DEVELOPMENT AND SCALE-DOWN OF ENZYME ANALYSIS METHODS FOR HIGH THROUGHPUT SCREENING OF IMPORTANT SOIL ENZYMES

by

Sheyda Shakoory

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APPROVED BY:

Assist. Prof. Dr. Emrah Nikerel .....
(Thesis Supervisor)

Assoc. Prof. Dr. Kazım Yalçın Arga .....

Assist. Prof. Dr. Bahar Soğutmaz Özdemir .....

DATE OF APPROVAL:..../..../2018

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ABSTRACT

DEVELOPMENT AND SCALE-DOWN OF ENZYME ANALYSIS METHODS FOR HIGH THROUGHPUT SCREENING OF IMPORTANT SOIL ENZYMES

Enzymes are generally accepted as the “Swiss-army knife” of sustainable bio-economy and as such, they represent an economical significance. Chief among those amylase, protease, phosphatase and urease for which various studies about their production methods is an attractive topic in biotechnology research.

The heart of industrial biotechnology is the availability of the producer (micro)organism. While the recombinant-route involving the use of advanced molecular techniques e.g. synthetic biology toolbox are of great interest, the immense undiscovered potential of nature and natural producers is very appealing, not only for economical purpose, but also from scientific point of view, allowing to discover possibly new mechanism even for already known processes. To materialize this potential, fast and affordable, high throughput screening methods that will allow identification of producers of desired enzymes among large number of environmental samples, need to be developed. This requires in turn, detailed study of enzyme properties, modification of available methods or development of novel methods if necessary.

This thesis aims to set up such high throughput methods for four aforementioned enzymes. The developed, scaled-down methods are based on a combination of spectroscopic and colorimetric approaches, with automatization and miniaturization, on 96-micro titer well plates (MTP). The methods are first tested using type strains for which the activity are known. Later, the developed methods are used to screen the in house available collection, consisting of 537 unknown microorganisms. Using this method new strong enzyme producers were discovered and reported. 87 isolates was observed as urease positive and 76 isolates was observed as amylase positive. Among those organisms 6ys29 is both urease and amylase positive. 15 isolates was detected as protease producers. 5ys19, 7ys60, 7ys66 are both protease and urease, 6ys29 is amylase and urease positive microorganism. 7ys48 is protease and amylase positive. 6ys59, 6ys33, 5ys5 are phosphatase and amylase, 7ys39, 5ys23, 7ys76, 5ys42 are urease and phosphatase positive isolates.

ÖZET

ÖNEMLİ TOPRAK ENZIMLERININ YÜKSEK VERİMLİ TARAMA IÇIN ANALIZ YÖNTEMLERININ GELIŞTIRILMESI VE ÖLÇEKLENDIRILMESI

Enzimler genelde sürdürülebilir biyoekonominin "İsviçre çakısı" olarak kabul edilir ve bu nedenle ekonomik önem arz ederler. Bunların arasında amilaz, proteaz, fosfataz ve üreaz özellikle önem taşir ve biyoteknoloji araştırmalarında üretim yöntemleri yer tutar.

Endüstriyel bioteknolojinin kalbi üretici (mikro) organizmanın varlığıdır. Rekombinant DNA teknolojilerin ve sentetik biyoloji gereçlerinin kullanılması büyük ilgi görüyorsa da, doğanın ve doğal üreticilerin henüz keşfedilmemiş potansiyeli, yalnızca ekonomik amaçla değil, aynı zamanda zaten bilinen süreçler için bile yeni mekanizmaları keşfetmeye uygun olması sebebi ile ilgi çekicidir. Bu potansiyelin hayata geçirilmesi için çok sayıda çevresel numune arasında arzu edilen enzim üreticilerinin tanımlanmasına imkân verecek hızlı ve ucuz yüksek verimli tarama yöntemleri geliştirilmelidir. Bu da enzim özelliklerinin ayrıntılı olarak incelenmesi, mevcut yöntemlerin değiştirilmesini veya gerektiğinde yeni yöntemlerin geliştirilmesini gerektirir.

Bu tez, adı geçen dört enzimlerin ölçülmesi veya üreticilerinin taranması için yöntemler geliştirmeyi amaçlamaktadır. Bu yöntemler ölçek küçültme çerçevesinde, 96-kuyulu plakalarda otomasyona müsait, spektroskopik ve kolorimetrik yaklaşımların birleşimine dayanmaktadır. Metotlar önce aktivitesi bilinen tip suşları kullanılarak test edilmiş, daha sonra 537 bilinmeyen mikroorganizmadan oluşan bölümümüzde mevcut kültür koleksiyonunu taramak için kullanılmıştır. Bu yöntemi kullanarak yeni güçlü üreticiler keşfedilmiş ve özellikleri raporlanmıştır. 87 izolat üreaz pozitif olarak ve 76 izolat amilaz pozitif olarak gözlendi. Bu organizmalar arasinda 6ys29 hem üreaz hem de amilaz pozitiftir. Proteaz üreticileri olarak, 15 izolat tespit edildi. 5ys19, 7ys60, 7ys66 proteaz ve amilaz pozitifken, 6ys59, 6ys33, 5ys5 fosfataz ve amilaz, 7ys39, 5ys23, 7ys76, 5ys42 üreaz ve fosfataz pozitif izolatları olalarak keşfedilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

g	Gram
h	hour
kg	Kilogram
M	Molarity: n/V, moles per volume
mL	Mililiter
mM	Milimolar
nm	nano meter
α	Greek letter alpha
β	Greek letter beta
μ	Greek letter mu micro, 10^{-6}
μL	Microliter
DNS	Dinitrosalycyic acid
ELISA	Enzyme-linked immunosorbent assay
HTS	High throughput screening
MTP	Micro titer plate
MUB	modified universal buffer
OD	Obtical density
pNPP	p-nitrophenyl phophate
rpm	revolution per minute
SmF	Submerged fermentation
SSF	Solid state fermentation

1. INTRODUCTION

1.1. INDUSTRIAL SOIL ENZYMES

Enzymes are important bio-catalysts accelerating biochemical reactions, in several industry such as food, pulp and paper, detergent, pharmaceutical, molecular biology, biofuel, textile, baking and animal feeds. The application of enzymes is not limited to be an intermediate but also they are used in the final products, e.g. in beer, wine, vinegar, and cheese production and also in leather and linen industry [1, 2]. The emergence of biotechnology necessitates not only the widespread use also large scale production and the analysis of these enzymes. These are typically extracted of produced from a variety of sources such as plants, animals, tissue, and microorganism such as bacteria, fungi and yeast [1].

The following soil enzymes are used in several industries, and are therefore of commercial value: Phosphatase, amylase, protease, urease, chitinase, dehydrogenase, beta glucosidase, amidase and cellulase. Beta glucosidase decomposes the organic matter, proteases mediate ammonification or N mineralization process in soil [3], amylase degrades the complex carbohydrates into monosaccharides such as glucose [4], urease hydrolyses urea to NH_4^- and phosphatase converts the organic phosphate to inorganic form. All these enzymes contribute to soil health [5].

These are typically produced by so-called Plant Growth Promoting Rhizobacteria (PGPR), which play vital role in carbon, nitrogen and mineral cycles. Nevertheless, the production of the soil enzymes are not limited to bacteria only, they can as well be produced by animals and plants. Isolation and identification of these (micro)organisms are central to develop the toolbox for white biotechnology.

1.1.1. Amylase

Amylase is an enzyme that degrades starch. Starch is found in all fruits, seeds, cereal, and tuberous crop and the main sources of starch are rice, wheat, potato, cassava, and maize. Starch is synthesized in plants during photosynthesis process in plastids in leaves and

amyloplasts which are localized in roots, tubers and seeds. Starch consists of amylose and amylopectin which they are glucose polymers [6].

Amylose has long linear α(1-4) glycosidic bonds and is a glucose polymer formed by the amorphous region at granules. The degree of polymerization is determined the number of glucose in amylose, which is different in each plant species, e.g. degree of polymerization of potato is 1000-6000 while for maize and wheat this is between 200 and 1200. Amylose is the most important energy source in plants because they are slowly digested [6, 7].

Amylopectin, in contrast, has short α (1-4) linear bonds consisting of 10 to 60 glucoses and α (1-6) side chain consist of 15 to 45 linear glucose units. Amylopectin is soluble in water and it forms the crystalline region in starch granules especially in cereal starches. Collectively, starch is the substrate that α-amylase act upon it to cleave terminal glucose residues and α (1-6)glycosidic linkages [7, 8].

Starch degrading enzymes, amylases, are classified into two main groups based on their active site. First group, endoamylaseshydrolyze internal α (1-4)glycosidic linkages, localized at internal part of amylose or amylopectin. As an example, α-Amylase (E.C.3.2.1.1.) is an endoamylase, termed also as 1,4-α-D-glucanglucanohydrolase; glycogenase, they hydrolyze starch to produce glucose and maltose. As a result of hydrolysis of α-glycosidicbonds, α-anomeric monosaccharaides or oligosaccharides produced [7]. The enzyme α-amylase is typically active at presence of calcium as a metal co-factor. The starch hydrolyzing enzymes and the starch modifying or starch transglycosylating enzymes are subclass of α-amylases.

The second class, exoamylases are active on external part of glucose units of amylose or amylopectin.*β*-Amylases (E.C.3.2.1.2) are acting on non-reducing end of branched polysaccharides and cleaves α (1-4) glycosidic bond such as glycogen and amylopectin. Amyloglucosidase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20) are the exoamylases cleaving both α (1-4) and α (1-6) glycosidicbonds [7].

Amylases are second most frequently used enzyme (after protease) in detergent industry. Hydrolyzing starch to monosaccharides and starch liquefaction, α- amylase is the promising enzyme to convert starch to oligomaltodextrins, glucose, and maltose, however alkaline pH and presence of calcium ion (damaging the washing machines, due to water hardness) for amylase activity are not desirable properties of this enzyme [8].

1.1.1.1. Amylase sources

α-Amylases have different sources ranging from plants, animals to microorganisms. They are typically isolated from rice, cassava mash waste water, and barley. However microbial sources are typically preferred in comparison to other sources due to e.g. rapid growth, scalability of the production, being cost effective as they need less nutritional compounds and less space. Furthermore, microorganisms can further be engineered to produce α-amylase with thermostable or pH stable properties. Various bacteria and fungi produce α-amylase, chief among those are *Bacillus amyloliquefacience* and *B. licheniformis, B. stearothermophilus, B. subtilis, B. cereus*as bacteria used for commercial manufacture of α-amylase in several industries. Noting that liquefication and saccharification processes require high temperatures (100-110˚C), *B. stearothermophilus, B. megaterium*are promising sources forthermostable α-amylase production. *Serratia marcescens, Thermomonospora curvata, Thermomonospora vulgaris,* are most wellknown bacterial sources for amylase production. *Streptomyces agglomeratus*, *Streptomyces coelicolor* are also reported as amylase sources [6-8]. In addition to thermostable producers, halophilic micoorganisms are also used for enzyme production as they tolerate the high saline conditions. *Chromohalobacter sp*., *Halomonasmerdiana*, *Halobacillus sp.*, *Bacillus dipsosauri*, and *Halorculahispanica* are halophilic sources for this enzyme.Apart from bacteria, the most important fungal sources of α-amylases are *Aspergillus sp*. and *Penicillium sp.* while the most commercially important fungal sources known *A. niger, A. awamori*, and *A. oryzae*. *Penicellium fellutanum, Aspergillus niger*, *Aspergillus awamori*, and *Aspergillus oryzae* are fungal amylase producers [7, 8].

1.1.2. Protease

Proteases are the most economically important industrial enzymes and play significant commercial role. Protease commercial applications includes detergents, protein as supplements, leather, photography, food, baking, pharmaceutical, and dairy industries. About 60 percent of enzymes sales in word wide is belong to proteases[9].

Proteases are classified into two major groups that include exo- and endo-peptidases based on the region of their enzymatic action. Endo-peptidases cleave peptide bonds on non-

terminal amino acid while exo-peptidases cleave peptide bonds near to amino or carboxyl terminus (named as aminopeptidases and carboxypeptidases respectively). The exo-peptidases are further subdivided into four major groups as serine proteases, aspartic proteases, cysteine proteases, and metalloproteases [10].

Neutral proteases act at pH between 5 and 8 and they are typically used in brewing and food industries. For alkaline proteases, the optimal conditions are pH 10 and temperature 60˚C, widely used in detergent industry. Fungi produce larger portfolio of enzymes in terms of pH range. *Aspergillus oryzae*as an example produces active protease in both acid, neutral and alkaline ranges. These are useful in cheese manufacture, reduce the bitterness of food protein hydrolysates, and food protein modification, respectively. The fungal alkaline proteases from *Tritirachium album* and *Conidio boluscoronatus*are also used in detergent industry. Viral proteases are generally endo-peptidases and they play important role by acting on protein processing of viruses which cause cancer and AIDS [10, 11].

1.1.2.1. Protease sources

Similar to amylases, proteases are also obtained from various sources, including plant, animal, and microbial. Papain, bromelain, and keratinases are plant proteases, papain can be isolated from latex of *Carica papaya* fruits, and bromelain extract from stem and juice of pineapples. Plant keratinases are heavily used in degrading hair and wools to produce lysine, an essential amino acid. Animal proteases such as trypsin, chymotrypsin, pepsin, and rennin are produced in large amounts. Trypsin, produced by *Bacillus sp.* and *Aspergillus sp*., is a serine protease which degrades food proteins and is used as bio-pesticides and in bating step in leather processing. Chymotrypsin is pancreatic enzymes and they used in milk protein deallergenizing. Pepsin is found in approximately all vertebrate animals stomachs and it is an acidic protease. Pepsin becomes active after hydrochloric acid treatment to release from zymogen. Renin or chymosin is produced in ruminant mammal stomachs and have large application area in dairy industry to improve the taste of curd. Rennin gets active at the presence of pepsin[9, 12].

The microbial sources for protease are attractive and mostly preferred sources because they have extensive biochemical diversity and they are capable to be used in protein engineering, they are cost and time effective and they need less time and limited space and are easy to

handle in terms of cultivation. Bacterial sources produce mostly alkaline and neutral proteases. Almost *Bacillus sp.* are well known to produce neutral and alkaline proteases. Bacterial sources are mainly owned to Bacillus species such as, *B. alcalophilus, B. ciculanas, B. coagulans, B. firmus, B. intermedius, B. lentus, B. subtilis, B. pumilus, B. licheniformis, B. sphaericus, B. thuringiensis B. proteolyticus and B. mojavensis*. There are sources for termostable protease production such as *Bacillus stearothermophilus*, *Bacillus thermoruber*, *Thermus aquaticus*, *Torula thermophila*, *Staphylothermus marinus, Thermoactinomyces thalpophilus,* and *Thermobacteroides proteolyticus* are bacteial soures of protease. Protease fungal sources such as, *Penicillium griseofulvin*, *Rhizopus oryzae*, *Tritachium album, Fusarium graminearum, Entomophthora coronata, Aspergillus* speceis such as *A. candidus, A. flavus, A. fumigatus, A. melleus, A. niger, A. oryzae, A. sulphureas, A. sydowi* and *A. sojae*. Yeast sources for protease production including *Candida lipolytica, Yarrowia lipolytica, Aureobasidium pullulans, Streptomyces rectus, Streptomyces griseus, Streptomyces moderatus, Streptomyces diasturicus, Streptomyces corchorusii* and *Streptomyces pactum.* [10, 13, 14].

1.1.3. Alkaline Phosphatase

The enzyme alkaline phosphatase (EC 3.1.1) has been first described in 1907 by Suzuki and his colleagues as an enzyme widely found in nature and then named it as phytase. They realized that during germination in rice seeds inorganic phosphate appears instead of phosphate ester which was related to the presence of a phosphatase enzyme. In further studies Levene and Medigreceanu found phosphatase in organ tissue of dogs such as intestinal mucosa and kidneys. Phosphatase is characterized as a metalloenzyme that can hydrolyze phosphate esters to alcohol and phosphoric acid [15, 16].

Alkaline phosphatase is typically measured using spectrometric, chemiluminescent, or fluorometric methods. Spectrometric method generally is based on degradation of disodium phenyl phosphate as substrate. Fluorometric method has specific fluorophor substrate monophosphoric ester whith yellow color appearance upon degradation with phosphatase. Chemilumiescent method, also known as fast alkaline phosphatase method, is the simplest and fastest method (F-Alp method) using luminometer as detector [17, 18].

1.1.3.1. Alkaline phosphatase sources

Several microorganisms produce alkaline phosphatase, including several *Candida spp, Candida glabrata, Candida tenius, Candida albicans, Candida maltosa, Saccharomyces boulardii, Saccharomyces cerevisiae, Yarrowia lipolytica, Schizosaccharomyces pombe,* are yeast sources for phosphatase production. Actinomycete such as, *Nocardiopsis dassonvilleli* and *Oerskovia xanthineolytica*are reported as phosphatse producers.

*Bacillus subtilis,B. cereus, B. megaterium, B. licheniformis, B. anthracis, Bacteroides ruminicola, Klebsiella aerogenes, Micrococcus sodonensis, Sphingobium*sp. Similarly, *S. yanoikuyae,* and *Bacteroides thetaiotaomicron,* are bacterial sources for phosphatase.

Aspergilus clavatus, Aspergillus flavus, Aspergillus oryzae, *Aspergillus niger*, *Penicillium digitatum, Magnaporthe oryzae, Aspergillus kawachii, Fusarium oxyporum, Pucciniastriiformis,* are fungal alkaline phosphatase sources [16, 19].

1.1.4. Urease

Sumner in 1926 isolated urease from jack bean, also known as ureaamido-hydrolase (EC 3.5.1.5), has crystallized structure, and enzymes are protein. Ureases are well known as nickel metalloenzymes which investigated by Dixon et al in 1975. They have identified the three dimensional structure and self-buffering ability of this enzyme. Urease converts urea to ammonia and carbon dioxide at the resulting of this reaction pH is increase up to “9.2” the optimum pH condition is around 7.0 and for acid urease is 2.0.

$$H_2N - CO - NH_2 + H_2O \xrightarrow{urease} CO_2 + 2NH_3 \qquad (1.1)$$

Determination of urease activity has been the subject of several studies, given the economic importance of this enzyme. These are generally classified in three sections as manometric, colorimetric and titrimetric. Skujins and MacLaren in 1969 measured urease activity by using ^{14}C-urea which is radiopharmaceutical and measured the CO_2 resulting from urease activity. M. W. Wheatherburnin 1967 performed Berthelot method to determination amount of ammonia by reacting it with phenol hypochlorite and alkaline hypochlorite producing

blue color. Natarajan in 1995 also modified Berthelot method to measurement of urease activity. Urea in wine was detected by high performance liquid chromatography (HPLC) method and its level is monitored upon urease activity. Potentiometric assay has been employed to measure urea in milk. Lastly, infrared spectrometry in a multi component method is used to detect the urea content in food samples [20-24].

1.1.4.1. Urease sources

Similar to the other enzymes, microbial sources has been used to produce urease. Examples include, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium roqueforti* as fungal sources which can be utilized to produced urease. Actinomycete sources such as *Streptomyces lydicus*, *Streptomyces agglomeratus* has also been used as urease producers.

From the bacteria side, the reported producers include *Bacillus pasteurii*, *Lactobacillus fermentum*, *Helicobacter pylori*, *Klebsiella pneumaniae*, *Klebsiella aerogenes*, *Proteus vulgaris*, *Bacillus subtilis*, *Rhizobium meliloti*, *Psychrobacter cruhalolentis*, *Enterobacter aerogenes*, *Pseudomunas syringaeare*, *Arthrobacter oxydans*, *Athrobacter mobilis*, *Staphlococcus saprophyticus*, *Ureaplasma urealyticum*, *Providencia stuartii*, *Sporosarcina pasteurii*, [25-27].

1.2. ENZYME APPLICATION

The main applications of enzymes are typically listed as detergent, textile, pulp and paper, fuel alcohol, pharmaceutical, cosmetic, leather , waste water treatment, even smaller industries e.g. cork treatment (taste of cork in wine cause consumers reject the product). In general, food industry including baking, texturizing, flavoring, clarification of juice, lipid emulsifier, and bread stalling as well as and poultry and animal feed are the second largest sectors where enzymes are used in various applications [1][28].

In feed industry enzymes were used as animal feed additives like xylanase and β-glucosidases for monogastric animals (e.g. chicken) which are not able to digest cereals or other plant-based feed. Among those enzymes, phytase is an additive enzyme to help consumption and digestion of phytate and release phosphorus normally bound in phytic acid

up to 80-90 percent. The use of phytase as additive allowed the banning the bone-meal, used formerly as source of inorganic phosphorus, after the “Mad Cow” disease.

Another commercially significant field of application of enzymes is pharmaceutical and cosmetic industry. Enzymes cocktails of lipase, amylase, and protease enzymes are used for treatment of pancreatic insufficiency. In toothpastes and mouthwashes oxidative enzymes use to inhibit pathogenic bacteria in mouth causing plaques, gingivitis and caries.

Similarly, products containing enzymes degrading starch and protein for example from food residues to glucose, protease and glucose oxidase are other enzymes which use in personal care segment, including enzymes have application in cosmetic such as shower gel, hair dying and hair waving [2, 29].

Enzymes play also important roles in bioremedation of environmental pollution caused by several industries, e.g. textile.An example to this is the souring step in cotton production, requiring high temperatures and alkaline pH. Using pectinase in souring step led to the decrease of the temperature and pH for cotton manufacturing. Similarly, cellulase, laccase, peroxidase, α-amylase are also used in stone-washing, bleaching, excess dye removal, de-sizing respectively [1, 2].

1.2.1. α-Amylase applications

One of the α-Amylase applications is industrial manufacture of glucose and fructose. First step is gelatinization in order to dissolve starch in water. Second step liquefaction which means convert starch into short chain dextrins which require thermo stable α-amylase. Last step is convert starch-hydrolystate syrup to 95 percent concentration of glucose syrup by using glucoamylase and pullulanase which cleaves α (1-4) from non-reducing ends and α (1-6) glycosidicbond respectively which termed saccharification. At presence of fungal α-amylase maltose and by using pullulanase and β-amylase high maltose syrup obtained. The final step is preparing fructose syrup by using glucose isomerase. In baking industry using α-amylase cause to increasing loaf volume and postpone staling, make the desirable texture, and increase shelf life. Branching enzymes and pullulanase are also use for this purpose, to obtain the better result to make the desirable baking products branching enzymes should be

used with α-amylase, β-amylase, cellulose, and lipase. α-Amylase also use in laundry or dishwashing, animal feeds, paper, biofuel and textile industries [1, 6, 30].

1.2.2. Protease Applications

Alkaline proteases are the most commercially important type of proteases which have applications in leather, dairy, detergent, food, and pharmaceutical industry. Traditional Leather processing has drawbacks such as being expensive and hazardous chemical like sodium sulfide which strongly create environmental pollution. Use of protease in de-hairing process is an attractive alternative choice because they can be active and stable in extremely alkaline conditions and also it help to improve the leather quality. In food industry proteases use in dairy as cheese making, baking industry in order to hydrolysis gluten, and use in manufacture of soy sauce and other soy products, debittering in products such as clinical nutrition supplement and health products which protein hyrdolysates is use on their production, in addition, synthesis and resolution D,L-amino acid, aspartame as an artificial sweetener is synthesis by protease from *Bacillus thermoprotyolyticus* [10, 31].

The most commercially important field of application for proteases is in detergent industry due to their stability and activity at high alkaline pH. Protease also is well-known in mineralization of nitrogen presence in source. Protease use in detergent industry by removing protein contain stains and also in pulp and paper industry as biofilm removal. In addition, protease use in leather industry at de-haring and bating steps. Papain and bromelain proteases was used for skin care such as dermal peeling products, after a while to reduce the cost bacterial protease from *B. subtilis* trade name subtilisin use for hydrolysis epidermal cells [1, 10, 12].

1.2.3. Urease Applications

In alcoholic beverages removing urea by acidic urease is highly important to prevent the reaction between urea and ethanol and production of ethyl carbomate which is carcinogenic compound. Urease also have medical application for example it use in inborn metabolic errors diagnosis that cause mental retardation. Urease also use for water reclamation for space stations and long term flights, remove urea from wastewaters and fertilizer wastewater

effluents. Urease combination with activated charcoal and ion exchanged and artificial kidney for remove urea from blood help to reduce dialysate.

Urease also use as urea indicator in resins, glues, solvents, detergent, cosmetic and liquid soap. Analysis of urea, creatinine, and arginine, carbon dioxide or ammonia production, and measurement of IgG are other applications of urease. Urea use as fertilizer, however waste materials should be clear from urea before release in environment. Urease use for urea remove from urine from animals, inherent metabolic errors detection, artificial kidney, food and beverage for example using acid urease for removing the urea from alcoholic beverage to controlling the reaction between urea and ethanol, estimation urea in blood[32, 33].

1.2.4. Phosphatase Application

Alkaline phosphatase has application in food industry, for example it can use in food quality control and drinking water. In heat treatment in milk and dairy products alkaline phosphatse presence as endogenous enzyme in milk, alkaline phosphatase activity assay used as indicator for estimate the effectiveness of heat processing in pasteurization. Phosphatase also has application in molecular biology laboratories by removing the phosphate group and labeling in ELISA, blotting and sequencing and nonisotopic probing[19, 28].

1.3. ENZYME PRODUCERS

There is wide range of enzyme producer sources in nature such as animals, plants, microorganism’s bacteria, fungi, yeast, etc. After development biotechnology recombinant sources added to list of enzymes producers. However, they are not suitable for food, need to know how, and methods are expensive recombinant technique had limited applications. Therefore, natural producer hosts are noteworthy to screening and characterization. Desired properties of hosts are fast producers, highly active enzymes, resistant to environment stress, large portfolio of enzymes.

1.4. ENZYME PRODUCTION METHODS

Current methods for enzyme production are chiefly submerged fermentation (SmF), solid state fermentation (SSF) and immobilization. Submerged fermentation is adequate for secondary metabolite extraction. The main mobile phase is liquid whereby the organism is in highly moist conditions. Solid state fermentation, in contrast, is highly cost effective due to use of agro industrial residues such as wheat bran and high productivities yet suffers from heterogeneity in culture media. The fermentation conditions such as pH, moisture, aeration, oxygen transfer can be easily adjusted in submerged fermentation. Solid state fermentation is preferred with microorganisms (typically fungi) mildly affected from little to no moisture content in their growth and production. The agro-residues are valuable due to their rich nutrient content, high volume, which can be converted into value-added products using SSF [34].

Amylase by submerged fermentation method by using potato starchy waste as a agro-industrial residues from *Bacillus amyloliquefaciens* was produced at optimum temperature 50˚C after 48 hours incubation. The temperature for amylase production is between 45-60˚C and the optimum was 50˚C and the optimum pH 7.0 for produce extracellular amylase from *B. amiloliquefacience,* optimum pH and temperature was 6.0 and 50˚C respectively and optimum moisture content was 90 percent in fungus *Thermomyces lanuginosus* was studied in SSF process. Optimum pH and temperature for *Bacillus sp*. was at 60˚C and 6.5 and for *Aspergillus oryzae* was at 50˚C and 5.0-6.0 respectively. As a consequence the fermentation condition depends on the microbial sources and the production method [35, 36].

Protease produce during post-exponential phase. Protease production can be influenced by culture condition such as carbon and nitrogen sources therefore use of easily metabolized nutritional sources such as glucose and amino acids could increase the yield of protease production. In solid state fermentation the optimum pH for alkaline protease is 9.0-11.0 and the optimum temperature for *Bacillus spp*. at 50˚C. The optimum moisture of content was 140 per cent and the xylose and maltose as carbon source and yeast extract as nitrogen source supported maximum activity of protease in SSF process [37-39].

1.5. HIGH THROUGHPUT SCREENING

High throughput and ultra-high throughput screening (HTS and uHTS) methods are miniaturization-based methods focusing on 6, 12, 24, 48, 96, 384, 1536, 3456 well plate format of samples. All devices are typically designed for robotic, cost effective assays, reducing time and chemical and biological material consumption especially when the large sample collections and large compound library needs to be analyzed or screened for the desired feature [40, 41].Plate based assays decrease volume of assays and micro fabricated devices are developed for continuous flow assays.HTS or uHTS is useful when large numbers of chemical or reactions and natural producer library required to analysis. Biochemical screens include enzymatic assays, protein-protein interactions and protein stabilization, and cell-based screens include target based, and phenotype based screening are two main type of high throughput screening [40, 42]. This approach has wide area of application in drug discovery and pharmaceutical industry, also it use in enzyme analysis and explore ligands for receptor.

A method suitable for HTS should:

(i) be simple to apply,
(ii) be suitable for parallelization,
(iii) comprise all assay steps at a single plate or well,
(iv) contain as few steps as possible (even at the expense of accuracy) in each assay,
(v) result in almost same or comparable outcome with low throughput assay counterparts.

Focusing on enzyme screening methods, most of available screening methods are unfortunately not suitable for HTS method as they have difficult steps such as filtration, evaporation, centrifuging, separation and washing steps which are also are not fit to automatization. Therefore HTS studies aims to generate homogeneous assays to circumvent all the challenges in miniaturization and automation.

In each HTS assay for enzyme screening, three main features are present: a selective reaction specific to the enzyme, indicator and a detection system. Next to these are the buffers and additional agents to start, stop or enhance the reactions or indicators are needed. As for various detection systems, fluorescence correlation spectroscopy, fluorescence polarization

(FP), homogeneous time resolved fluorescence resonance energy transfer (HTR-FRET), Scintillation proximity assay (SPA) electrochemi luminescence (ELC) fluorescence correlation spectroscopy (FCS) can be listed. Typically, fluorescence based methods are preferred in lieu for biochemical assays since they have high sensitivity and speed Figure **1.1** [40, 43].

In enzyme assays recent approaches based on chromogenic and fluorogenic high throughput screening has been reported. For instance, adrenaline is a chromogenic assay. As a result of reaction between remain sodium periodate from enzymatic reaction and adrenaline red color appears. This method used in lipases and esterases measurement. Fluorescein bisothiocyanate (FITC) other example of high throughput screening assay, used in kinase, phosphatase and protease activity assays by using labeled protein such as FITC-casein which is fluorescein protease substrate [44].

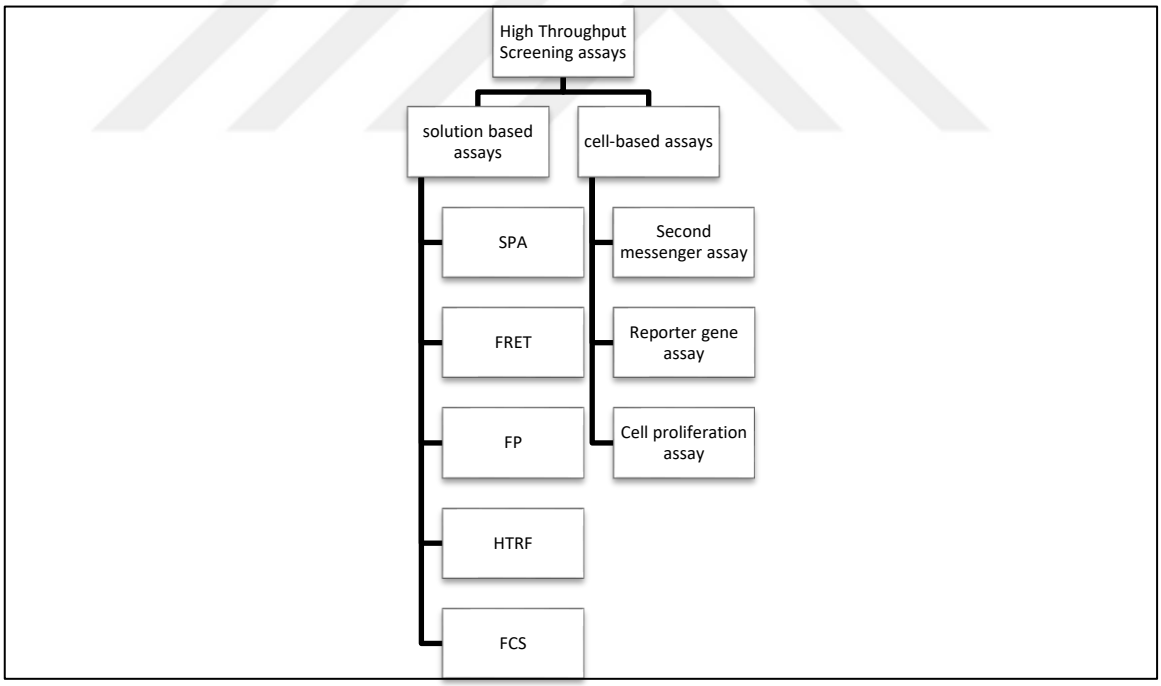

Figure 1.1. High throughput assay tools

1.6. ANALYTIC METHODS FOR ENZYMES

According to definition of international union of bio chemistry enzymes activity is the ability of enzyme which can catalyzes the conversion of 1 micromole of substrate per minutes under optimized standard condition, called unit. Change in absorbance, change in substrate amount, and increase reducing groups like sugar, at the specific time such as 10, 30, and 60 minutes gives units that distinguished the quantitative activity of enzyme. In enzymatic analysis there are important factors that affect the reaction in good or bad way, such as substrate concentration, temperature, pH, time of assay, amount of enzyme etc., which should be optimized to obtain high activity. Calorimetry, titrimetric, polarimetry, radiometry, potentiometry, conductometry, viscosimetry, colorimetry, absorption photometry, fluorometry, luminometry, turbidimetry, are main methods which use enzyme activity assays[1].

Calorimety method is based on temperature change during reaction, include change temperature during time and change local heat, recovering by electric cooling or heating and measured evolved electric.

Absorption photometry is promising method for enzyme assays because this method is easy to apply, suitable price, can get quantitatively data and it is sensitive. Samples absorbed visible or UV lights after substrate and enzyme react, change in amount of substrate or product measured spectrophotometricaly.

Conductometry in titrimetric enzymatic reactions is based on replacement of the one ion to another one cause change in conductivity of solution which measured by conductometer.

Polarimetry method uses isomerases, the enzyme reactions that cause change in optical rotation and in principle, however this method is not common.

Viscosimetry measures the change in viscosity during enzymatic reaction. For example, cellulase activity was measured in the past by viscosimetry while colorimetric method replaced the former method recently.

Luminometry method is based on light emission by detectors like fluorescence, phosphorescence and chemiluminescence. Luminometre is the instrument designed for measuring the number of photons emitted during a specific reaction.

Titrimetric method is based on measurement of acid pH change during enzymatic assay in sample of interest by using pH indicator such as phenol red, thymol blue etc.

Fluorimetric method is based on fluorescence material and its sensitivity is nearly 1000 folds compared higher when compared to absorption photometry method. It can detected the lowest amount of enzyme in organs or tissue, yet requires advanced equipments[1].

1.6.1. Amylase Activity Assay

The amylase activity assayed by three main methods such as, dinitrosalicylic acid method (DNS) which is based on measuring reducing sugar, and starch-iodine method. Nelson-Somogyi (NS) methods based on Nelson arsenomolybdate and Somogyi copper reagents. Nelson discovered arsenomolybdate to determination reducing sugars in 1944 [6, 45].

Dinitrosalicylic acid reagent monitors the reducing sugar concentration during the enzymatic reaction. Starch is to be used as substrate in this method which is prepared in phosphate buffer pH 7 in or acetate buffer pH 5. The incubation time and temperature also is change between 50˚C to 70˚C and 20 or 10 minutes respectively. Dinitrosalicylic acid reagent used to terminate reaction after incubation for 10 minutes at 100˚C. The optical density of the mixture is monitored by reading its absorbance at 540 or 575 nm [30, 46, 47].

Alternatively, Nelson-Somogyi method is based on Nelson arsenomolybdate and Somogyi copper reagents. The reaction mixture incubated for 10 minutes at 50˚C and Somogyi copper is added to terminate the reaction. The mixture incubated at 100˚C in a water bath for 40 minutes and then left at room temperature to cool down. Nelson arsenomolybdate added incubated for 10 minutes, followed by centrifugation at 13000 rpm for 1 minute. The absorbance of the supernatant is measured at 610 nm [48].

Starch iodine method is used to determine of α-amylase activity. The assay can be both solid and liquid phase. In liquid based method, after gelatinization, the starch solution is incubated with enzyme at 60˚C for 30 minutes and HCl is used to stop the reaction. Iodine is used as the reagent binding to the starch granules, resulting in the blue color at case of presence of starch. The absorbance is typically measured at 620 nm. Solid based starch-iodine test is performed by preparing starch-agar containing 2 percent starch and 1.5 percent agar. This method is mostly used for screening new isolates to identify the α-amylase producer

organisms. The agar-starch is poured in plates and a circle shape is cut from the center of the agar, then the inoculum is added to that circle. After 24 hour incubation, iodine reagent cover the agar plate to show any clear zones[49, 50].

1.6.2. Protease Activity Assay

Protease activity measurement is performed mainly on two ways: qualitative and quantitative method. Protein agar plate assay, radial zone diffusion assay and thin-layer enzyme assay are qualitative protease screening methods. In contrast, the quantitative assay techniques are spectrophotometry, fluorescent oligopeptide energy transfer assay, X-Ray based and enzyme-linked immunosorbent assays (ELISA) [51].

The frequently used protease activity assay is based on liberation of tyrosine from casein substrate upon protease activity. As such, it is nonspecific method. In this method 0.65 percent casein solution is prepared in 50 mM phosphate buffer and 5 mL of this solution is incubated with 1 mL enzyme. After incubation at 37°C for 10 minutes 5 mL trichloroacetic acid (TCA) solution is added and incubated for 30 minutes at 37ºC to terminate the reaction. The addition of TCA typically results on precipitation of some particles. This is followed by centrifugation at 10000 rpm for 10 minutes. After incubation, 2 mL of the supernatant transferred to another vial. 5 mL sodium carbonate and 1 mL Folin'sreagentare added to each tube. After incubation at 37°C for 30 minutes the absorbance read at 660 nm [52, 53].

The method described above have been modified frequently in terms of substrate change, time of the assay, volume of the samples and the reagents, the buffer used, pH, the temperature, the reagent concentrations. For example in Arima et al., 5 mL of 1.2 per cent Hammarsten casein incubated with 1 mL enzyme solution for 20 minutes at 35°C. Then 5 mL of TCA (0.44 M) added to stop the reaction, samples filtrated. 2 mL of supernatant added to 5 mL (0.55 M) sodium carbonate and 1 mL Folin's reagent, after 20 minutes at 35°C the absorbance was measured at 660 nm.[54]

Protease activity also measured by using other substrates such as benzoylarginine amide, benzyloxy carbonyl phenylalanyl leucine, and N-caseinate. These substrate are mostly used in soil protease activity measurements [3, 55-57].

1 g soil, 6 mL Atkin-Pantin buffer (pH 8 0.2 mol/L), and 6 mL Z-FL (2 mmol/mL) and 0.4 mL toluene incubated in shaker at 30°C for 5 hours. Then 9 mL TCA (0.11 mol/L), sodium acetate (0.22 mol/L) and acetic acid (0.33 mol/L) the mixture should be filtered and after filtration 2 mL of mixture and 1 mL buffer and 1 mL ninhydrin reagent L-8500 boiling and after cooling read the absorbance at 570 nm [56].

To measure proleolytic activity, two types of substrate such as congo red and nigrosin were used. 20 mg of substrate dissolved in 2 mL 0.05 M Tris-HCl buffer pH 8.5 (this buffer contain 0.01 M $CaCl_2$) at 40-50°C for 20 minutes. Then 1 mL proteinase solution was added and incubated for 60 minutes. Absorbance measured at 490 nm in case of substrate was congo read an at 570 nm if the substrate was nigrosin [58].

1.6.3. Phosphatase Activity Assay

Phosphatase activity can be measured by several methods and different substrate can be used. For example the use phenyl phosphate, glycerophosphate, naphtylphosphate and p-nitrophenyl phosphate has often been employed in the literature. The method which described by Tabatabai and Bremner in 1969, has been by far the most preferred method of alkaline and acid phosphatase activity assays recently also the modified types. This method use p-nitrophenyl phosphate as substrate and the protocol is used for soil phosphatase determination. Modified universal buffer MUB, toluene and p-nitrophenyl phosphate and enzyme container sample were incubated at 37˚C for 1 hour. To terminate the enzymatic reaction, calciumchloride and sodium hydroxide added to reaction mixture and samples filtered. After that the yellow color measured by colorimeter or spectrophotometer (410 nm).

In other study, which is modified in buffer that use enzyme assay instead of modified universal buffer MUB buffer use Tris-H_2SO_4 buffer (50 mM pH 8.0) 4 mL and enzyme 1 mL and p-nitrophenyl phosphate 1 mL are incubated at 37˚C for 1 hour after incubation TrisNaOH buffer (0.1 M pH 12.0) 1 mL add to stop enzymatic reaction. Samples were centrifuged and the yellow color measured at 410 nm [59, 60].

$$p-nitrophenyl\ phosphate\ +\ H_2O \xrightarrow{phosphatase} p-nitrophenol + H_3pO_4 \qquad (1.2)$$

1.6.4. Urease Activity Assay

Urease assay based on NH_4^+ –N which released during reaction the urease enzyme and 0.2 urea solution and THAM buffer at pH 9 incubated for 2 hours at 37°C in the case of soil samples toluene could be added. Then by using chloroform fumigation for 1 hour chloroform fume discharged from the samples and urease activity can be measured. Urease from jack bean activity assay is performed by incubation THAM buffer pH 7, urea solution and jack bean extract at 37°C for 30 min [61].

Weatherburn in 1967 investigated new method to determination of amount of ammonia by reacted it with phenol hypochlorite[22]. This method performed by preparing two main reagents phenol nitroprusside and alkaline hypochlorite. 5 mL phenol nitroprusside and 5 mL alkaline hypocolorite incubated with 20 µL ammonium sulfate at 37°C for 20 minutes. Absorbance read at 625 nm. In modified method urea solution 50 mM and 100mM potassium phosphate buffer pH 8 and urease were incubated at 37°C for 30 minutes in shaker at 700 rpm. 100 µL of the reaction mixture, 500 µL 5 per cent phenol nitroprusside, 500 µL 0.2 alkaline hypochlorite and 150 µL water shake at room temperature for 10 minutes. Then absorbance measured at 625 nm.

Other Wheaterburn modified method performed by preparing 125 mM urea solution with phosphate buffer pH 6.5 and incubated 200 µL with 50 µL of urease for 3, 9, and 15 minutes. At each incubation time 1 mL phenol solution added to stop the reaction, 750 µL 0.9 per cent hypochlorite solution added and the reaction mixture incubated for 20 minutes at 37°C. Absorbance measured at 630 nm [27, 62, 63].

The urease activity measurement for microorganism screening based on titrimetric method, solid medium. Urea agar contain, urea 20 g/L, potassium phosphate monobasic 2g/L, peptone 1g/L, phenol red 0.012g/L , sodium chloride 5g/L, glucose 1g/L which autoclave at 105 for 10 minutes. The interest microorganism incubated in urea agar and after 8, 12, 24 and 48 hours of incubation result can be observed. This method is also performed by preparing urea broth medium which is contain urea 20 g/L, potassium phosphate monobasic 9.1 g/L, potassium phosphate dibasic 9.5 g/L, yeast extract 0.1 g/L and phenol red indicator [64, 65].

1.7. MICROBIAL GROWTH

The objective of study microbial growth is to observe different phase of growth and then plot the standard growth curve and also calculate the specific growth rate. This could be useful for identifying microorganism properties then provide optimum growth condition. There are main factors that affected growth process which includes pH, temperature, oxygen, carbon dioxide, ions, light, osmotic pressure, organic or inorganic substances, nutrients such as carbon and nitrogen sources, minerals, vitamins and amino acids. Bacteria and archaea reproduce by binary fission, multiple fission, budding and sporulation which mean they have asexual reproductivty. Filamentous and heterogeneous cells growth is more complicated than homogeneous, and required cellular automaton and swarm system to detection. Bacterial Growth in batch growth has four main stages, lag phase, logarithmic phase or exponential phase, stationary phase and death phase.

Lag phase depends on physical and nutritional condition of new culture medium could be vary in terms of length. However, generally in this phase cells have no growth in size or number, for this reason this phase called adaptation phase.

Exponential phase is principal phase as cells number increase constant rate by binary fission. At the first moment of this phase instantly after lag phase acceleration phase happens which cells have maximum growth rate. Generation or doubling time is the stage that number of cells doubled each units of time and its length could be vary from minutes to days. Specific growth rate based on biomass concentration calculated in this phase where the growth curve is approximately linear.

At stationary phase there are metabolically active cells which has decrease in speed of division. Secondary metabolites released from cells and microorganism sporulate to survive. Culture condition such as pH, accumulation of toxic substances and nutritional deficiency can affect the length of stationary phase or lead to acceleration death phase.

Death phase is the part of growth after stationary phase which cells decline and cells died at constant rate. In this phase some species can survive by sporulation [66].

Ultrasensitive flow cytometry and automated colony counting by image recognition are methods for growth rate measurement. However in flow cytometry method cells have to

stain by fluorescence substance and colony counting method is not precise enough. To find out different phases of growth and concentration of culture medium optical density OD is an approach based on the optical absorbance at distinct wavelength is preferred because it is simple and rapid answer method [66].

1.8. THE AIM OF THIS THESIS

The aim of this thesis is to develop a series of methods to screen and/or measure the activity of some commercially important soil enzymes. The developed methods will be based on miniaturization, based on 96-well plates. The developed methods is to be further used to screen in house culture collection find candidate production hosts that are fast producers of highly active enzymes. Additionally, finding an organism with a broader enzyme portfolio is also desired.

2. MATERIALS

2.1. CHEMICALS

Agar	Sigma Aldrich 500g
Ammonium sulfate	Sigma Aldrich 1kg
Casein form bovine milk	Sigma Aldrich 500g
Dextrose glucose	Sigma Aldrich 1kg
Folin & ciucateu's phenol reagent	Sigma Aldrich 500 mL
Iodine	Sigma Aldrich 100g
Magnesium sulfate	Sigma Aldrich 500g
4-Nitrophenyl phosphate disodium salt	Acros 10g
Peptone from meat bacteriological	Fluka 500g
Phenol red	Sigma Aldrich 10g
Potassium iodide	Sigma Aldrich 1kg
Potassium phosphate dibasic	Sigma Aldrich 1kg
Potassium phosphate monobasic	Sigma Aldrich 1kg
Sodium carbonate	Sigma Aldrich
Sodium chloride	Sigma Aldrich 1kg
Sodium hydroxide	Sigma Aldrich 1kg
Starch soluble	Sigma Aldrich 1kg
Sulfuric acid	Sigma Aldrich 2.5 L
Trichloroacetic acid	Sigma Aldrich 1kg
Trizma® base	Sigma Aldrich 1kg
Urea	Sigma Aldrich 1kg
Yeast exctract	Sigma Aldrich 1kg

2.2. EQUIPMENTS AND DEVICES

96 well plate	Isolab

Autoclave	Hirayama HV-85
Autoclave	Tuttnauer 5050 ELV
Balance	Shimadzu AUW220D
Balance	Radwag PS 4500/C/1
Balance	Desis
Biological Safety Cabinet Class II	SafeFast Elite
Biological Safety Cabinet Class II, Type A2	HFsafe-1200
Biological Safety Cabinet Class II, Type A2	Labculture®-ESCO
Bottles (100, 250, 500, 1000 mL)	Isolab
Cryo tubes	Isolab
-80 Freezer	Sanyo
Fridge	Arçelik
Graduated cylinders (50, 250, and 500 mL)	Isolab
Incubatopr Shaker	New Brunswick Innova® 44
Incubator Shaker	Satorius Stedim CERTOMAT® IS
Magnetic stirrer	Benchmark
Micropipette Tip (10,200,1000) µL	CAPP
Multichanel micropipette 300 µL	Eppendorf
pH Meter	Mettler-Toledo
Scanner	Canon
Spectrophometer	ThermoScientific
Vortex	Scilogex MX-S
Water Bath	Grant SUB Aqua 12 Plus
Water source	Arium®pro Sartorius
Water source	Arium®advance Sartorius

2.3. PREPARED SOLUTIONS

Casein solution 0.65 per cent

Folin & Ciocaltea’s solution 0.5 M

Iodine solution 5 mM

4-nitophenyl phosphate disodium salt solution 5 mM

Potassium phosphate buffer 50 mM pH 7.5

Sodium Carbonate solution 500 mM

Starch agar

Trichloroacetic acid solution 10 per cent

Tris-H2SO4 buffer pH 8

Tris-NaOH buffer pH 10

Tris-NaOH buffer pH 12

Tryptone soy broth medium 30 g/L

Urea agar

3. METHODS

3.1. CULTIVATION OF CULTURE COLLECTION

537 unknown microorganisms form culture collection located at Department of Genetics and Bioengineering at Yeditepe University are used in the present study. 5 µL of microorganisms taken directly from -80ºC glycerol stock were inoculated in 250 µL of 30 g/L tryptone soy broth medium (TSB) in 96 micro titer well plate, in 4 replicates and are incubated for 18 hours at 37°C and shaken at 70 rpm. The growth was monitored by measuring the optical density (OD) at 540 nm in spectrophotometer and recorded as 0, 3, 6, 9, 12, 18th hours. The OD data is further used to construct the growth curve.

All assays performed as 4 replicates. For example first column, rows A to D and E to H two different microorganisms was assayed. In all plates of assays column 11, rows E to H and column 12 rows A to D was belonged to reference microorganisms. In column 12 rows E to H, the culture medium without any organism was assayed as negative control, as presented in Figure 3.1.

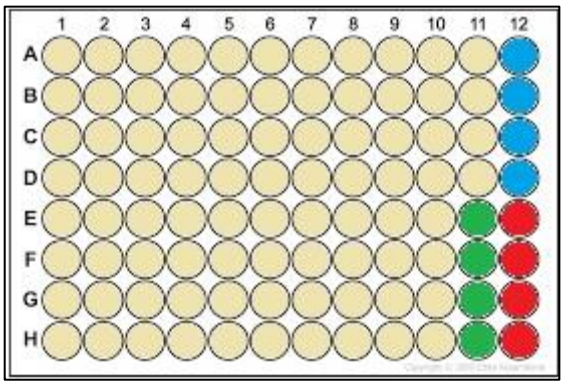

Figure 3.1. Plate design for assays

3.1.1. Culture Collection Known Microorganisms

To have positive or negative controls for both growth and enzyme production, 3 different organisms are used *Bacillus subtilis*, *Virgibacillus pantothenticus*, and *Escherichia coli* as *B. subtilis*, *V. pantothenticus* are known to produce and secrete these soil enzymes, while *E. coli* grows but does not produce or secrete these extracellularly. Tryptone soy broth (TSB) used as negative control in all assays. All the enzyme measurements (protease, phosphatase, amylase and urease activity assays) are performed separately and the corresponding procedure is described separately below.

3.1.2. Specific Growth Rate

Specific growth rate was calculated as following[66]. Starting from the mass balance for biomass:

$$\frac{\mathrm{dx}}{\mathrm{dt}} = \mu \mathrm{x} \tag{3.1}$$

Where, x is the biomass concentration, t is time and μ is the specific growth rate. Upon rearrangement and integration from initial time t_0 to an arbitrary final timet_f, the following equation is obtained:

$$\int_{x_0}^{x_f} \frac{dx}{x} = \int_{t_0}^{t_f} dt \tag{3.2}$$

integrated of first equation microbial cell concentration at the time t:

$$x_t = \mathrm{x}_0 \mathrm{e}^{\mu \mathrm{t}} \tag{3.3}$$

With e being the base of natural logarithm. This can be further arranged into:

$$lnx_t = lnx_0 + \mu t. \tag{3.4}$$

When natural logarithm of biomass plotted against time, the linear portion of the curve corresponds to the exponential growth, with slope being μ^{max}.

$$y = mx + \text{n} \quad (3.5)$$

3.2. MEASUREMENT OF AMYLASE ACTIVITY

Amylase activity determination was based on solid medium (agar), color change and image recognition. First starch agar prepared containing, magnesium sulfate heptahydrate 0.5 g/L, starch 5 g/L , ammonium sulfate 2 g/L, agar 15 g/L, potassium phosphate monobasic 1 g/L, potassium phosphate dibasic 1 g/L, and autoclave at 121°C for 3 minutes. After that 150 µLwas pour immediately in each wells. 20 µL of each inoculum was pour in center of starch agar. They were incubated at 37°C for 5 hours and then 25 µL of iodine solution was added to detection the presence of amylase. After adding iodine immediately took photos of samples [50].

3.3. MEASUREMENT OF PHOSPHATASE ACTIVITY

For preparing substrate first step is Tris-H_2SO_4 buffer (50 mM pH 8.0) preparation after adjusting the pH to 8.0 adjusting volume to 500 mL by distilled water. After that 5 mM substrate solution prepare by measuring 0.4639 g 4-nitrophenyl phosphate in 250 ml Tris-H2SO4 buffer. 100 µL of 50 mM Tris- H_2SO_4 buffer and 25 µL of 5 mM substrate solution was added to 25 µL of inoculum. Samples were incubated at 37˚C for 3 hours. 100 µL of Tris-NaOH buffer (0.1 M pH 12.0) add to stop enzymatic reaction. Absorbance was measured at 410 nm. Phosphatase activity was calculated as following [60].

PNpp has been treated with excess phytase enzyme aiming full conversion of PNpp to PNp. Standard calibration was prepared by adding 5 g/L phytase to 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mM p-nitrophenyl phosphate and the mixture was incubated for 3 hours. After incubation 100 µL of Tris-NaOH buffer (0.1 M pH 12.0) was added and the absorbance measured at 410 nm.

$$activity\ [U/mL] = \frac{\mu mole\ of\ pNP \times total\ volume\ of\ assay}{time\ of\ assay \times volume\ of\ enzyme} \quad (3.6)$$

3.4. MEASUREMENT OF PROTEASE ACTIVITY

150 µL casein solution 0.65 per cent which prepared by 50 mM phosphate buffer pH 7.5 incubated at 37˚C for 5 minutes to adjust casein solution temperature to 37˚C. 60 µL of inoculum was added and incubated at 37°C for 60 minutes. After incubation 50µL of reaction mixture was transferred to other 96 well plate and 125 µL sodium carbonate and 25 µL Folin's reagent was added. They incubated at 37˚C for 30 minutes and absorbance was measured at 660 nm by spectrophotometer. Protease activity units per mL was calculated as following [52].

$$Protease\ activity\ \left[\frac{\mathrm{U}}{\mathrm{mL}}\right] = \frac{\mu\text{mol of tyrosine released} \times \text{total volume of assay}}{\text{time of assay} \times \text{volume of enzyme} \times \text{volume use for colorımetric determination}} \quad (3.7)$$

Protease activity also measured for those microorganisms that shown desirable protease activity in MTP based assay, by increased volume. 130 µL casein was incubated with 25 µL supernatant at 37˚C for 10 minutes. After incubation 130 µL TCA (110 mM) added to mixture and incubated at 37˚C for 20 minutes. The mixture was centrifuged at 10000 rpm for 5 minutes. 250 µL supernatant was added to 625 µL sodium carbonate (0.5 M) and 125 µL Folin's reagent and incubated at 37˚C for 30 minutes. Absorbance was measured at 660 nm.

Standard calibration was performed by preparing L-tyrosine (1.1 mM) and diluted this solution to 5 different concentration (0.055, 0.111, 0.221, 0.442, 0.553 µmoles tyrosine) with distilled water. After that 125 µL sodium carbonate and 25 µL Folin's reagent was added in 50µL of each diluted L-tyrosine and incubated at 37˚C for 30 minutes. Absorbance was measured at 660 nm.

3.5. MEASUREMENT OF URAESE ACTIVITY

The urease activity measurement was based on titrimetric method, solid medium (agar) and image recognition and color changes. Urea agar contain, urea 20 g/L, potassium phosphate monobasic 2g/L, peptone 1g/L, phenol red 0.012g/L , sodium chloride 5g/L, glucose 1g/L which autoclave at 105 for 10 minutes, then 150 µL of urea agar poured in 96 well plates. 20 µL of inoculum was pour in center of urea agar. They were incubated at 37°C for 18

hours, the photo was took and recoded as 0^{th}, 3^{th}, 6^{th}, 9^{th}, 12^{th}, 18^{th} to analyses the color change related to change in pH during the time of assay. Results was obtained by image recognition [25, 26, 67].

3.6. STATISTICAL CALCULATIONS

Arithmetic average and the standard deviation of n samples are calculated as

$$\bar{x} = \frac{1}{\mathrm{n}} \sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{x_i} \tag{3.8}$$

$$s = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (\bar{x} - x_i)^2} \tag{3.9}$$

To assess whether any measured difference is statistically significant, Student's t-test is applied: first the t statistics is calculated, from which a p-value is obtained using t-table with $n - 1$ degrees of freedom. The t-statistic is calculated using

$$t = \frac{x_i - \mu}{s/\sqrt{n}} \tag{3.10}$$

In case the difference between two groups (e.g. reference strain vs. candidate strain), two sample t-test is used with the following t-statistics:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \tag{3.11}$$

4. RESULTS

4.1. SPECIFIC GROWHT RATE

The screened organisms showed maximum specific growth rate between 3rd to 9th hours of fermentation. In 3th, 6th , 9th hours they showed further distribution additionally growth and in contrast 9th, 12th, 18th hours showed that generally they are not able to grow at this period which shown in Figure **4.1**.

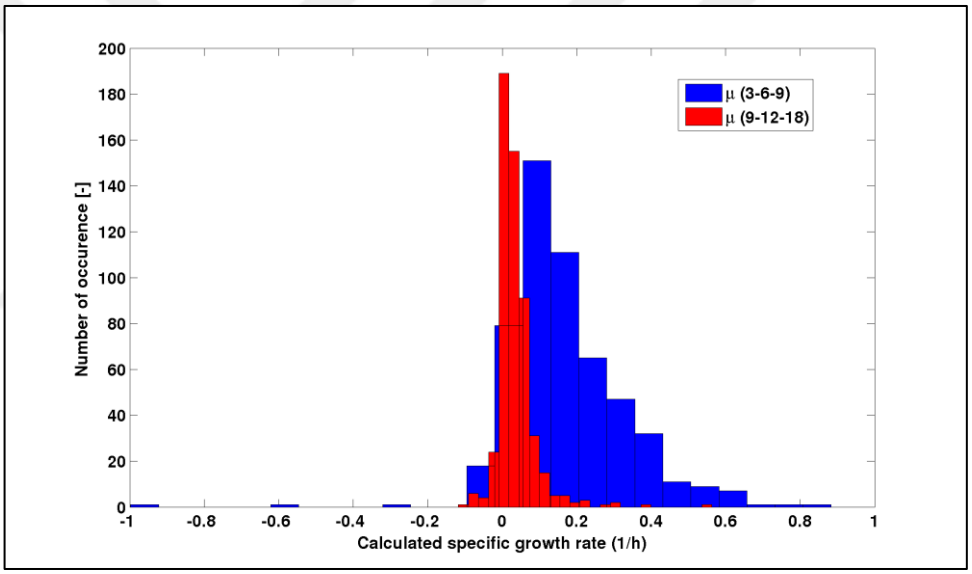

Figure 4.1.Specific growth rate distribution of screened collection.

4.2. PROTEASE ACTIVITY MEASUREMENT

Protease activity was measured and there were 15 organisms which has significant protease activity greater than *V. pantothenticus* and *B. subtilis* which shown in.Table **4.1**. All the stock library protease activity results shown in Figure **4.3**. and also plotted with specific growth rate shown in Figure **4.4**. The maximum protease activity 0.04 U/mL was belong to 2ys29 with (p-value=0.092) *V. pantothenticus* and (p-value=0.106) *B. subtilis*. In future study these

organisms will re-analysis in order to confirm the protease productibility. As expected TSB medium has been shown less activity than microorganisms Figure **4.3**.

Protease activity was assayed for 15 microorganisms, as strong protease producers based on low throughput method and the results shown in Table **4.2** indicates that all of them exhibited high protease activity as expected. 7ys48 has been shown highest activity 0.907 U/mL among other hosts.

Table 4.1. 15 Organisms which exhibited greater protease activity than *B. subtilis* and *V. pantothenticus*.

Strain code	**Protease activity U/mL**	**Standard deviation**	**P-value *V.pantothenticus***	**P-value *B. subtilis***
1ys37	0.0278	0.0013	0.0178	0.009
1ys46	0.0272	0.0013	0.0478	0.024
2ys68	0.0324	0.0015	0.0001	0.00
2ys23	0.0334	0.0042	0.0231	0.022
3ys58	0.0277	0.0013	0.0198	0.010
3ys62	0.0294	0.0018	0.0058	0.004
3ys49	0.0271	0.0005	0.0131	0.001
3ys53	0.0286	0.0008	0.0002	0.00
5ys19	0.0294	0.0009	0.0001	0.000
6ys15	0.0283	0.0018	0.0248	0.018
7ys27	0.0298	0.0011	0.0001	0.000
7ys41	0.0296	0.0029	0.0418	0.036
7ys48	0.0281	0.0016	0.0229	0.015
7ys60	0.0279	0.0009	0.0033	0.000
7ys66	0.0344	0.0033	0.0066	0.006

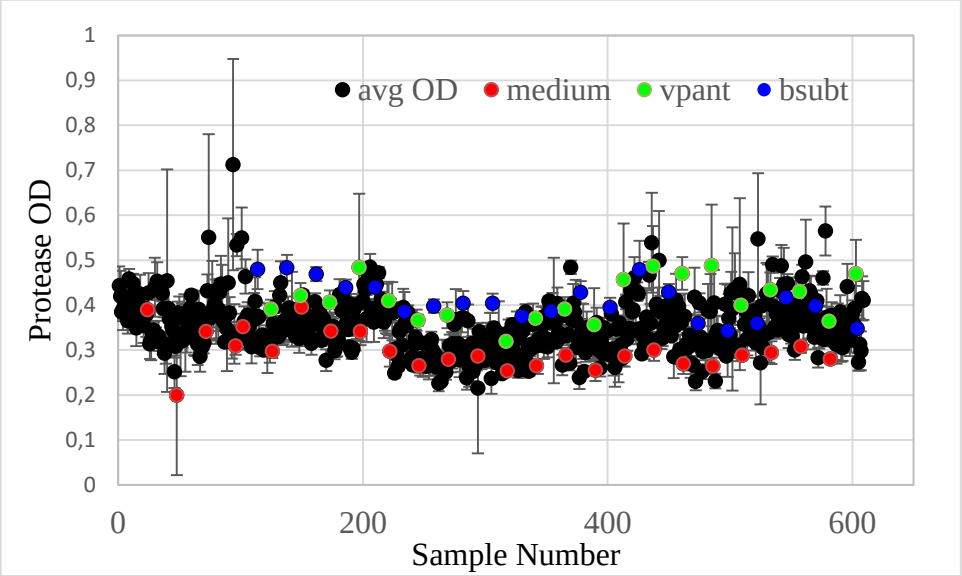

Figure 4.2. Protease OD of stock library, *V.pantothenticus* and *B. subtilis*.

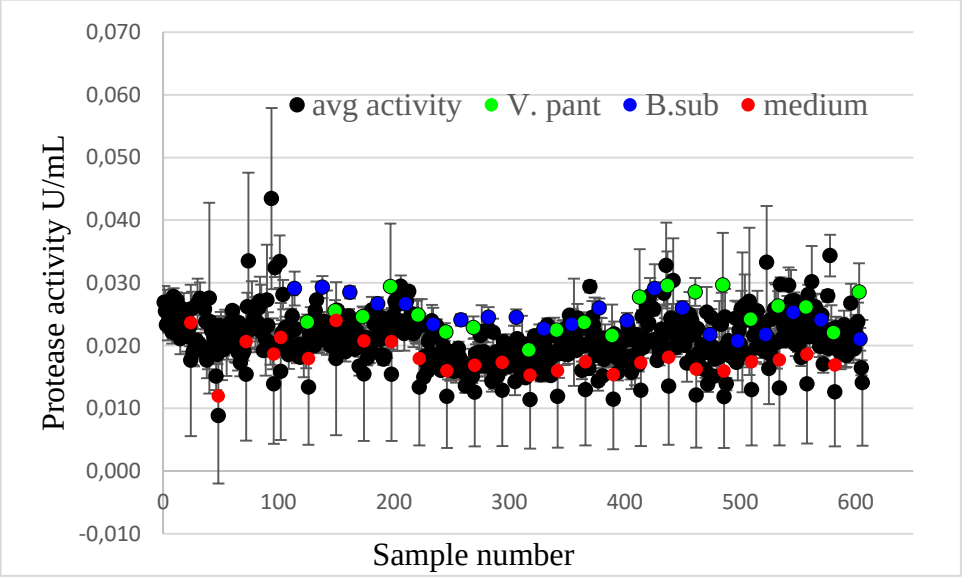

Figure 4.3. Protease activity of stock library, *V. pantothenticus* and *B. subtilis*

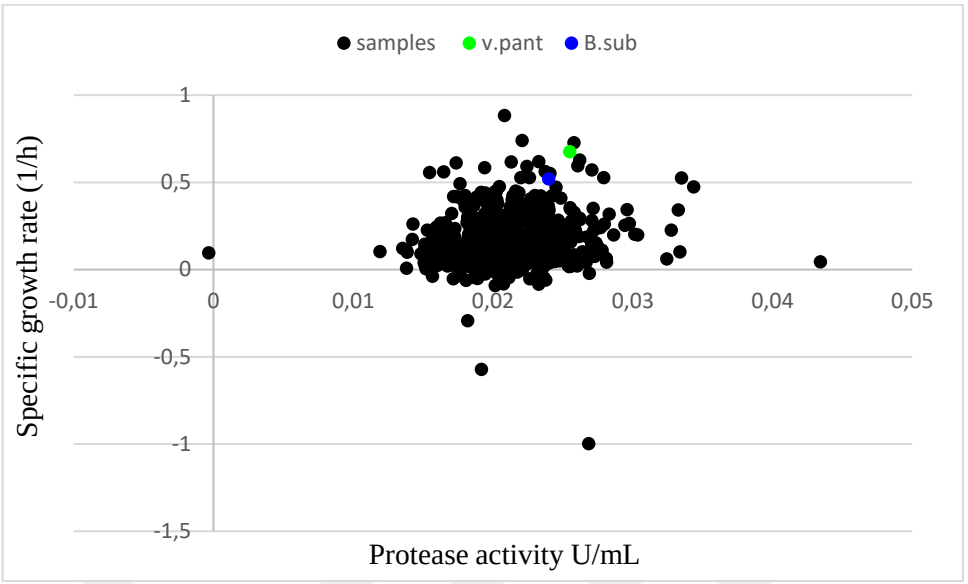

Figure 4.4. Protease activity plotted by specific growth rate

Table 4.2. Protease activity of candidate strong producers based on low throughput method

Organism	Protease activity U/mL 18th	Protease activity U/mL 24th
1ys37	0.580	0.637
1ys46	0.333	0.371
2ys68	0.501	0.542
2ys23	0.289	0.333
3ys58	0.367	0.362
3ys62	0.300	0.262
3ys49	0.425	0.463
3ys53	0.430	0.335
5ys19	0.474	0.434
6ys15	0.452	0.469
7ys27	0.270	0.376
7ys41	0.332	0.352
7ys48	0.907	0.855
7ys60	0.363	0.308
7ys66	0.368	0.376
V. pantothenticus	0.572	0.556

4.3. PHOSPHATASEACTIVITY MEASUREMENT

Alkaline phosphatase was measured and the absorbance at 420 nm was show in Figure **4.6**, in this figure stock library compared to the *V. pantothenticus*, *E. coli* as known organisms and TSB medium. Standard calibration was measured and the slope and intercept shown in Figure **4.5**. Phosphatase activity shown in Figure **4.7**. Alkaline phosphatase activity was plotted with specific growth rate shown in. Figure **4.8**. Protease activity plotted against alkaline phosphatase activity in order to observe that where the protease activity is high phosphatase activity might be low Figure **4.9**.

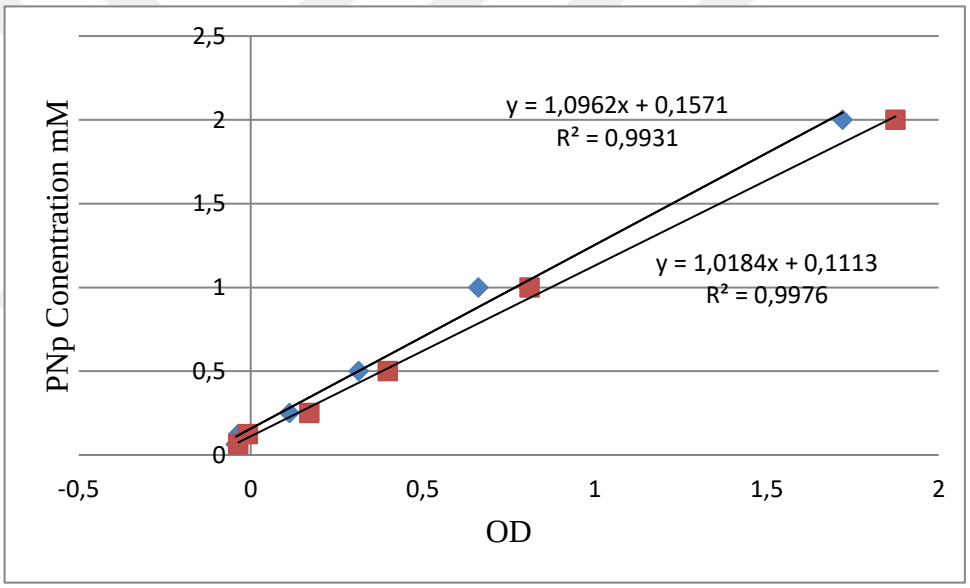

Figure 4.5. Standard calibration of PNp at two randomly selected different cases

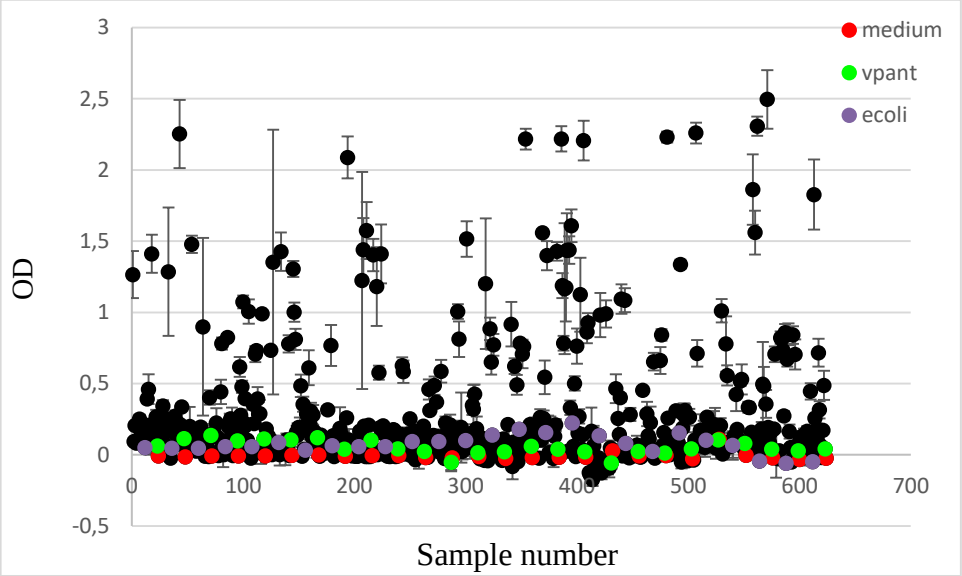

Figure 4.6. Alkaline phosphatase OD 410nm.

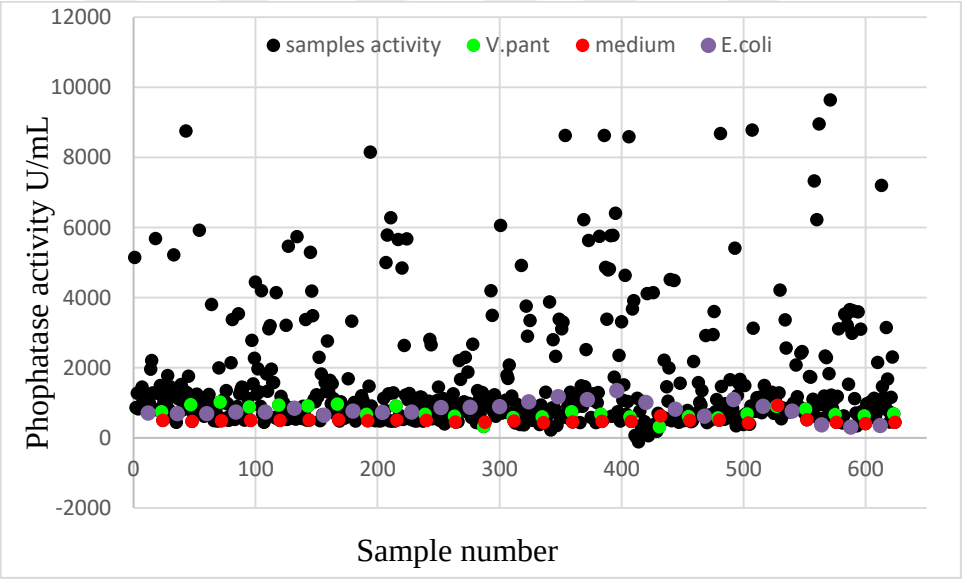

Figure 4.7. Phosphatase activity U/mL of stock libray, V.pantothenticus and E. coli

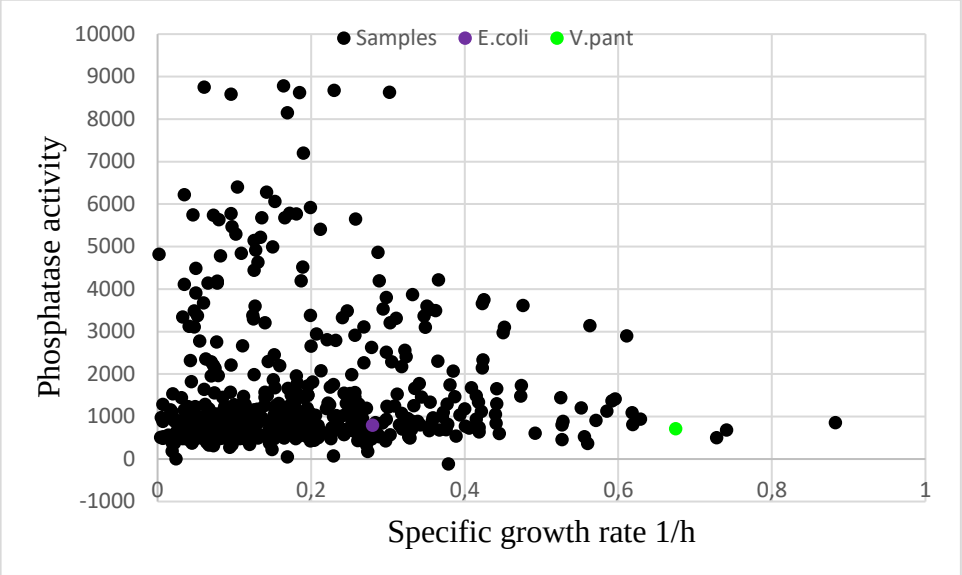

Figure 4.8. Phosphatase activity plotted with specific growth rate 1/h

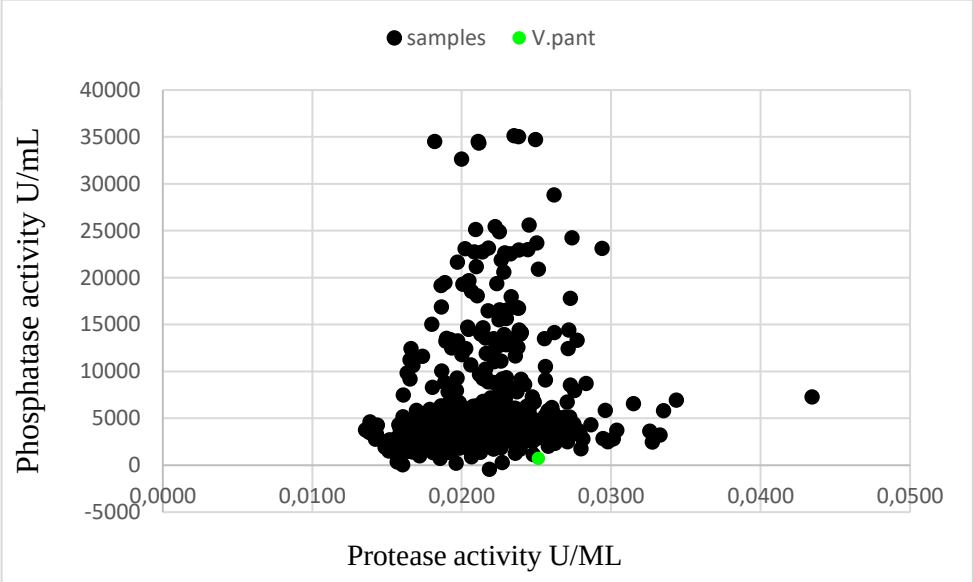

Figure 4.9. Alkaline phosphatase activity plotted by protease activity U/mL.

4.4. AMYLASE ACTIVITY MEASUREMENT

Starch agar method was performed and due to production of amylase after pouring iodine reagent white or cream color observed which confirmed the hydrolysis of starch. In negative strains blue color observed resulting presence of starch Figure **4.10**.

76 isolates was observed as amylase positive 7ys62,7ys57, 7ys54, 7ys52, 7ys48, 7ys49, 7ys47, 7ys45, 7ys42, 7ys37, 7ys33, 7ys31, 7ys32, 7ys28, 7ys23, 7ys24, 7ys21, 7ys22, 7ys19, 7ys20, 7ys17, 7ys18, 7ys16,7ys14, 7ys15, 7ys12, 7ys13, 7ys10, 7ys8, 7ys9, 7ys6, 7ys7, 6ys76, 6ys74, 6ys71, 6ys59, 6ys33, 6ys52, 6ys29, 6ys20, 6ys21, 6ys14, 6ys2, 5ys79, 5ys5, 4ys36, 4ys37, 4ys30, 4ys31, 4ys26, 4ys24, 4ys17, 4ys13, 4ys3, 4ys2, 3ys57, 3ys52, 3ys4, 2ys57, 2ys79, 2ys77, 2ys53, 2ys26, 2ys75, 2ys18, 2ys28, 1ys54, 1ys58, 1ys41, 1ys81, 1ys59, 1ys77, 1ys4, 1ys8 and 1ys20.

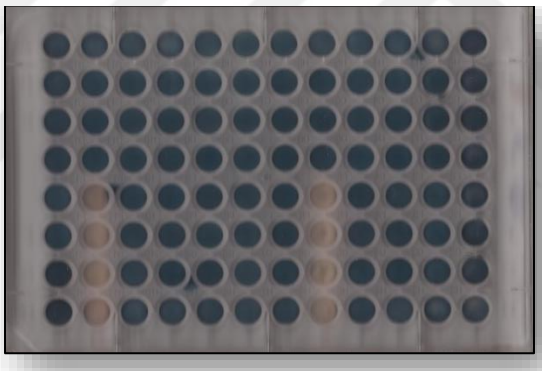

Figure 4.10. Starch iodine test in 96-well plates the blue color indicates presence of starch

4.5. UREASE ACTIVITY MEASUREMENT

After incubation samples which plated in urea agar contain phenol red pH indicator for 18 hours at 37°C, the urease positive isolates colonies exhibited change color from orange to pink which indicate release ammonia and increase the pH to alkaline, as a consequence of hydrolysis of urea Figure **4.11**.

87 isolates was observed as urease positive 7ys60, 7ys66, 7ys68, 7ys76, 7ys39, 7ys50, 7ys59, 6ys79, 6ys81, 6ys70, 6ys68, 6ys55, 6ys34, 6ys36, 6ys39, 6ys12, 6ys29, 6ys27, 6ys25, 6ys10, 6ys8, 6ys6, 6ys4, 5ys49, 5ys50, 5ys51, 5ys52, 5ys53, 5ys54, 5ys60, 5ys25, 5ys45,

5ys42, 5ys23, 5ys19, 4ys81, 4ys64, 4ys62, 4ys60, 4ys58, 4ys56, 4ys38, 4ys15, 4ys10, 4ys7, 4ys8, 2ys42, 2ys60, 2ys45, 3ys3, 3ys5, 2ys39, 2ys21, 2ys33, 2ys16, 2ys81, 2ys9, 2ys24, 2ys71, 2ys3, 2ys41, 2ys49, 2ys69, 2ys65, 2ys6, 2ys31, 2ys22, 2ys13, 2ys17, 2ys35, 2ys62, 2ys5, 2ys36, 2ys32, 2ys43, 2ys80, 2ys8, 1ys53, 1ys43, 1ys35, 1ys63, 1ys32, 2ys37, 2ys19, 1ys26, 1ys28 and 1ys48.

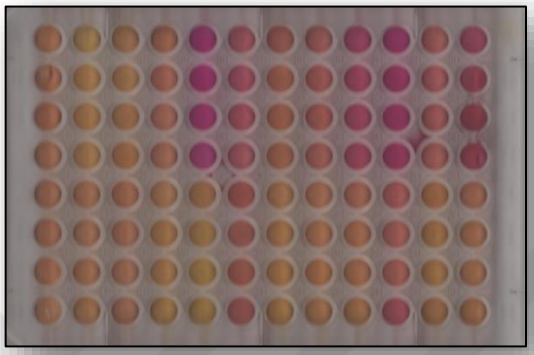

Figure 4.11. Urea agar in 96-well plate the pink color indicates increase in pH and yellow indicates color decrease pH

An interesting approach is to investigate which (combinations of) enzymes do the organism produce. There is no microorganism which produce all of 4 enzymes. There are 119 microorganism that produce none of those enzymes “protease, phosphatase, urease and amylase”. 31 microorganism was detected as 3 enzymes producers, as an example 17 of them produce protease, phosphatase and amylase, 13 microorganism are phosphatase protease and urease producers. 136 microorganisms was found as 2 enzymes producers. For example: 9 amylase and protease, 37 microorganisms amylase and phosphatase, 36 microorganisms protease and phosphatase, 1 microorganism protease and urease and 53 microorganisms are both phosphatase and urease producers. These results are summarized in Figure **4.12** as venn diagram.

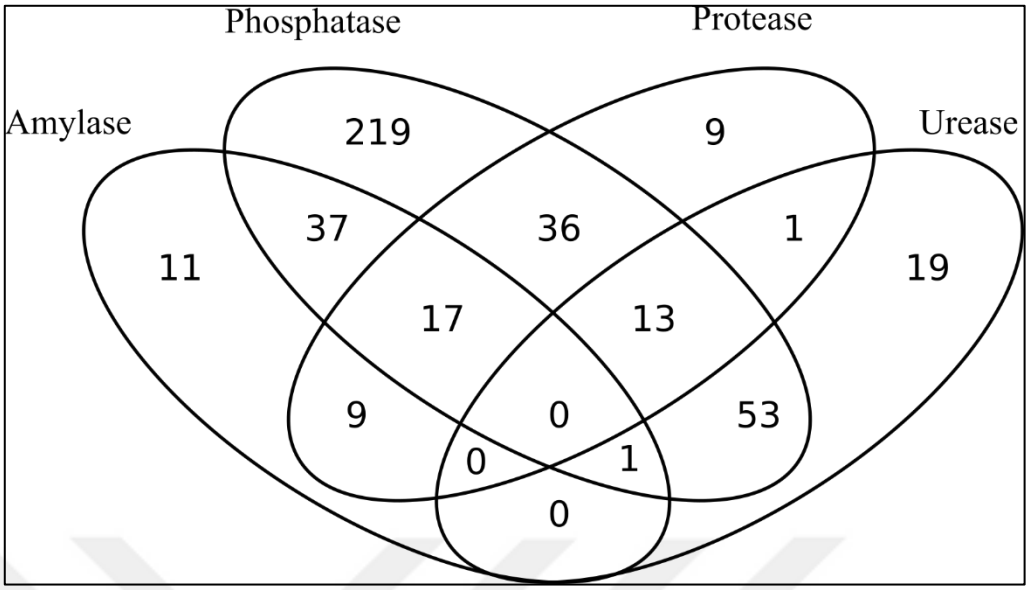

Figure 4.12 Venn diagram illustrating, number of good producers for each enzyme

5. DISCUSSION

This thesis aims to develop a fast and affordable method to screen and detect producers of commercially important 4 soil enzymes. Such an attempt assumes several trade-offs between speed, precision, throughput, overall feasibility and cost. Along this line, there has been a number of choices throughput the study, e.g. omitting the use of TCA to stop enzymatic activity. One important choice is the selection of the enzymes. Amylase and protease are commercially important due to their wide range of industrial applications, e.g. dairy, meat, flavor, juice and cheese industries as well as textile, leather, feed, pharmaceutical, cosmetic, detergent industries. Similarly, urease, has important role in urea and creatine (bio)sensors, food and beverage, bioremediation, and phosphatase is used in bioremediation, in particular precipitation of heavy metal ions, pharmaceutical industries [1, 32].

Another important choice was the focus on the organism, the developed method focuses on enzymes secreted extracellularly. In doing so, screening on crude enzymes, without the need of purification was possible and whole cell can be used for further analysis (e.g. advanced molecular and genetic characterization). The methods also include cultivation and calculation of maximum specific growth rate, and as such, they limited to (micro) organisms that can be cultivated. Lastly, two different approaches were used to quantify the activity. Protease and phosphatase were assayed by quantitative spectrophotometric method on liquid media, while amylase and urease were assayed by qualitative colorimetric methods on solid media.

An important feature of the developed method is the minimal requirement of pre-processing in provided (soil) samples, such as incubating the sample in buffer. One important item in method development is the timing of the assay for maximum observable separation among samples. For example, in urease screening assay, the incubation time is 3 hours although color change starts since first hour, since longer incubation delivers false positives, due to e.g. evaporation. Similarly, in amylase screening assay 5 hours is enough to identify high and low activity, although maximum activity obtained at a later time [25, 49, 50].

To take into account variation due to assay volume pipetting etc. errors, each sample is assayed 4 times, side by side. For example 1ys1 was placed in the first column, rows A to D. For even more accurate and certain results location of each replicate in the plate should

be randomized. A side point is the preparation of the agar in the well plates, one needs to avoid air bubbles.

In urease assay, colorimetric method is chosen since it involves minimum number of steps the choice on solid media is to avoid abrupt color change in culture media. Additionally, the medium contains peptone which is hydrolyzed during cultivation, resulting in an increase in free amino acid in medium, indirectly causing increase in pH, therefore false positive urease activity. To solve this, control medium without urea should be taken along to see an increase in pH and correct for urease activity[68]. One important note is the pH change upon sterilization due to heat, steam. Potassium buffer can be used to keep the pH more stable however too much stability is also not desired, since these results, in turn, in false negative. It would be better to freshly prepare urea agar and use it immediately without sterilization or sterilize with filter or UV and add phenol red at last step. One important point to notice is that the urease activity is followed titrimetrically. In case of microbial growth the pH typically decrease, rendering the color as yellow. One needs to consider that in the extreme case of growth and high level of production, the color will turn into pink. Another point to consider is to plate to plate variation. Because of this, the pH calibration should be repeated in each plate.

Protease activity assay was based on tyrosine measurement liberated during degradation of casein by protease. Trichloroacetic acid addition was omitted to avoid centrifugation step and to limit the assay volume. This omission results that even blank (no enzyme) shows blue color after some time, pointing the false positives and confirming the effect of TCA. The false positivites are recovered by always comparing the samples to the negative (blank) and positive (*B. subtilis* and *V. pantothenticus*) controls.

Casein is used as substrate in protease assay. It has the advantages of being cheaper however the disadvantages is that casein solution is not stable for long time and needs to be freshly prepared for each assay.

Skim milk agar plate is also the method for protease screening by creating a clear zone which indicates protein hydrolysis. However, this is not preferred here since seeing the clear zone and measuring the relative area in 96-well plate is almost impossible. Additionally skim milk method is qualitative and not accurate enough.

According to literature information protease isolate from *Bacillus subtilis* 168 exhibited 8.670, 12.060, and 19.734 U/mL after 8, 14, and 24 hours of incubation respectively[69]. Protease from *V. pantothenticus* was reported 200-250 U/mL at pH 9.5 which is the optimum pH[70]. In soil samples from different lands the protease activity 0.39, 0.98, 0.24 and 1.64 μmole tyrosine equivalent/g soil/hour was reported[23].

The maximum protease activity in this study exhibited from 2ys29 0.04343 U/mL. While the minimum activity is observed with TSB medium (blank) with 0.01799 U/mL (dynamic range 0.02544 U/mL). *Bacillus subtilis* and *V. pantothenticus* exhibited protease activity 0.02587 and 0.02514 U/mL respectively.

Phosphatase assay was based on modified Tabatabai 1994 method [59], most popular method in literature for acid and alkaline phosphatase activity assay. The modifications in this work are simply decrease of sample volume and parallelization for high throughput as this method was already suitable for high throughput screening with few easy steps. A significant deviation among the results comes from air bubbles in micro titer plates resulting from pipetting. Utmost care or automatization via robots would be minimize this error and related false positives and negatives.

Phosphatase activity in crude extract from *Aspergillus fumigatus* was reported 0.29 U/mL. in soil phosphatase activity measured 23.5 g/Kg/hr, 134.4 g/Kg/hr and 140.6 g/Kg/hr, after spent wash process, also reported in literature [19]. In present study the highest phosphatase activity belonged to 7ys75 with 38568 U/mL and the lowest was belong to 5ys59 which was -462 U/mL and 5ys55 which was 27 U/mL, with a large dynamic range of 39031 U/ml. In comparison the literature data with the current study there was a drastically difference in addition the absorbance 410 nm is 10 fold more than compare with the literature. This probably due to the fact that the organisms are cultivated in this assay, increasing the number of microorganisms, which is far less in actual numbers in the soil itself.

Amylase activity is measured by the starch-iodine assay in agar medium. The purple color of iodine remain constant while if there is amylase activity the color fade away after just few minutes, therefore after pouring iodine on plate of assay the picture should be taken without delay before missing the color. Similar to the urease case, the calibration curve needs to be prepared in each plate. Alternatives are liquid starch medium, in which the colors is highly

instable and the Nelson-Somogyi method based on arsenomolybdate contains highly toxic chemicals.

Amylase activity was reported 136 U/mL in optimal condition such as pH 6.5 and at 30°C and 146 U/mL where sucrose used as a carbon source[71]. α-Amylase activity was reported using soluble starch as substrate at 48th hours of incubation 72.3 U/mL [72]. In agar based studies the diameter of clear zone measured as percentage distribution. However in this study amylase screening was only qualitative therefore the comparison with literature was not possible[72].

To fermentatively characterize microorganisms, they are grown on 250 µL TSB liquid medium at 37°C in shaker for 18 hours. From optical density data specific growth rate was calculated and 1ys50 has the highest specific growth rate with 0.883 (1/h). In this study also *B. subtilis*, *V. pantothenticus*, and *E. coli* were grown. The specific growth rate of *B. subtilis*, V. *pantothenticus* and *E. coli* were reported as 0.481 (1/h), 0.8-1.4 (1/h) and 1.68 (1/h) respectively[73, 74]. While in this study the specific growth rate of *B. subtilis* average of 19 experiments 0.520, *V. pantothenticus* 0.675 and *E. coli* 0.280 (1/h).

6. CONCLUSION

High throughput screening method was developed based on miniaturization, based on 96-well plates. In house collection has been screened for growth and enzyme production 1ys50 has highest specific growth rate with 0.883 (1/h). Strong producers of 4 enzymes were detected, 5ys19, 7ys60, 7ys66 are both protease and urease producers. 6ys29 is amylase and urease positive microorganism. 7ys48 is protease and amylase positive. 6ys59, 6ys33, 5ys5 are both phosphatase and amylase producers. 7ys39, 5ys23, 7ys76, 5ys42 are both urease and phosphatase positive isolates. Protease producer candidates verified at Erlen scale. The proposed method identifies the desired organisms with good accuracy.

In future study enzyme barcode for classification, develop methods for other commercial enzymes develop HT storing (well plate) stock library and incorporate robotics are planned to study.

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