning site (MCS) for linearization as the phytase sequence did not contain the enzyme's recognition site.

A gradient PCR reaction was performed with *B. subtilis* gDNA to find the optimum Tm of the primers. The performance of Tm between 50-60 °C was analyzed and according to the gel electrophoresis results (Figure 5.1), it was observed that the primers contained a wide range of temperature scale in which the clearest bands were between 50-53 °C and 55-56.9 °C. PCR was performed with the selected Tm; (50°C) using the *Bacillus* gDNA as the template. The resulting products were loaded on to agarose gel for confirmation and purification (Figure 5.1). Wells number 2 and 4 were cut off from the agarose gel and purified. At the end of the purification, 20 ng/µL and 11 ng/µL DNA were purified from gel by using 20 µL nuclease free water. At the end of this step, the purified DNA contained phytase gene with *XhoI* RE recognition sites at both ends.

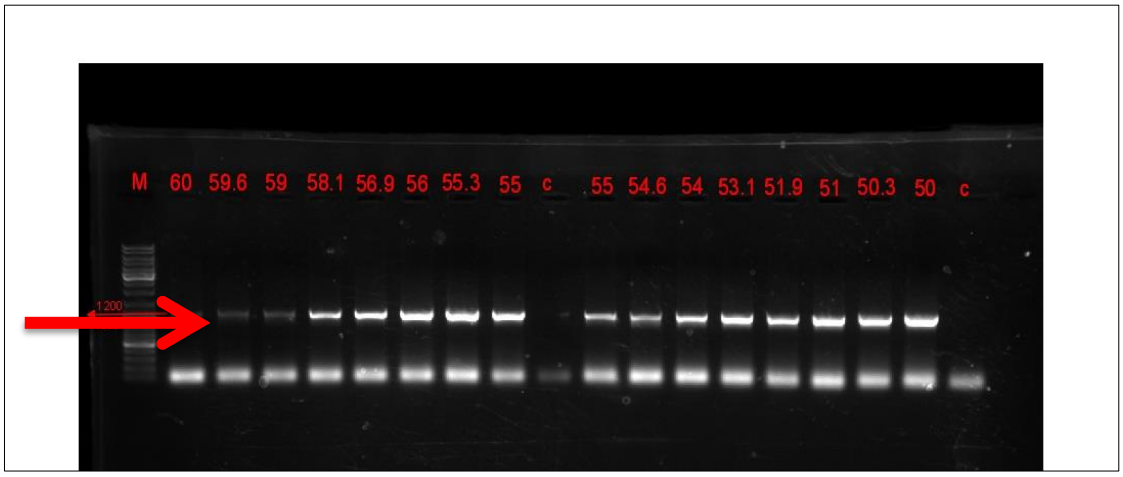

Figure 5.1. Results of gradient PCR amplification of phytase gene encoding region from *B. subtilis* 168. The arrow indicates the expected PCR product (1200 bp). (M: ladder mix, c: control)

5.2. CONSTRUCTION OF THE EXPRESSION VECTOR IN *E. coli*

Fast Digest *XhoI* restriction enzyme was used for linearization of pBE-S DNA. Following the digestion, In-fusion cloning was used for insertion of phytase gene from the previous step into the pBE-s DNA plasmid. After the In-fusion reaction, the constructed vector was named as pBE-phy and it was transformed into *E. coli* by heat-shock and grown on ampicillin selective media (Figure 5.2). For the control of transformation, pUC19 plasmid was transformed and blue-white screening showed high efficiency. The ampicillin resistance of the grown colonies indicated that the plasmid was successfully inserted in *E. coli*.

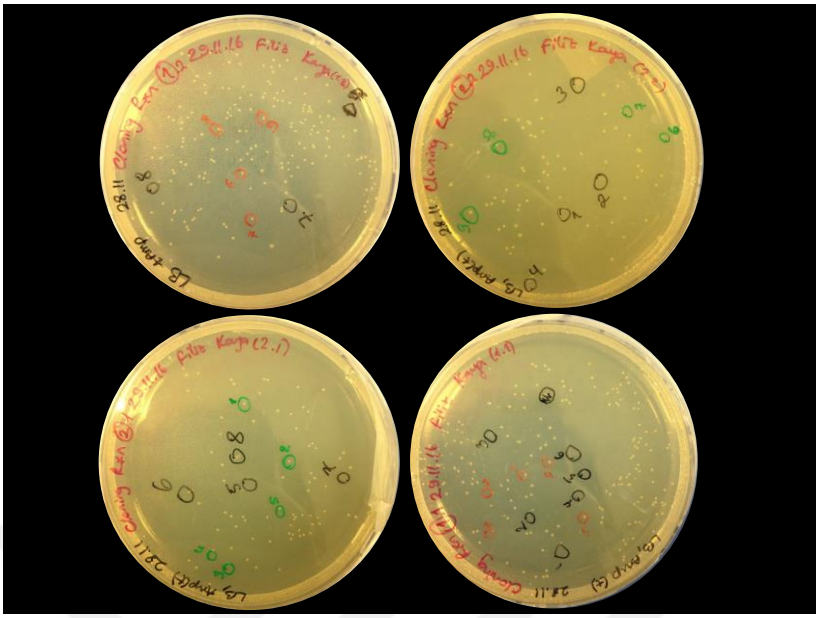

Figure 5.2. *E. coli* colonies grown on LB agar with ampicillin after transformation with pBE-phy plasmid.

5.3. VALIDATION OF TRANSFORMATION: COLONY PCR

Observed colonies were checked with colony PCR method to validate whether the inserted plasmids contained the desired phytase gene, with the primer set FW1Primer and RV1Primer (Table 4.1). To check all the transformed colonies, the PCR was performed in 3 parts. The agarose gel image (Figure 5.3) showed that out of 50 colonies only 7 colonies contained the pBS-phy plasmid where rest of them could have been transformed with pBE-S DNA plasmid considering that all of the colonies were grown on selective media. According to gel electrophoresis results, *B. subtilis* colonies belonging to the observed bands (white boxed) were chosen for plasmid purification. The purification results (Table 5.1) exhibited high concentration of plasmid DNA within a good range of purity (1.8-2.0).

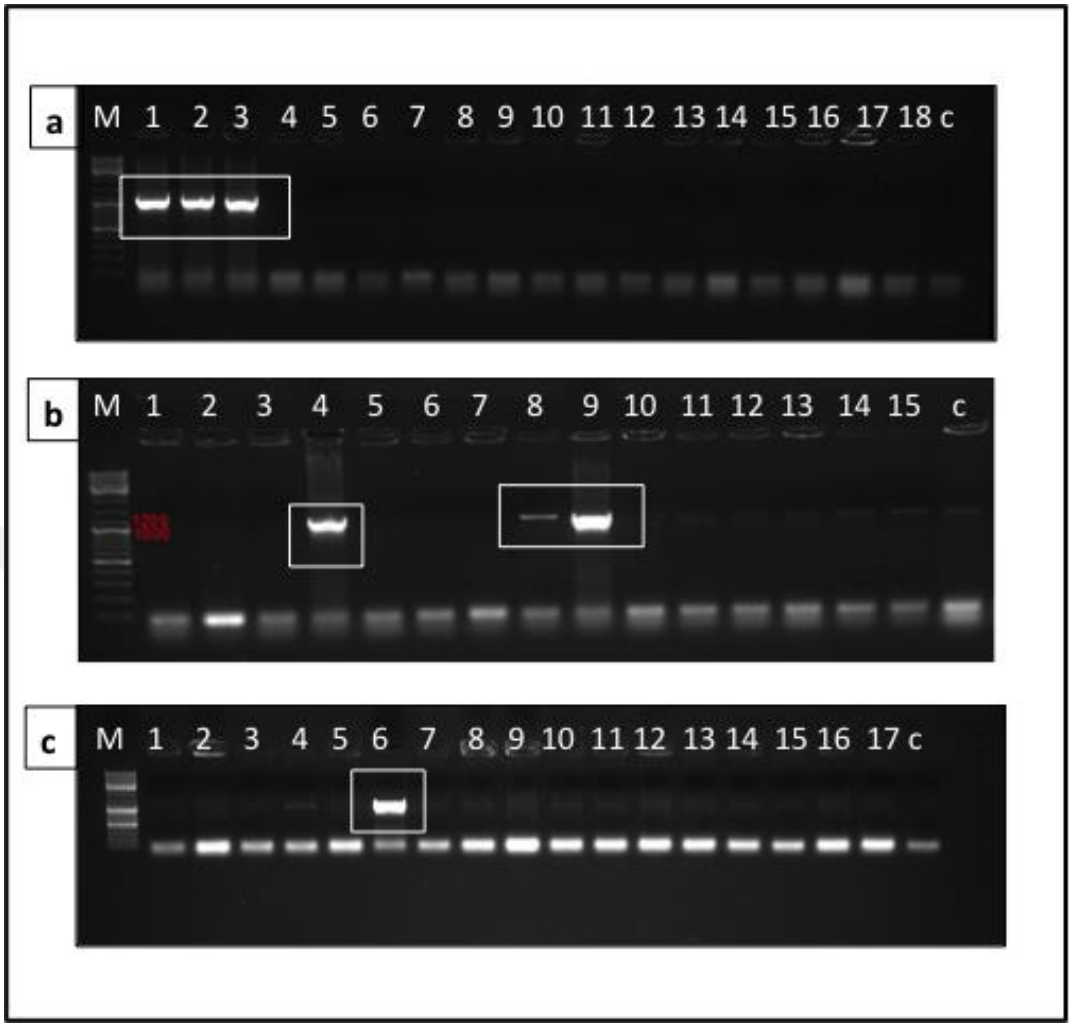

Figure 5.3. Gel electrophoresis results of the colony PCR. (a, b, c) First lane M contains DNA ladder mix, numbered wells contain pBE-s DNA+phytase cloning reaction, and last well contains control reaction. The bands shown in frames indicate the expected PCR product (1200 bp).

Table 5.1. The DNA concentration and purity results of the isolated plasmids.

Well number	**DNA concentration** (ng/µL)	**DNA purity (A230/A260)**
a.1	358	1.85
a.2	339.3	1.86
a.3	391.4	1.87
b.4	442.2	1.8

b.8	323.3	1.8
b.9	353.4	1.8
c.6	271	1.85

5.4. LINEARIZATION OF THE pBE-phy VECTOR WITH *MLU*I AND *ECO*52I RESTRICTION ENZYME DIGESTION

After purification of plasmids, number a.3 and a.4 were chosen for digestion by *Mlu* I and *Eco52* I based on their concentration and purity. A sequential digestion was performed, both for 3 hours at 37°C. Agarose gel image (Figure 5.4) belongs to the digestion of plasmids by *Mlu* I and *Eco52* I. All bands at the top were cut off from the agarose gel and purified. DNA ate concentration of 35.1 ng/µL, 35.5 ng/µL, 37 ng/µL and 37.3 ng/µL were purified from gel by using 30 µL elution buffer. Band numbers a 4.1 and b 4.2 were chosen for the next cloning reaction.

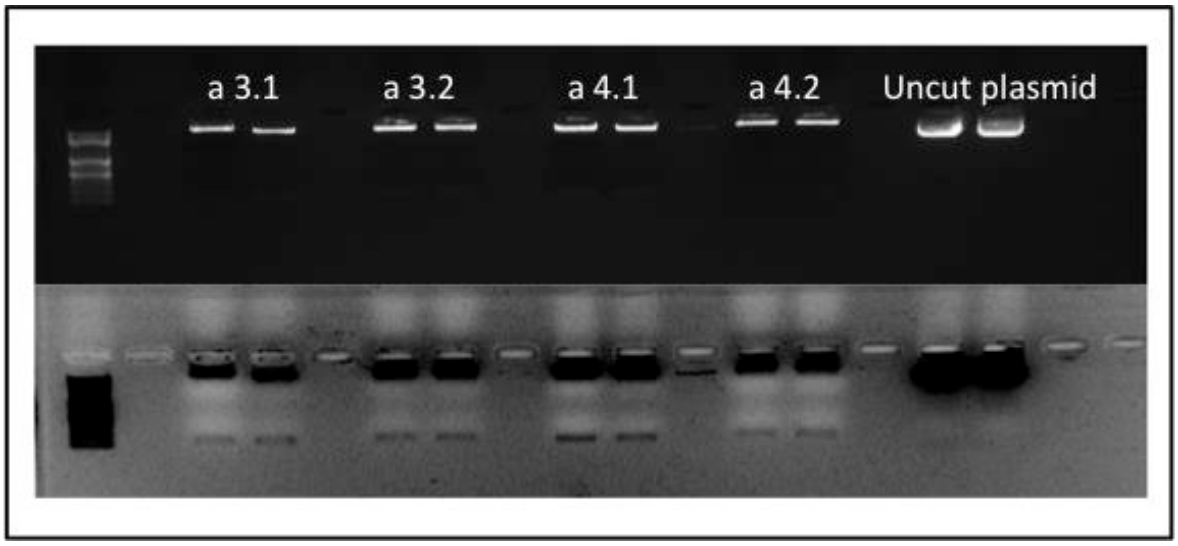

Figure 5.4. Agarose gel image of digested plasmid by Mlu I and Eco52 I. (1,5 per cent agarose gel, run for 10 min at 100V) (digested plasmid: 7,000 bp, uncut plasmid: 7,087 bp)

5.5. PREPARATION OF SIGNAL PEPTIDE LIBRARY

In order to prepare a signal peptide (SP) library fused to the previous target gene phytase, a commercial kit mixture including 173 SPs was used for cloning. The mixture consists of inserts homologous to a specific region in the pBE-S DNA plasmid, which was used for the construction of the pBE-phy plasmid. In-fusion reaction was set up to insert SP DNA mixture into the digested pBE-phy plasmid. When the plasmids from the previous step were linearized with 2 restriction enzymes, the original secretory signal peptide sequence *AprE* was removed and the SP inserts were placed here. Besides the cloning reaction, negative and positive control reactions were spread on LB amp+ plates to check the transformation efficiency. Transformed cells were incubated overnight at 37°C, next day colonies were observed showing that the transformation was successful (Figure 5.5). The size of the library can only be confirmed by counting the number of colonies. In this case around 200 colonies was counted which was much lower than expected than the recommended number of 2,000 or more for a sufficient plasmid library [62] .

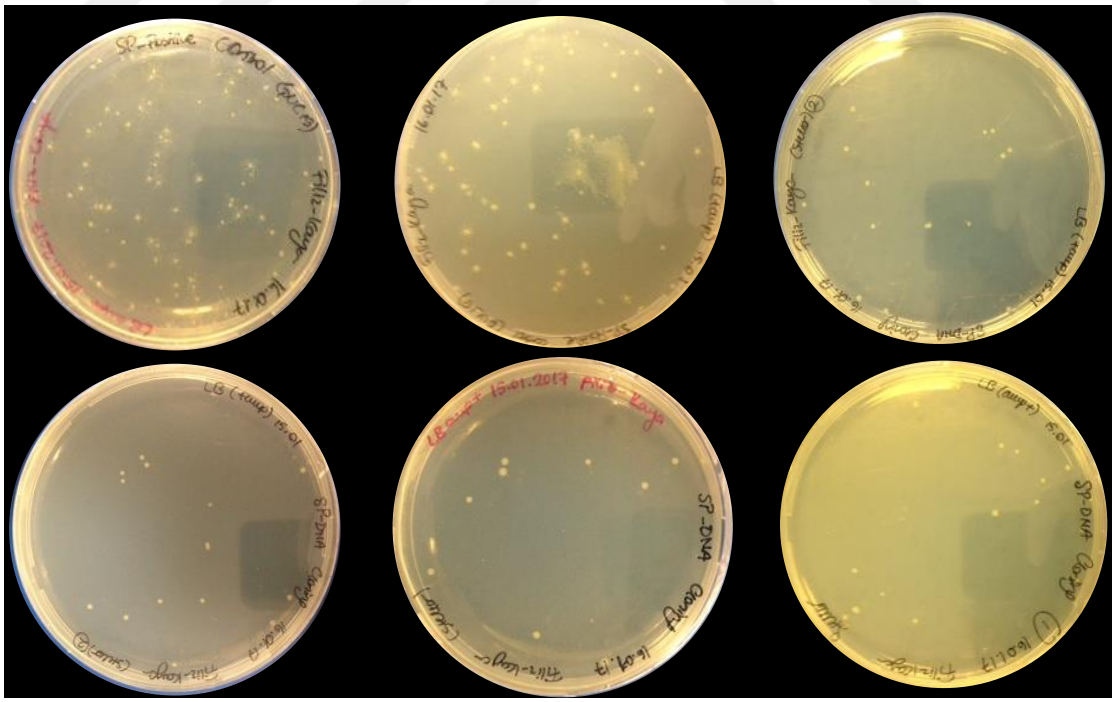

Figure 5.5. *E. coli* cells plated on LB agar after transformation with pBE-phy-SP plasmid.

Colonies were pooled using 5 ml LB culture medium and then purified with a final concentration of 155.3 ng/µL with 1.87 purity. Originally, 20 µg of plasmid DNA was expected, however DNA concentration of our sample was only 7.7 µg. This pool of plasmids contained the signal peptide library prepared with a SP mix of 173 signal peptides.

5.6. TRANSFORMATION OF *B. SUBTILIS* RIK1285 WITH THE SIGNAL PEPTIDE LIBRARY

Freshly prepared competent *B. subtilis* RIK 1285 cells were transformed with the SP library prepared in the previous step. To check the competency of *B. subtilis* cells, transformation was done with both pBE-S DNA and pBE-phy-SP plasmid, and then spread on LB plates containing kanamycin (10µg/mL) as controls along with the transformants. Also, a loop of *B. subtilis* competent cells was spread on non-selective LB plates to check viability. After an overnight incubation at 37 °C, the colonies were observed (Figure 5.6). The expected number of colonies with an efficient transformation is 3 to 8 x 10^2 colonies/µg whereas only 29 colonies were observed which is below the expected number. Petri plates were stored at +4°C for further studies.

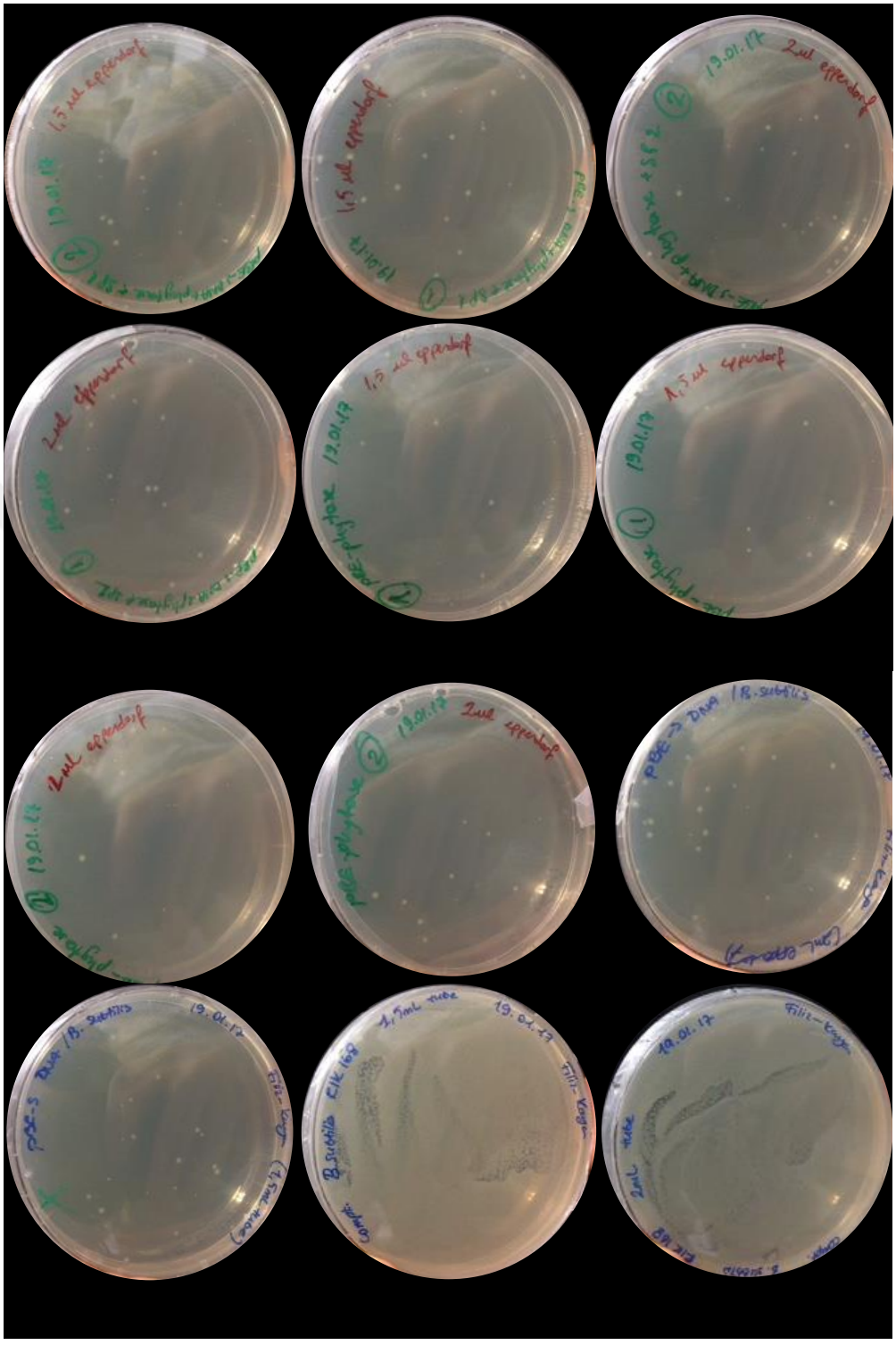

Figure 5.6. *Bacillus subtilis* RIK1285 cells plated on LB agar after transformation with SP library.

5.7. PSM AGAR PLATE ASSAY FOR COLONY SELECTION

One of the common qualitative methods for detecting the produced phytase enzyme is to use phytate containing agar plates. In a case, a bacterium is producing extracellular phytase and the enzyme is capable of hydrolyzing the phytate in the plate. As a result, a clear zone is formed around the colony. After the transformation of *B. subtilis* RIK1285, the colonies were screened with PSM agar plate assay for selection of the transformants with extracellular phytase secretion (Figure 5.7). After the incubation, the colonies with a clear zone around were recorded as successful. All of the colonies formed a clear zone except number 29. According to the clear zone area indicating phytic acid degradation around the colonies, SP6, SP12 and SP27 were nominated with the highest scores.

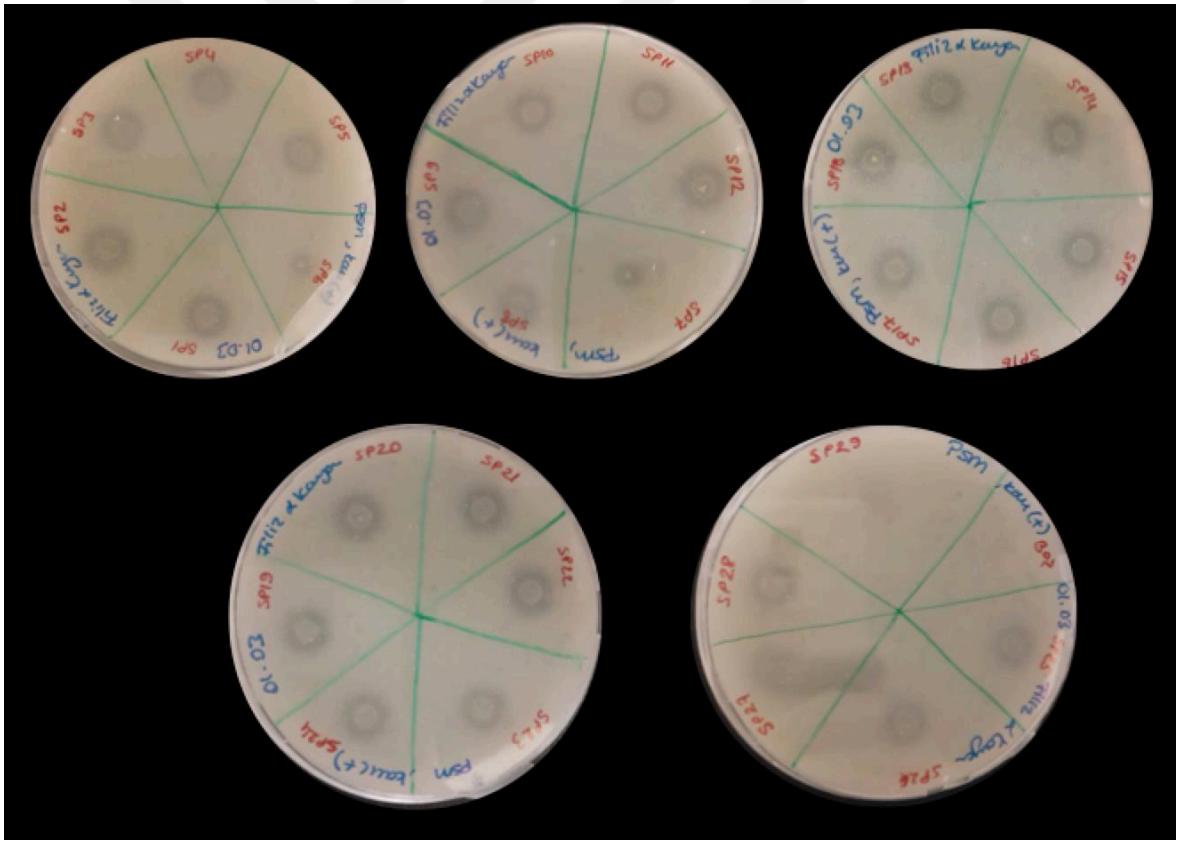

Figure 5.7. *B. subtilis* RIK1285 colonies containing pBE-phy-SP plasmids grown on PSM agar plates for selection (48 hours of incubation)

5.8. PHYTASE ACTIVITY ASSAYS

5.8.1. Determination of Activity Change Over Time

To determine the phytase activity change over time, 3 transformants producing phytase (SP6, SP12, SP27) were grown in rice bran medium and phytase activity was measured at 24, 48, 72, 96 and 120th hours (Figure 5.8). For the rest of the activity measurements samples from 48th hour were used.

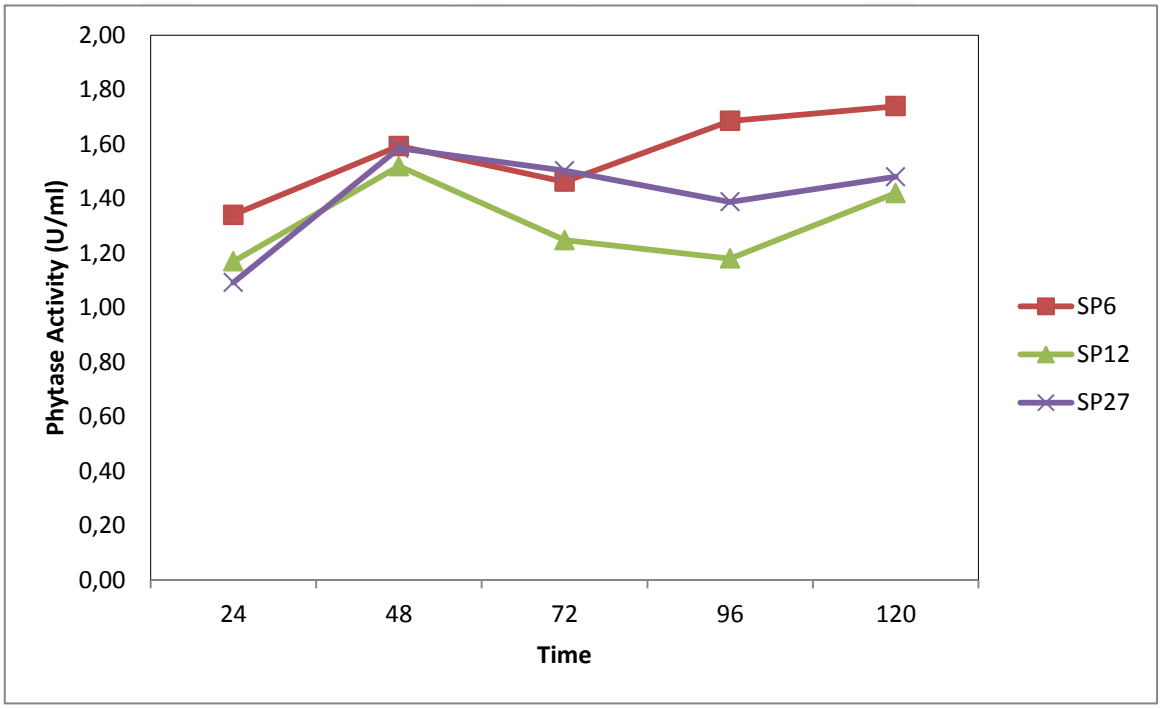

Figure 5.8. Phytase activity change at different hours.

5.8.2. Determination of Activity Change Over Time Using Different Growth Media

In order to analyze the effect of the growth medium on phytase production, different media (LB, YPD, LB+rice bran, IP6 salt medium and rice bran medium) were used for cultivation and production. The results showed a significant increase in production when rice bran was included in the medium (Figure 5.9) and for the rest of the analysis, rice bran medium was used for cultivation and production.

Also a color difference was observed between the cultivations after 48th hours of inoculation (Figure 5.10). Activity results revealed that the pink color change occured when phytase production increased.

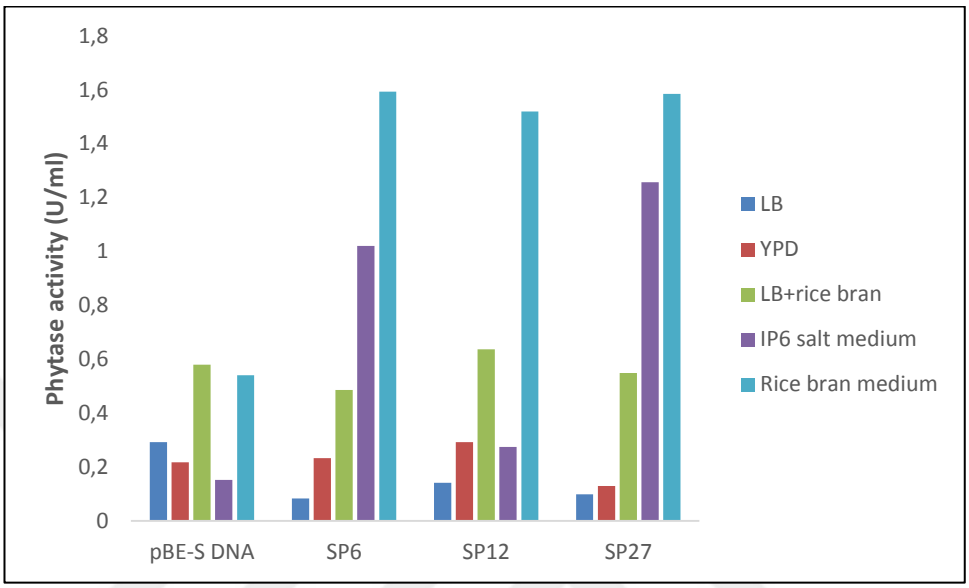

Figure 5.9. Phytase activity change with different growth media at 48th hour.

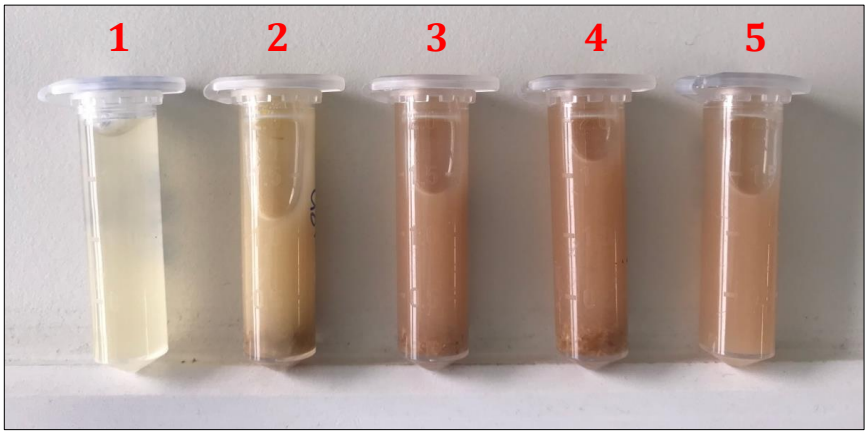

Figure 5.10. Supernatants of cultures belonging to *B. Subtilis* RIK1285 transformants grown in different media. 1: LB (pBE-S DNA), 2: Rice Bran Medium (pBE-S DNA), 3: Rice Bran Medium (SP6), 4: Rice Bran Medium (SP12), 5: Rice Bran Medium (SP27).

5.8.3. Phytase Activity Determination of the Constructed Transformants

After determination of the optimal growth medium as rice bran medium, the enzyme activity assay was applied for determining the phytase activity of all of the pBE-phy-SP transformed colonies (Figure 5.11). At this point of the analysis, a blank belonging to each culture was prepared separately which was subjected to phytase assay to calculate the amount of free inorganic phosphorus already in the culture medium. One colony transformed with pBE-S DNA was analyzed as a background check since it wasn't expected to produce any phytase.

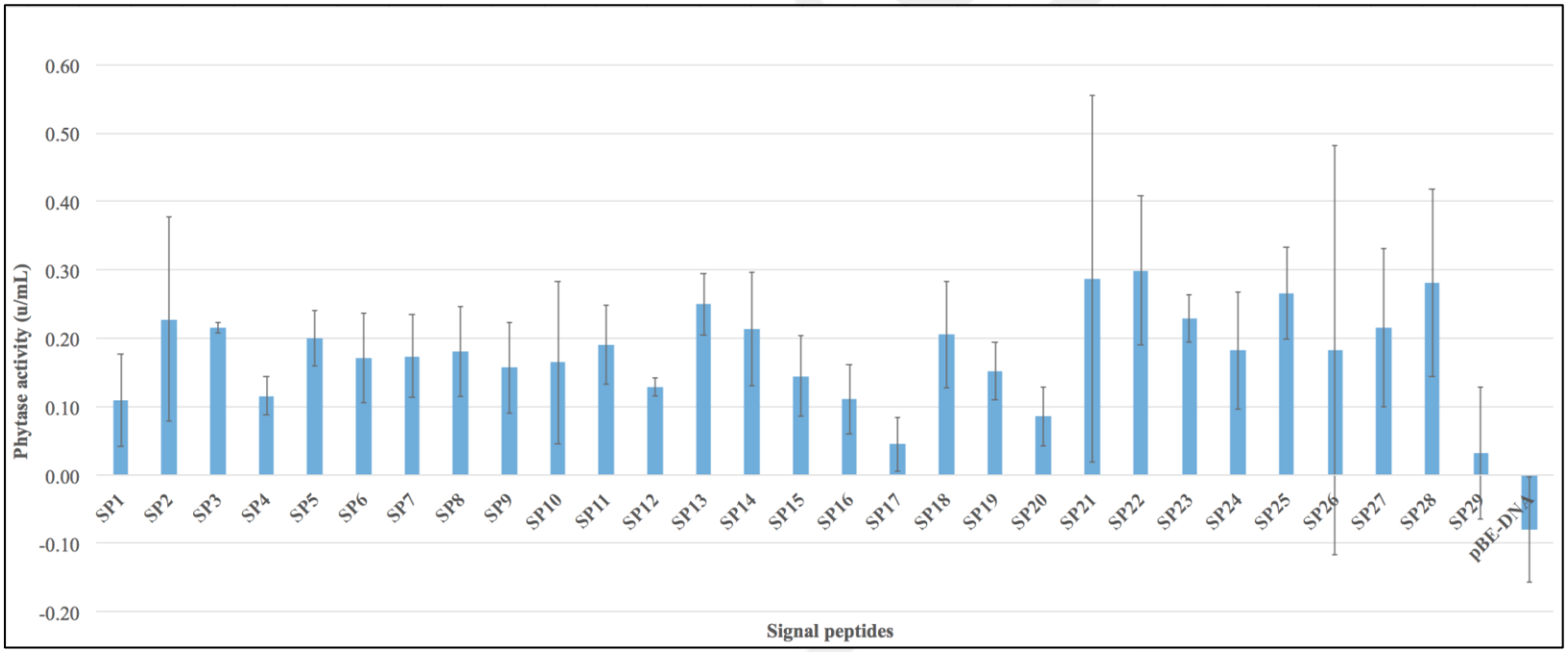

Figure 5.11. Phytase activity results of transformed *B. Subtilis* colonies.

5.9. SDS-PAGE ANALYSIS

SDS-PAGE was performed for observation of the produced proteins. The supernatant of the bacterial cultures were loaded to polyacrylamide gel after TCA-acetone precipitation. One of the colonies, SP12, with high phytase activity was chosen for identification and samples were taken from 24th and 48th hour cultures. The same *Bacillus subtilis* (SP12) grown on LB medium was also used for comparing the effect of different growth media. Expected band size was around 41 kDa. Figure 5.12 represents the 12% SDS gel stained with Coomassie. Many protein bands were observed in the samples obtained from rice bran medium, but almost no protein was observed in samples from LB growth. The protein band obtained around 41 kDa was denser which shows successful phytase production.

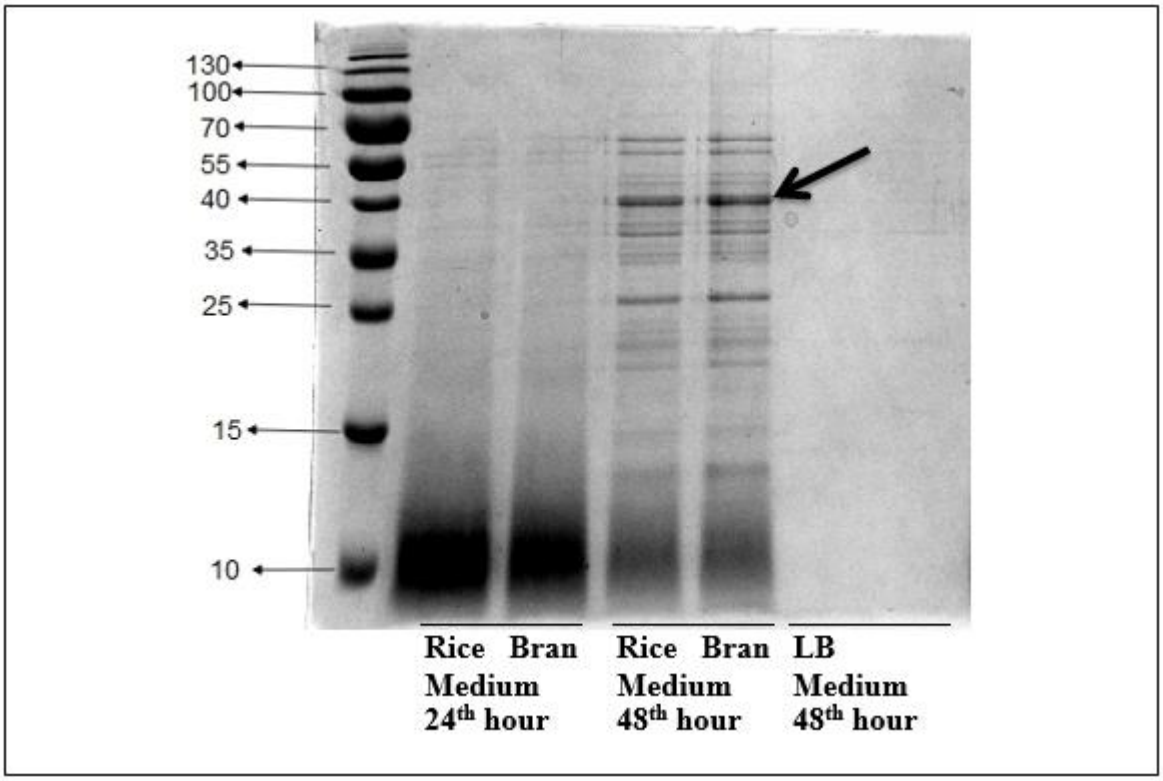

Figure 5.12. SDS-PAGE results belonging to TCA-Acetone precipitated supernatant samples from the cultivated *B. subtilis* strain with pBE-phySP12. The arrow indicates the expected 41 kDa of phytase.

5.10. SEQUENCING RESULTS

For the analysis of the signal peptide sequence, 6 colonies among the total 30 were chosen according to their activity results. The colonies with highest and lowest activities were chosen for comparison to check whether the source of the activity difference was the signal peptide (Table 5.2). The plasmids of the chosen colonies named pBS_phy_SP5, pBS_phy_SP12, pBS_phy_SP13, pBS_phy_SP17, pBS_phy_SP20 and pBS_phy_SP22 were isolated with Nucleospin Plasmid Isolation Mini Kit. One way sequencing was performed with the primer SP_phy_F using Sanger Sequencing Technique. The primers were designed to recognize around 270 bp upstream of the start of the phytase gene, to include both the signal peptide and the beginning of the phytase sequence.

Table 5.2. The names and the phytase activities of the selected colonies.

Plasmid Name	Phytase Activity (U/ml)	Std. dev.	Signal Peptide
pBS_phy_SP5	0,200	0,0404	lipB
pBS_phy_SP12	0,129	0,0131	dltD
pBS_phy_SP13	0,249	0,0447	lipB
pBS_phy_SP17	0,045	0,0394	lipB
pBS_phy_SP20	0,085	0,0426	lipB
pBS_phy_SP22	0,299	0,1090	lipB

Sequencing result were analyzed and aligned with the phytase sequence (NC_000964.3) to check the correct insertion of phytase via Jalview program. The results showed that the phytase was inserted correctly into the pBE-S DNA plasmid (Figure 5.13). The sequencing result of pBS-phySP17 was too short for alignment so it was not included. Determination of the signal peptide was done by comparison of 173 signal peptides with the sequencing results manually. It was determined that 5 of the plasmids included the lipB (86 bp) (Figure 5.14), and one included dltD (63 bp) signal peptide (Figure 5.15).

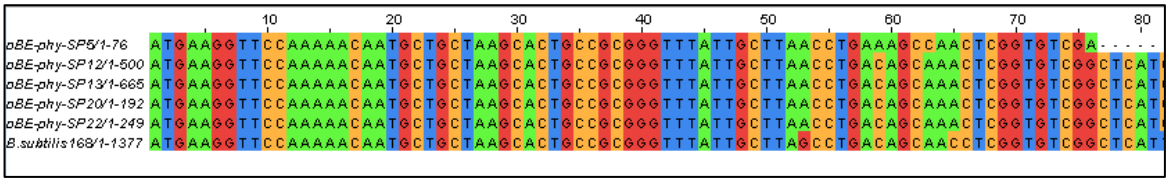

Figure 5.23. Plasmid sequencing results, aligned with *B. subtilis* 168 phytase sequence.

```
pBE-phy-SP13      51 CTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAGTCTACTCTGAAC    100
lipB-SP            1 ----------------------------------------------------      0

pBE-phy-SP13     101 TTAAGCAAAAGGAGAGGGACGCGTATGAAAAAAGTACTTATGGCATTCAT    150
                                             ||||||||||||||||||||||||||
lipB-SP            1 ------------------------ATGAAAAAAGTACTTATGGCATTCAT     26

pBE-phy-SP13     151 TATTTGTTTATCGCTGATTCTATCTGTTTTAGCCGCTCCGCCGTCTGGCG    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
lipB-SP           27 TATTTGTTTATCGCTGATTCTATCTGTTTTAGCCGCTCCGCCGTCTGGCG     76

pBE-phy-SP13     201 CAAAAGCTGCGGCCGGTGCACATATGGAGCTCGGTACCCTCGAGATGAAG    250
                     ||||||||                                  phytase gene
lipB-SP           77 CAAAAGCT----------------------------------------      84

pBE-phy-SP13     251 GTTCCAAAAACAATGCTGCTAAGCACTGCCGCGGGTTTATTGCTTAACCT    300

lipB-SP           85 ----------------------------------------------------      84
```

Figure 5.14. Plasmid sequencing results. Alignment of pBE-phy-SP13 with lipB SP sequence. Green arrow indicates the start of the phytase gene sequence.

```
pBE-phy-SP12      51 CATCTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAGTCTACTCTG    100
dltD-SP            1 ----------------------------------------------------      0

pBE-phy-SP12     101 AACTTAAGCAAAAGGAGAGGGACGCGTATGAAAAAGCGTTTTTTCGGTCC    150
                                                 |||||||||||||||||||||||
dltD-SP            1 ---------------------------ATGAAAAAGCGTTTTTTCGGTCC     23

pBE-phy-SP12     151 AATTATTTTGGCGTTTATTCTATTCGCAGGCGCCATCGCAGCGGCCGGTG    200
                     ||||||||||||||||||||||||||||||||||||||||||||
dltD-SP           24 AATTATTTTGGCGTTTATTCTATTCGCAGGCGCCATCGCA----------      63

pBE-phy-SP12     201 CACATATGGAGCTCGGTACCCTCGAGATGAAGGTTCCAAAAACAATGCTG    250
                                                 phytase gene
dltD-SP           64 ----------------------------------------------------      63

pBE-phy-SP12     251 CTAAGCACTGCCGCGGGTTTATTGCTTAACCTGACAGCAAACTCGGTGTC    300

dltD-SP           64 ----------------------------------------------------      63
```

Figure 5.15. Plasmid sequencing results. Alignment of pBE-phy-SP12 with dltD SP sequence. Green arrow indicates the start of the phytase gene sequence.

6. DISCUSSION

Recombinant studies are one of the basic tools in biotechnology used for finding out about a protein or making modifications for a better version of itself to increase the production. Phytase is an important enzyme with economical and ecological benefits by its ability to reveal inorganic phosphates from phytic acid and recently recombinant studies have become popular for its production. If an efficient phytase producing recombinant system can be found, the addition of the produced enzyme can help balancing the phosphorus cycle by decreasing the inconsumable phosphorus amount in the manure or by increasing the feed efficiency. In this study, the aim was to produce extracellular phytase from *B. subtilis* RIK 1285 with signal peptide modifications. Firstly, a plasmid was constructed, which could extracellularly secrete phytase protein acquired from *B. subtilis*, and then the effect of signal peptides and growth media on secretion of the produced phytase was investigated.

B. subtilis 168 was used as the source of the phytase gene due to its properties as model gram-positive bacteria and the entire genome being sequenced and studied. The fact that it is considered as GRAS makes *B. subtilis* a good candidate for producing enzymes that will be used directly since the products are endotoxin free. Also, choosing the source and the host as the same organism makes it possible to skip codon optimization. However, one important factor when a protein is secreted outside the cell is that its own proteases may destroy the product, so the chosen host strain in this study, *B. Subtilis* RIK1285, was deficient in two types of proteases [88].

The cloning method for both steps was chosen as In-fusion technique, which is classified as a seamless, single step directional cloning technique because it can clone multiple fragments into the chosen vector with a single reaction without the need of restriction enzyme digestion. In this technique, the ligation procedure is not required but instead the In-fusion Enzyme that generates single stranded 5’ overhangs is used for fusing the DNA fragments with vectors. The only necessity for this technique is a 15 bp homology at the termini of the sequences between the inserts and linearized vectors, and then these overhangs are annealed at the sites of complementary DNA.

The shuttle vector pBE-S DNA from the TAKARA Bio® was used as both cloning and expression plasmid. This plasmid is specifically designed for recombinant *B*. *subtilis* studies with its ability to replicate in *E*. *coli* as well and express in *B*. *subtilis*. It is an advantage because *B*. *subtilis* has low copy number that complicates the construction process. The plasmid pBE-S DNA owns different origin of replications (ori) and antibiotic resistance for *E*. *coli* and *B*. *subtilis,* pUB and ColE1 ori respectively, which helps selecting transformants from different hosts. Another important property of a vector is its promoter region that is located upstream of the gene and determines when the gene will be expressed. Generally in systems that aim production, controllable promoters, in other words, inducible promoters are used but in this case, the vector pBE-S DNA included a native *Bacillus subtilis* derived subtisilin promoter called *aprE* promoter, which is encountered frequently in *B*. *subtilis*. Though this promoter is classified as constitutive, it is only capable of expression when the bacteria reach the end of transient phase of growth. *B*. *subtilis* originated P_{aprE} belongs to a highly expressed alkaline protease gene, and thus investigated for optimization of expression [81]. The reason of preferring P_{aprE} may be due to the fact that even though the systems are designed for increasing the production when the cells starts production from the early stages of its life, the metabolic burden increases too much and prevents the cells from growing and producing.

There are methods for proving the presence of the protein such a SDS and western blotting, but product specific assays are applied to observe whether the product works actively. For a correct and easy evaluation of a recombinant study, methods related directly with the protein are chosen and in most cases, fluorescent tags are used. PSM agar plate assay was used for observation of the extracellular phytase activity, although this method is not accurate for detecting the amount of enzyme produced, it was used as a first step to find out whether the bacteria produce phytase.

The selected colonies were grown in different growth media to compare whether different media had any effects on growth and production. Among different media used, rice bran medium gave the best results where simple but basic media such as LB or YPD showed no phytase production, similar with other studies [62]. The phytase activity was analyzed by measuring the amount of inorganic phosphates. For elimination of background activity and the phosphate already available in culture medium, each activity was considered with its

own blank. The source of the background activity could be the phosphorus belonging to the medium components. The utilization of agro residue rice bran as a substrate for bacterial growth is a well-known and cheap carbohydrate source, abundant agricultural waste/residue/by product. It was shown in previous studies that the carbon source had an effect on initialization of phytase production. The bran used in the medium was recommended by other studies. Some bacteria, especially *Bacillus,* can use it as a long-term carbon source via hydrolysis [82]. Usage of different types of bran such as wheat, rice was observed in which bacterial cultures were used for production of an expected product. Especially for phytase production, the presence of phytate or phytate containing material inoculation triggers the production of phytase because the only way of the bacteria reaching its carbon is to produce phytase [83]. Also, it is known that *B. subtilis* has a tendency to synthesize hydrolysis enzymes depending on the resources found in its environment to take advantage, so this could be a reason of higher production with bran including media [57]. According to this study, the overall results showed that the components of the growth medium could directly affect the production stage of the enzyme. Parallel to other studies [78-80], rice bran proved itself to be an essential component for phytase enzyme production since the highest phytase activity was achieved with rice bran liquid medium.

The result of this study consistent with other studies about the advantages of rice bran but the high standard deviation in the activity results belonging to rice bran medium were concerning. The high deviation could be explained by homogenization issue of bran in the culture media. The literature was scarce in this area and no evidence of a problem was recorded in other studies including bran. It could be said that as beneficial as it is, bran usage could be deceptive for the first stage of a recombinant production. An easier to handle medium should be used for selecting the phytase producing bacteria, and then complex media including rice bran can be suggested for the later stages of large scale production.

A pink color change was observed in some of the cultures grown in rice bran medium. As it can be seen clearly in Figure 5.10, some colonies such as pBE-phy-SP6 took a more pinkish color. The color change of the medium occurred around 48^{th} hour and it is important to note that phytase activity correlated well with the color change. Generally, the color change indicates the presence of indoles (IAA- indole-3-acetic acid) produced in

similar *Bacillus* originated phytase production [30,75,84,85]. Further assays could be conducted in the future to record whether the transformants are also capable of producing IAA since indoles is classified as a phytohormone and is a desired property in PGPR [86]. In further studies, indole assays can be applied and the relationship between IAA and phytase can be investigated.

After the activity measurements of all colonies, one was chosen for further quantification. In order to prove the presence of the produced phytase, strain annotated SP12 was chosen and the supernatant was used for SDS-page analysis. The literature shows that *Bacillus subtilis* phytase has a size around of 41 kDa [87] which correlates with our result shown in Figure 5.13. For comparison of the growth time, medium samples were taken from *Bacillus* grown in LB and rice bran liquid medium. As expected, there wasn't any protein bands representing phytase in LB medium samples which also was confirmed with no phytase activity. Since only the supernatant of the cell suspension was used, no other bands were present in the SDS-page. Also, the presence of phytase was compared at its 24^{th} and 48^{th} hours and it was observed that the protein band representing phytase is much thicker in 48^{th} hour, which shows that the production starts around that hour. Some studies indicated that they reached the same result in the SDS-page in 24^{th} hour samples where other studies indicated different hours [62]. This difference could be explained with the fact that different growth parameters might be affecting the result by creating a different timeline of growth. Our results indicated an intact one band of protein at the expected size. As a remark, the plasmid also included a His-tag that could be used for purification and isolation of the phytase but it was not used during this study. Even column purification could be applied for easy purification if necessary.

The phytase activity of the original source *B. subtilis 168* was analyzed and no extracellular activity was recorded (data not shown). Some studies indicated that the promoter region of the gene was not operable in *B. subtilis* [62]. For further investigations, the promoter region can be analyzed, or the activity assays can be repeated to understand whether the phytase is produced intracellularly. Also in the future work, intracellular phytase can be analyzed to check whether there is any phytase secreted inside the cell. Intracellular and extracellular phytase levels may change if there are no signal peptides cloned.

One of the focus points of this study was signal peptides (SP) that determine the final destination of the produced enzyme and have great significance in industrial biotechnology. *B. subtilis* has the ability to express proteins extracellularly and many signal peptide optimization studies revealed that it could enhance secretion therefore cut down the downstream processes [55]. Generally among various SPs, the proteins carrying a sec-type SP get secreted outside the cell. However, if protein doesn't have a transport signal, they cannot leave the cytoplasm. Generating a signal peptide library makes it possible to find the most suitable secretory signal, which is very important for the extracellular secretion of the target protein considering that each production can be optimized with a different SP, in some cases, non-native SP. A downside of using a system where peptides are cloned as a pool is that it's not possible to know whether all of the 173 signal peptides are successfully inserted. In this work, the plasmid:SP DNA mixture ratio was prepared according to the recommended procedure (TAKARA) but number of colonies obtained was low. A pitfall of preparation of signal peptide library is that during the cloning process there is no way to understand which signal peptides are successfully inserted into the plasmid other than picking each colony for sequencing their plasmid. All of the colonies were pooled which creates a possibility of encountering the same signal peptide within these colonies.

In the *B. subtilis* transformation step, freshly prepared competent cells were used which directly affects the efficiency. Other studies indicated that they had approximately 3,000 number of colonies while only 29 colonies were observed in this study [62]. Even though the colony observation was much lower than expected with SP library, the colony counts of the control groups showed that the transformation step was actually successful.

As a final step, 6 colonies including the ones with the highest and the lowest activities were selected and these plasmids were sequenced. The pairwise alignment results confirmed that the cloning was successful and phytase gene was inserted correctly. Surprisingly, 5 of the colonies shared the same SP sequence, lipB, and only one colony was fused with dltD and both of these signal peptides belong to the sec-type. As said before, signal peptides only determine the final destination of the product. So the activity of the enzyme is actually not affected, but the amount of enzyme secreted may increase according to its SP. The meaning of different activity results of the colonies sharing the

same SP could either mean that the activity analyses were not spot-on, the small amount of supernatant culture used for analysis might have caused deception. It should be noted that *B*. *subtilis* is known to carry low number of plasmid copies and maybe the occurrence of different activities with the same signal peptide is the consequence. Another reason could be due to the low efficiency at both transformation steps. SP library formation and transmission of this library into *B*. *subtilis* cells might have affected the overall process and only one of the signal peptide was able to be cloned. A secondary screening should be performed to check whether a higher activity can be reached. For further investigation, both SP library preparation and *B*. *subtilis* transformation steps should be repeated.

7. CONCLUSION AND FUTURE PROSPECTS

- A vector expressing phytase was successfully constructed and cloned into the host organism *B. subtilis* RIK1285.
- The transformed colonies showed different activity results in a variety of media. The results showed that rice bran had a significant effect on production of phytase.
- To find the most compatible signal peptide for an extracellular production with higher yields, a SP library was prepared. However, only 2 signal peptides were detected with different enzyme activities; lipB and dltD.
- It was shown that rice bran was effective for production but for identification or detection, but it was found out to be not suitable for identification or detection of phytase.
- This study can be used as a basis for development of transformants with higher phytase activity.
- In the future, the developed phytase secreting *B. subtilis* strains can be applied to plants to test their growth-promoting activity.

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