DESIGN OF ARTIFICIAL BACTERIA-YEAST CONSORTIA FOR PRODUCTION OF PROTEASE, AMYLASE, AND LIPASE & OPTIMIZATION OF PROCESS CONDITIONS

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ABSTRACT

DESIGN OF ARTIFICIAL BACTERIA-YEAST CONSORTIA FOR PRODUCTION OF PROTEASE, AMYLASE, AND LIPASE & OPTIMIZATION OF PROCESS CONDITIONS

Enzymes are proteins that act as biocatalysts for many industrial processes. Among the hydrolytic enzymes, protease, amylase, and lipase have an important role due to their wide range of industrial applications. The bottleneck of enzyme technology is that its inability to meet industrial demand at a satisfactory and cost-effective rate. Enzyme cocktails have more precise regulation, faster and effectiveness in the degradation of complex substrates than individual enzymes. Besides, enzyme cocktail fermentations, rather than combining single enzymes, have advantages such as decreasing fermentation time and cost. Currently, several methods for improving enzyme production are available, including synthetic biology and statistical approaches based on single strain fermentations. However, the clarification of microbial interactions is still required to increase the productivity of the desired product. Microbial consortia, the coexistence of two or more microorganisms, is a promising approach that provides the opportunity to both concomitant production and high activity.

This thesis aims to develop the concomitant production of the aforementioned three enzymes by artificial microbial consortia composed of bacteria and yeast families. The effect of microbial interaction on enzyme cocktail production was evaluated with two different consortia designs. In addition, the design and optimization of the factors affecting the enzyme cocktail production were carried out with the Response Surface Methodology. In this context, twenty different experiments were conducted using a quadratic polynomial model with Central Composite Design. As a result, both synergistic interactions of microorganisms and optimization of process conditions made it possible to improve fermentation performance.

ÖZET

PROTEAZ, AMİLAZ VE LİPAZ ÜRETİMİ İÇİN YAPAY BAKTERİ-MAYA KONSORSİYUM TASARIMI & PROSES KOŞULLARININ OPTİMİZASYONU

Enzimler, birçok endüstriyel proses için biyokatalizör görevi gören proteinlerdir. Hidrolitik enzimler arasında proteaz, amilaz ve lipaz, geniş endüstriyel uygulama alanları nedeniyle önemli bir role sahiptir. Enzim teknolojisinin darboğazı, endüstriyel talebi tatmin edici ve uygun maliyetli bir oranda karşılayamamasıdır. Enzim kokteylleri, karmaşık substratların bozunmasında tek tek enzimlerden daha kesin, daha hızlı ve etkili düzenlemeye sahiptir. Ayrıca enzim kokteyli fermantasyonları, tek enzimleri birleştirmek yerine, fermantasyon süresini ve maliyetini düşürme gibi avantajlara sahiptir. Şu anda, sentetik biyoloji ve tek suş fermentasyonlarına dayalı istatistiksel yaklaşımlar dahil olmak üzere enzim üretimini geliştirmek için çeşitli yöntemler mevcuttur. Bununla birlikte, istenen ürünün üretkenliğini artırmak için mikrobiyal etkileşimlerin açıklığa kavuşturulması gerekmektedir. Mikrobiyal konsorsiyum, iki veya daha fazla mikroorganizmanın bir arada bulunması, hem eş zamanlı üretim hem de yüksek aktivite imkanı sağlayan umut verici bir yaklaşımdır.

Bu tez, bakteri ve maya familyalarından oluşan yapay mikrobiyal konsorsiyumlar tarafından yukarıda bahsedilen üç enzimin birlikte üretimini geliştirmeyi amaçlamaktadır. Mikrobiyal etkileşimin enzim kokteyli üretimi üzerindeki etkisi iki farklı konsorsiyum tasarımı ile değerlendirildi. Ayrıca Tepki Yüzey Metodolojisi ile enzim kokteyli üretimini etkileyen faktörlerin tasarımı ve optimizasyonu gerçekleştirilmiştir. Bu kapsamda Merkezi Kompozit Tasarım ile ikinci dereceden bir polinom modeli kullanılarak yirmi farklı deney yapılmıştır. Sonuç olarak, hem mikroorganizmaların sinerjik etkileşimleri hem de proses koşullarının optimizasyonu, fermantasyon performansının iyileştirilmesini mümkün kılmıştır.

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α	Greek letter ‘alpha’
β	Greek letter ‘beta’
μ	Greek letter ‘mu’
gg^{-1}	Grams per grams
gL^{-1}	Grams per liter
h	Hour
L	Liter
μL	Microliter
M	Molarity
mL	Milliliter
nm	Nanometer
v/v	Volume per volume
wvol	Working volume
A_{Max}	Validation experiment to maximize amylase
Amy	Amylase
ANOVA	Analysis of variance
B/Y	Bacterium/Yeasts ratio
$CaCl_2.2H_2O$	Calcium choride dihydrate
CCD	Central composite design
C_{OPT}	Validation experiment to maximize enzyme cocktail
dH_2O	Distilled water
DNS	Dinitrosalicyclic acid
F-C	Folin & Ciocalteu’s phenol reagent
HCl	Hydrochloric acid
kDa	Kilodalton
Lip	Lipase
L_{Max}	Validation experiment to maximize lipase
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
Na_2CO_3	Sodium carbonate

NaCl	Sodium chloride
P_{Max}	Validation experiment to maximize protease
pNP	p-nitrophenol
pNPP	p-nitrophenyl palmitate
Prt	Protease
RPM	Revolutions per minute
RSM	Response surface methodology
TCA	Trichloroacetic acid
TSA	Trypti soy agar
TSB	Typtic soy broth
UmL^{-1}	Units per mL enzyme
X_1	Starch
X_2	Olive oil
X_3	Inoculum ratio

1. INTRODUCTION

1.1. INDUSTRIAL ENZYMES AND THEIR APPLICATIONS

Enzymes are proteins that act as biocatalysts in living organisms, regulating the rate at which biochemical reactions progress without undergoing any modification. Humanity has been utilizing enzymes in nature since the birth of civilization. In Greek, "enzymos" means to ferment, to swell, similar to yeast, through the production of various products such as linen, leather, wine, cheese, yogurt, and indigo through the addition of preparations such as papaya fruit (papain) or cattle rumen [1]. However, with the advancement of biochemistry in the 19th century, the nature and function of enzymes began to be explained. Dr. Christian Hansen isolated enzyme rennet from saline solution in calves' stomachs in 1874 [2]. In 1879, Eduard Buchner discovered that sugar could be fermented in the absence of living yeast cells in a mixture using the zymase enzyme. In 1914, trypsin was used as the first commercial enzyme for protein degradation in detergents [3]. In 1930, enzymes were first used to clean and produce fruit juices. Glucoamylase and α-amylase and were first used in starch hydrolysis in the early 1960s, replacing the traditional acid hydrolysis method. In America, particularly cellulosic and amylolytic enzyme additives attracted attention for use in ruminants due to their beneficial effects on ruminant ratios between 1950 and 1960 [4].

Since enzymes can catalyze nearly all biochemical reactions, industrial catalysis has become increasingly reliant on them. Enzymes are appealing for industrial applications due to their ability to perform highly distinct, complex, and biocatalytic reactions but also their economic and eco-friendly benefits. Due to the specificity of enzymes for their substrates (referred to as the lock and key model), the majority of them function as hydrolases in natural substance degradation. Industrial enzymes have historically been used in a wide variety of industries, including beverage-food, feed, detergents/cleaners, textile, pulp and paper, biofuel production, and leather degreasing [1]. Food and beverage industries account for nearly 35 percent of total revenue, while detergent and feed industries account for 25 percent and 20 percent, respectively. Additionally, their global market reached 5.1 billion dollars in 2009 [5], 6.3 billion dollars in 2017, and is expected to expand at a compound annual growth rate (CAGR) of approximately 6.8 percent through 2024 [6,7]. This growth is due to increasing demand for especially food and feed enzyme production

in proportion to the increasing population all around the world. Additionally, the industrial enzyme market is dominated by six companies: Novozymes, AB Enzymes, BASF, DSM, DuPont, and Roche, which collectively account for 75 percent of the total enzyme market [8].

The advantages of microbial enzyme production include ease of optimization, obtaining exponential growth, high productivity, and cost-effective production using low-cost media [9]. Due to these advantages, industrial enzymes are produced 60 percent by fungi, 24 percent by bacteria, 6 percent by yeasts, and 10 percent by animals and plants [10]. Table **1**.1. contains a list of commercially available hydrolytic enzymes derived from microorganisms.

Approximately 80 percent of industrial enzymes are hydrolases, which perform in natural substances degradation by using water to break down chemical bonds. All hydrolases are classified as EC3 enzymes based on the properties of the enzyme catalyst (EC) reaction system [11]. Proteases remain the primary group, owing to their widespread use in dairy products; amylases are accepted as the second-largest group, and lipases are the third group in high industrial demand [12].

Table 1.1. Available commercial hydrolytic enzymes currently employed for various industrial applications [13]

Enzyme	Product Name	Microbial Sources	Specialties	Companies
Protease	MAXIREN ® ALCALASE ® NEUTRASE ®	*Kluyveronmyces lactis* *Bacillus licheniformis* *Bacillus amyloliquefaciens*	42.5ºC and pH 6.6 for optimum activity 60 ºC and pH6.5-8.5 for optimum activity 30-55 ºC and pH5.5-7.5for optimum activity	DSM for specialties Novozymes Novozymes
Amylase	AMG ® TERMAMYL VALIDASE GX ® MAXAMYL WL ®	*Aspergillus niger* *Bacilluslicheniformis* *Aspergillus niger* *Bacillus licheniformis*	Thermostable up to 80 ºC Thermostable up to 90 ºC 65 ºC and pH 4.5 for optimum activity	Novozymes Novozymes Valley Research GBiosciences
Lipase	LIPOLASE ® LIPOZYM RM NOVOZYM435 ® LIPOZYM TL IM ®	*Humicola languinosa* *Rhizomucor miehei* *Candida antarctica* *Thermomyces Lanuginosus*	60ºC and alkaline pH for optimum activity Immobilized lipase on Doulite ES 652 Immobilized on silica nanoparticles Immobilized lipase on silicate	Novo Nordisk Novozymes Novozymes
Cellulase	CELLULOCLAST ®	*Trichoderma reesei*	50-60ºC and pH 4.5-6.0 for optimum activity	Novozymes
Glucanase	CEREFLO ®	*Bacillus subtilis*	-	Novozymes
Pectinase	PECTINEX PECTINEX ® NOVOSHAPE ®	*Aspergillus aculeatus* *Aspergillus niger* *Aspergillus oryzae*	50ºC for optimum activity 50ºC and pH 4.5 for optimum activity 50ºC and pH 4.8 for maximum activity	Novozymes
Xylanase	RAPIDASE ® NOVOZYME280 ®	*Aspergillus niger*	10-50ºC and acidic pH for activity	DSM food specialties Novozymes
Multienzyme complexes of carbohydrates	CEREMIX VISCOZYM CATAZYME ACCELLERASE	*Bacillus amyloliquefaciens* *Aspergillus aculeatus* *Aspergillus niger* *Trichoderma reesei*	Produced by separate fermentations by blending standard enzymes 25-55ºC and 5.5 for activity 60ºC and pH 6 for optimum activity 50 and pH 5 for activity	Novozymes Novozymes Novozymes Dupont

1.1.1. Protease

Proteases are hydrolytic enzymes with a molecular mass of between 15-90 kDa that exhibit selectivity and specificity in the modification of proteins. They function by hydrolyzing the peptone bonds within the amino acid polypeptide chain [14,15]. Proteases are typically used commercially to meet the needs of industrial applications such as detergent, food, baking, dairy, and feed additives. Additionally, they account for nearly 60 percent of the global enzyme market [16].

Proteases are classified into two major groups, endo- and exo-peptidases, according to the region of enzyme action. Endo-acting peptidases hydrolyze nonterminal amino acids (within the molecule), whereas exo-acting peptidases hydrolyze peptide bonds near the amino (N-) or carboxyl (C-) terminus (known-, aminopeptidases and carboxypeptidases). Additionally, exo-acting peptidases are classified into four major groups, these include cysteine (EC 3.4.22) and aspartic (EC 3.4.23) proteases, as well as serine (EC 3.4.21) and metalloproteases (EC 3.4.24) proteases [17]. Serine alkaline protease is a significant member of the protease family that is frequently used in detergents, leather, and feed additives [18]. At the moment, proteases derived from *Aspergillus sp.* as well as *Bacillus sp.* have been used as feed additives as a component of an enzyme mixture [19].

Bacterial protease sources (particularly *Bacillus sp*.) are well-known for their ability to synthesize alkaline and neutral proteases [20]. The main *Bacillus sp.* are *B.subtilis, B.licheniformis, B.alcalophilus, B.amyloliquefaciens, B.brevis B. ciculanas, B.cereus, B.mojavensis, B.macerans, B.mycoides, B.megaterium, B.thuringiensis, B.polymyxa, B.intermedius, B. proteolyticus, B.lentus, B.pumilus* and *B.firmus* [21]. Despite the fact that fungal proteases have a slower reaction rate than those from bacteria, they exhibit high substrate specificity and resistance to harsh climatic conditions [22]. Several fungi that are capable of producing protease are *Aspergillus sp., Rhizopus sp., Penicillium sp., Trichoderma sp.,* and *Thermomyces sp.* [23,24].

1.1.2. Lipase

Lipases (EC 3.1.1.3, triacylglycerol hydrolases) are serine hydrolases that hydrolyze triglycerides into glycerol and fatty acids. They are acidic glycoproteins with a molecular mass of between 20-60 kDa that is classified as a subclass of esterases [25]. The term "long-chain" refers to triglycerides with 16-18 carbon atoms. Additionally, they have an interfacial activation, which means that their activity increases when an interface is present, such as when the substrates form an emulsion. Ca^{2+} is the most effective inducer of lipases, whereas EDTA is the most effective inhibitor [26]. Additionally, the nitrogen source peptone promotes lipase production [27]. Lipases are generally classified into three groups based on their specificity in defining industrial applications. Lipases with substrate specificity, lipases with regioselective activity (which includes non-specific, 1-3 specific, and fatty acid-specific lipases), and lipases with enantioselective activity [28,29].

After proteases and amylases, lipases are the most common industrial enzymes. They are used in the cleaning industry, food and feed, textile and leather degreasing, medicine, pharmaceutical, and cosmetic industries. In 1984, Claude Bernard discovers a substance (later named pancreatic lipase enzyme) produced by the pancreas that can saponify and emulsify oils. For a long time, animal lipases were used; however, as demand and supply increased, microbial lipase production increased [30].

Bacterial lipases are classified as intracellular or extracellular and are referred to as glycoproteins or lipoproteins. They are non-specific in the specificity of a substrate and few of them are thermostable. Bacterial lipase producers are listed as; *Bacillus sp. Streptococcus sp.* which are Gram (+), and *Actinobacter sp., Burkholderia sp., Lactobacillus plantharm, Pseudomonas sp*., which are Gram (-) producers [31]. Fungal and yeast lipases have been in the studies since the 1950s. They have garnered considerable attention as a result of their substrate specificity and physical and chemical conditions, and their producers include *Aspergillus sp., Geotrichum sp., Penicillium sp., Streptomyces sp., Candida sp.,* and *Yarrowia sp* [32,33].

1.1.3. Amylase

Amylases act glycoside hydrolases with a molecular mass of between 10-210 kDa [34] as the target on α-1,4-glycosidic bonds and classified into three subclasses based on their bonding type. α-amylase (EC 3.2.1.1) (alternative names: endoamylase, termed also as 1,4-α-D-glucanglucanohydrolase; glycogenase) help internal α-1,4-glycosidic bonds hydrolysis of starch in the final product as maltotriose, maltose, and glucose units [35]. β-amylase (EC 3.2.1.2) (alternative names: 1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase) is another form of amylases, act on non-reducing end and catalyze α-1,4 glycosidic bonds for separating maltose at a time and the optimum pH of β-amylase is around 4-5. γ-amylase (EC 3.2.1.3) (alternative names: Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase) acts both α-1,6 glycosidic and α-1,4 glycosidic bonds at the non-reducing end of amylose and amylopectin to obtain glucose as the final product. The optimum pH value of γ-amylase, known as the most acidic amylase, is around 3 [36].

Amylases were first identified as diastase (hydrolysis of starch into maltose), they were the first enzyme group that was found by Anselme Payen in 1833. Amylases play a significant role in industries (e.g.food and feed, detergent, bread making, pulp and paper, biofuel, textile) and comprise almost 30 percent of the enzyme market [37]. Presently, amylases replaced chemical starch hydrolysis in the industry of starch processing. Furthermore, bacterial amylases are used in medical, analytical chemistry, and clinical areas [38].

The major bacteria and fungi producers used as the production of amylase are identified following: Bacterial sources are; Bacillus sp. (particularly *B.amyloliquefaciens* and *B.licheniformis*), *Pseudomonas sp*., and *Clostridium sp.* [39]. The most commercially significant fungal sources of amylase production besides the bacterial sources are *Aspergillus sp.*, *Penicillium sp.,* and *Rhizospus sp.* (30). They are (specifically *Aspergillus sp*.) acidifying fungi that prevent bacterial contaminations due to their acidity tolerance (pH ≤ 3) [40].

1.2. ENZYME COCKTAILS AS TOOLS IN INDUSTRIAL APPLICATIONS

Considering their high substrate specificity, selectivity for the development of diverse biocompatible, biodegradable, and renewable resources with specific functions, enzymes have attracted attractive attention as high-efficiency biocatalysts [41]. Organisms participating in the carbon cycle produce synergistic enzymes that work in concert to degrade complex substrates [42]. The term "enzyme cocktail" refers to a mixture or combination of various enzymes that enhance the effectiveness and efficiency of any catalytic reaction. Enzyme cocktails including protease, amylase, and lipase take an important role as industrial applications due to their advantages created by their synergistic effects in hydrolysis, in particular in the feed industry to improve nutrient utilization and growth performance of animals; in the agro pulp industry, to develop an eco-friendly biobleaching technology, in the detergent industry to improve the cleaning agents, to increase efficiency leather soaking process, and to increase food quality in terms of wine and beer manufacturing. Baked and dairy goods stand out for developing flavor, digestibility, and nutritional value in the food industry [43–47].

The conditions of an optimal operating condition for each enzyme, single-use, and low stability maybe result in limiting their use in industrial processes [48,49]. Despite their challenges, because it is difficult for a single enzyme to catalyze complex reactions, enzyme cocktails provide advantages, which they enable the performance of extremely complex reactions, transformations, such as improving high catalytic efficiency via efficient transfer, removing product inhibition, and facilitating cofactor regeneration [50]. Additionally, the development of enzyme cocktail systems is increasingly driven by economic and environmental constraints, which calls for an incentive to develop alternatives to multistep synthetic methods [51].

Enzyme cocktail fermentation, rather than combining single enzymes, contributes significantly to the elimination of certain production challenges such as time and cost. Considering these advantages, *de novo* enzyme production from single organisms, development of selected organism via mutation such as mutants resistant to catabolite repression, constitutive mutants producing enzyme in the presence of repressor, or constitutive mutants that form enzymes without the addition of inducer, and microbial

consortium designs that take an advantage of the relationships between organisms are alternative techniques for concomitant enzyme production [52,53].

1.3. MICROBIAL CONSORTIA

Microorganisms that live in a complex community called a consortium in nature are composed of interactive species that participate in the global cycling of carbon, oxygen, and nitrogen [54,55]. The microbial consortia (or mixed cultures, or co-cultures) presents an alternative solution in the following circumstances;

(i) the need to optimize the utilization of a diverse and complex carbon source that is difficult to utilize in a single-strain fermentation,

(ii) the need to block pathways (i.e. glucose repression) that can result in low productivity with a single-strain fermentation, and

(iii) the need to eliminate by and/or intermediate products that can reduce growth and productivity in a single-strain fermentation.

Typically, microorganisms are grown in the form of a single-strain culture to generate a specific product depending on industrial purposes [54]. However, co-cultures of microorganisms that can survive under the same conditions can be used to boost the productivity and economics of the fermentation process [55].

The primary advantage of microbial consortia in the production of extracellular enzymes has been discussed previously in the literature [54,56–61]. Co-cultivation and strategies involving interspecific interaction aid in increasing enzyme production [56]. However, while co-culture systems increase the activity of some enzymes, this is not the case for all enzyme production. This demonstrates that co-cultivation triggers the induction of specific enzymes suitable for this system through the assignment of total extracellular protein, rather than simply increasing protein secretion [62]. This advantage could be attributed to the carbon sources that were used [63]. Consequently, increased productivity may be achieved through microbial interaction, as multi-step reactions can be carried out more quickly than would be possible with a single strain.

Despite numerous advantages over single-strain cultures, co-cultivation approaches for generation purposes continue to face some challenges and limitations. While some of these obstacles do not apply to small-scale fermentations, these are significant feasibility barriers that must be overcome before scale-up production, successfully. In this case, when designing a microbial consortium, the following criteria must be considered;

(I) The compatibility of strains is critical in any successful co-cultivation system [64]. Co-cultivation strains should grow at the same rate and have similar growth parameters, such as temperature, pH, oxygen, and media requirements. Also, strains should not produce toxic materials that are harmful to other members. These instances may be considered even when strains from the same species are used [65]. To design and improve a successful process, it is necessary to understand each microorganism's precise role and overall contribution to the fermentation process.

(II) When co-cultivation partners share the same resources for growth, it is referred to as exclusive competition and result in unstable growth of consortium members, which is an unfavorable fermentation technique for bioproduction of the industrial fermentation processes. Furthermore, each organism has unique nutritional preferences and requirements, complicating the application of this approach. As a result, nutritional divergence (or cross-feeding) within a co-cultivation system is an attractive solution for coexistence [66].

(III) Optimizing operating parameters (environmental parameters such as concentration of carbon sources, medium compositions, pH, agitation, temperature, aeration rate) to obtain the production of high value-added compounds, resulting in high yields and a cost-effective process. Another reason for optimizing the fermentation process for microbial consortia is that this strategy has been successfully applied in a variety of bioproduction processes and is a prerequisite for scale-up. [67].

(IV) Balancing optimum population ratios in microbial consortia throughout the co-cultivation process is a significant bottleneck for bioproduction. However, fine-tuning inoculum ratios is a key strategy to balance the strain-to-strain ratios between co-cultivation members in such a way that one strain does not destroy

the other. Although the subpopulation ratio frequently fluctuates or changes over the cultivation period, the inoculum ratio between co-cultivation members has a significant effect on bioproduction. Furthermore, the volume of culture may affect the viability of co-cultivation. When culture volume is increased, the stability of the population decreases, which may result in heterogeneity within the culture system [68].

Classification of Microbial Consortia

Microbial consortia are classified into three types based on the construction, function, and modes of interaction of the related microorganisms. Artificial microbial consortiums, natural, synthetic, and semi-synthetic microbial consortia are the classifications based on their construction modes. The artificial consortium is made up of wild-type microorganisms that can grow symbiotically and live in a closed system to produce value-added products [69]. Other consortium types include, respectively: a community of microorganisms living together in nature; a co-culture system containing metabolically engineered microorganisms; and a co-culture system containing both wild-type and engineered microorganisms.

The function mode of the co-cultivation system is based on substrate facilitation consortium, nutrient exchange consortium, and environment maintenance consortium. A consortium of substrate facilitators is formed in which one strain ensures hydrolysis to generate intermediate free sugars for the growth of other strains [70]. When one strain in a mixed population is unable to produce an essential life-supporting molecule for itself, which is called auxotrophy, a nutrient exchange consortium is effective. Environment maintenance consortium is important when one strain provides a suitable environment for its partner's growth (e.g. aerobic-anaerobic consortia) [68].

The co-culture system is based on their interaction (cross-feeding/talk) and mutual aid in growth. The interaction modes are classified as positive and negative interactions [56]. Figure 1.1. illustrates how individual microorganisms affect another member(s) of the population in co-culture systems. In a co-culture system, the division of labor based on cooperative interactions is referred to as synergistic division of resources or substrate cross-feeding (carbon and energy sources) and it represents generally possible efficient resource use. Synergistic growth and enzyme production are described as having occurred

when a co-culture grew or produce a related enzyme better than any of the corresponding single cultures [71]. Division of labor enables concurrent optimization of multiple tasks for complex material degradation via enzyme generation. Separating distinct cell reaction types in a co-culture system can facilitate parallel substrate utilization in the community and increase productivity by overcoming associated with catabolite repression in single-strain cultures [70,72,73]. According to the framework of this thesis, the accumulated short-chain intermediate-products, that are generated by extracellular degradation of carbon sources within the co-culture system, are metabolized by other members of the consortium. One microorganism in a community provides benefits to others at no cost or benefit to itself in the case of commensalism. Furthermore, mutualistic interactions are frequently observed in natural and synthetic co-culture engineering, where they are defined as relationships that benefit all participants. Mutualism is a type of cellular factory application that included syntropy, which is defined as the exchange of resource/metabolite or mutual-cross feeding [74,75]. Neutralism occurs when two species use dissimilar compounds (similar to nutritional divergence) and avoid producing a product that could inhibit the growth of others. Amensalism is a one-way interaction in which one organism suppresses the growth of its partners by producing toxic products, but these products do not affect the producer, as opposed to commensalism. Predation or parasitism (both of which are uncommon in microbial consortia) defined scenarios in which the growth is constrained by the depletion of the other, resulting in population dynamics that frequently exhibit enduring oscillations [68].

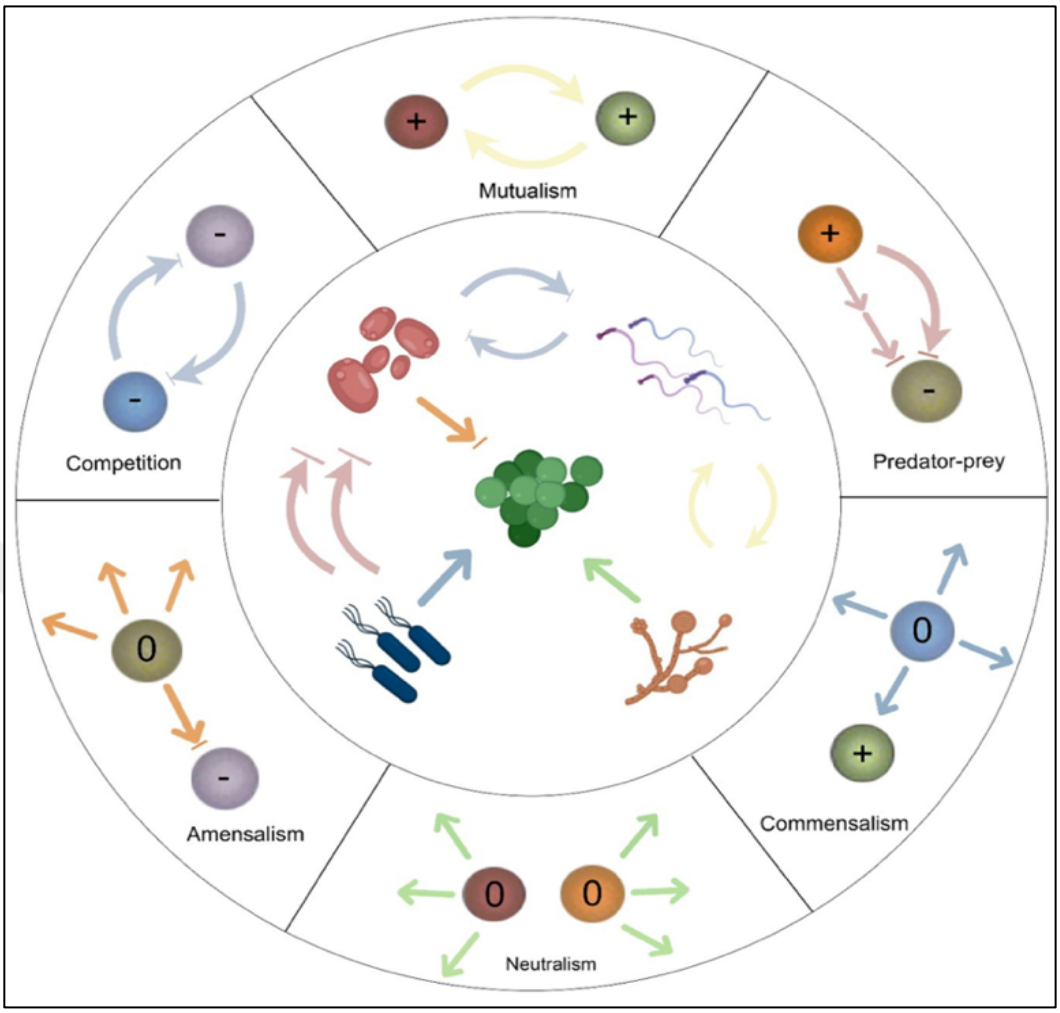

Figure 1.1. Effects of interactions among microorganisms in a community [55]
(0): Neutral interaction, (+): Positive interaction, (-): Negative interaction

Understanding the network and interactions among species in a microbial consortium has significantly aided in shedding light on the dynamics underlying effects on desired product yield. The major and sub-interaction profiles between the two species are indicated in Figure 1.2. Various techniques work their functions and interactions to aid in determining the roles of microorganisms as individuals or in combination with others. Alternative techniques for analyzing individual behavior of species in a mixed culture include microbiological methods (cell counting, flow cytometry), molecular biology methods (PCR-based), single-cell genomics (SCG), microfluidic devices, and fluorescence imaging [76–81]. Furthermore, microbial kinetics can be used to estimate populations of both known and unknown species that are involved in microbial growth and product formation [82].

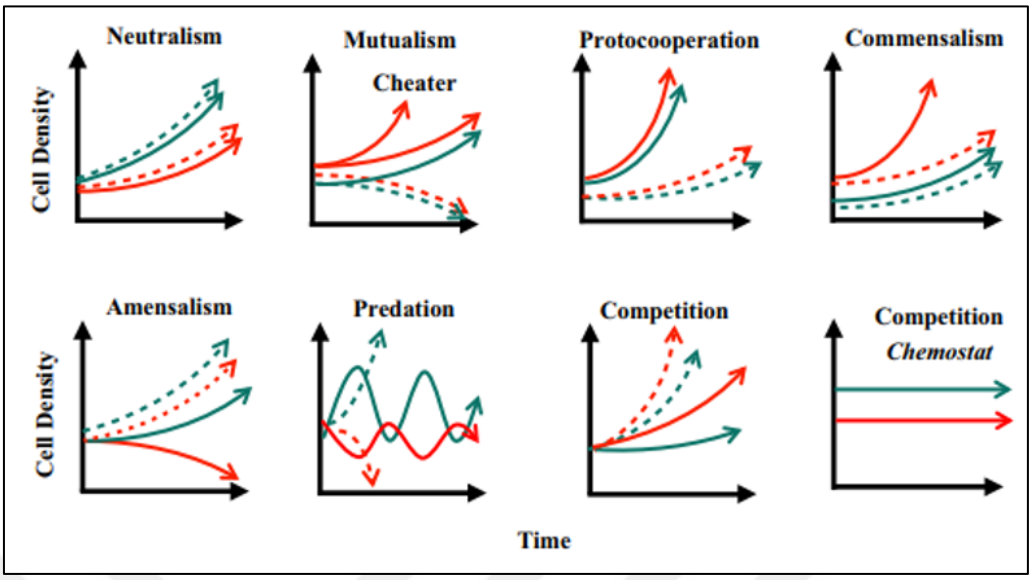

Figure 1.2. Profiles of the relationship between two species in a co-culture system. Green and red = species 1 and 2; dashed line = single-cultures; solid lines = co-cultures [83]

1.4. STATISTICAL OPTIMIZATION

1.4.1. Design of Experiments

Design of experiment, also known as statistical experimental planning, is a type of process analysis in which relevant parameters that are effective in the experiment are changed in a controlled manner to determine their effects on response [84]. The significance of planning and designing simulation experiments provides effective methods of predicting the effects of changes in the model's input on its output. Although experimental design methods can be used in various cases, they are most commonly used in biotechnological studies. Biotechnological processes can be quite complex, with numerous variables that can have a significant impact on the process's performance. As a result, experimental design methods can examine multiple levels of many factors affecting the experiment at the same time, and the state of a factor can be addressed at multiple levels of other factor(s) [85].

Statistical experimental design methods empirically define the regression model between one or more measurable input parameters. These methods offer significant benefits in terms of optimizing environmental conditions, increasing efficiency, reducing the number of experimental points, and lowering process costs. The first step of the procedure in a new

and/or unknown system should be the selection of factors that may affect the desired responses (e.g., productivity or product yield) and the determination of the experimental domain [86]. Following the determination of both factors and responses, one of the appropriate experimental design methods is selected based on the detail of the obtained information, cost, and time constraints [87]. As a result, the process is completed with the interpretation of the results in the approach of experiment design and optimization, favorable research planning, conducting planned experiments to obtain a measurement data matrix.

1.4.2. Factorial and Central Composite Design

Factorial design is one of the most common experimental design methods based on the fact that all of the variables in the experiment change at the same time. Furthermore, it is a method for determining the main effects of two or more factors as well as the effects of their interactions with one another [88]. The full factorial design is one of the most useful design when combinations of factors (k) are examined at two-level and the design comprises of 2^kexperiment. Fractional $(1/2\ , 1/4, .. 1/2^p)$ factorial design approach can be applied where the interactions between the parameters in the experimental design are negligible or the total number of experiment points is very high. In this design, 2^{k-p}experiments are described where p is a fraction size [89].

One must always consider that reducing the process cost and increasing the response yield in a bioprocess design, the Central Composite Design (CCD) is employed together with two-level full factorial or fractional factorial designs that allow either linear or quadratic models. The most important feature that distinguishes CCD from others is the use of axial (or star) points expressed as α-value [90]. Axial points necessitate two additional levels of experimentation, one at the minimum and one at the maximum for each factor. In this case, the quadratic effect is ensured by testing the factors at five levels. The axial points may differ according to the desired design features and the number of factors. The CCD is a favorable method for the full factorial three-level design because of demands a small number of experimental points. The general CCD comprising of three-part for k factors are given [91];

I. At a center point where performing a total n_{center} runs; $x_1 = \ldots = x_k$

II. A factorial (cube) points corresponding to a total $n_{factorial}$ points; $x_i = -1$ or $x_i = +1$ for $i = 1, \ldots, k$

III. An axial (star) points formed by $n_{ax} = 2k$ points are equal to α $(or\ -\alpha)$.

In these conditions, the total number of levels is $n_{factorial} + 2k + 1$. Consequently, $2^k + 2k + C_0$ is the total point where C_0 represent the number of center points [92].

Figure 1.3. depicts a graphical representation of a standard CCD matrix. The distance between the axial points in this design varies according to preference and the number of factors. Standard CCD matrix Central Composite Circumscribed has circular symmetry. In addition, five levels of factors are required in these designs. Designs, where the axial points are at the same level as the minimum and maximum (-1,+1) points of the factors, are called Central Composite Face-Centered. These designs are generally used in situations where factors are not suitable for five levels. In cases where factor levels should be evaluated within a narrow range, axial points are selected between minimum and maximum levels of factors and designed with Central Composite Inscribed. Furthermore, in order to obtain the most unbiased information possible from a trial, keep in mind that the experiment is rotatable and the values of the response variable obtained by the combination of factors are equidistant from the center point [84].

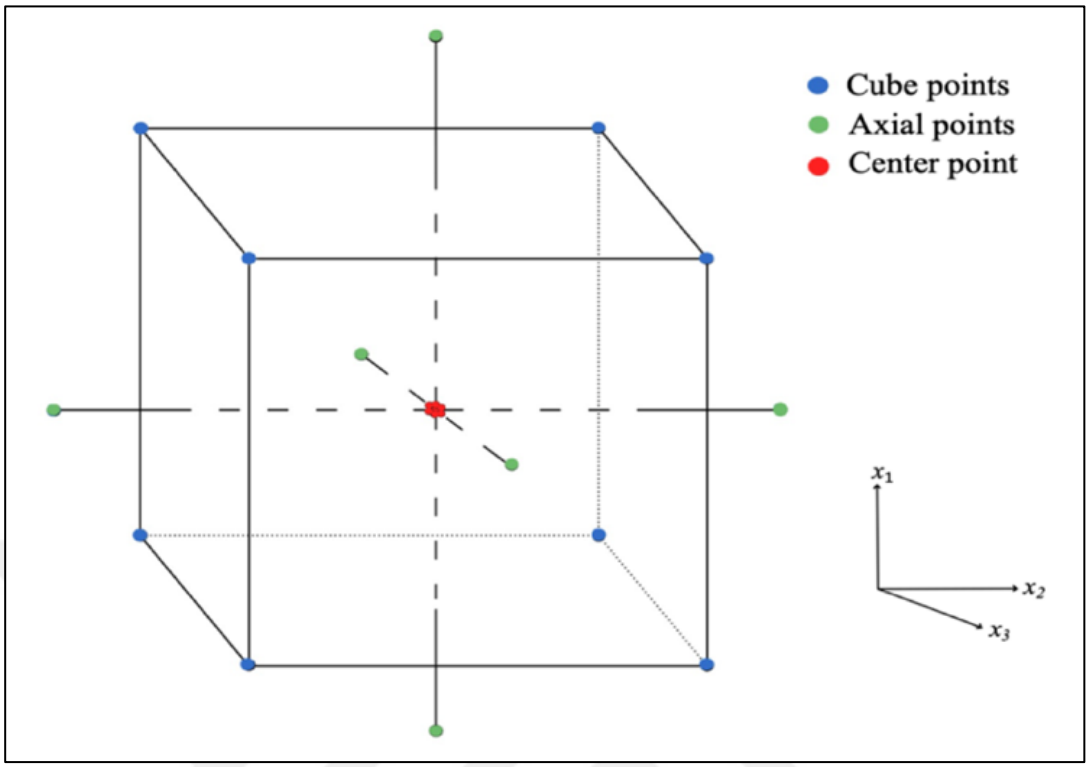

Figure 1.3. Central composite design in a three-variable case. The blue dots represent the cubic part, green dots form the axial part and red dots are the center part

Along with the CCD, several alternative second-order designs are followed; One of them is a 3^k factorial or fractional design. These designs are not used where k is greater than three due to the high number of design points required. Box-Behnken designs, which are a close substitute for CCD, are quadratic designs with insufficient 3k factors and rotatable capability. Combining incomplete block designs with factorials results in Box-Behnken design. The Taguchi method is used to determine the minimum number of experiments that must be conducted within the permissible factor and level range. When it is assumed that two-way interactions are negligible, Plackett-Burman design is used to study the main effects [93,94].

1.4.3. Response Surface Methodology

The response surface method (RSM) was developed and defined by Box and Wilson in 1951. The basis of the RSM is the modeling of surfaces defined with two or more variables employing mathematical and statistical techniques. This method aims to improve and

optimize bioprocesses, improve product performance and process by reducing variables [95]. Furthermore, the critical purpose of forming a response surface is to determine new production conditions to ensure a product quality level above that obtained under current conditions by predicting a region with certain properties (experimental range) and the optimum point of this region in a design consisting of many parameters that are effective on the results of an experimental study [93].

The RSM is created by performing a model regression analysis. The significance of a factor's effect or interaction with other factors on the response variables is determined by regression coefficients [96]. Linear regression is a statistical technique that examines the functional relationship between the dependent variable (response) and one or more independent variables. It is widely used in estimating model parameters. A linear regression model is any linear model, including polynomial models, regardless of the shape of the response surface it generates [87]. The quadratic polynomial equation used to estimate the response surface is given below.

$$y = \beta_0 + \sum_i . \beta_i x_i + \sum_i . \beta_{ii} x_i^2 + \sum_{i<j} . \sum_j . \beta ij \, x_i \, x_j + \varepsilon \qquad (1.1)$$

where; y is the response variable, β is regression coefficients, x is independent variables, and ε represents residual error. Since it is necessary to carry out experiments for at least three different levels of factors to define quadratic effects with the regression model, it is feasible to define all possible factor effects (linear or quadratic)[93].

In order to determine the behavior of the response corresponding to the levels of the experimental values, a mathematical equation is identified with the data related to the points of the experimental design. Accordingly, the β parameters of the equations should be estimated. Thus, the matrix notation for equation (1.1) is expressed following equation:

$$Y = X\beta + \varepsilon \qquad (1.2)$$

where; Y represents $nx1$ vector of measured values, X is an nxp matrix of independent variables and β represents $px1$ the vector of coefficients and ε represents an $nx1$ vector of random errors.

The method of least square (LSM) is a multiple regression technique used to fit a model to a set of experimental data producing the possible lowest residual. After the mathematical transformation, the solution of equation (1.2), that is parameters of vector b are obtained by the given equation:

$$\beta_{p\times 1} = (X^T X)_{p\times p}{}^{-1} (X^T Y)_{p\times 1} \tag{1.3}$$

where X^T is a $p \times n$ transpose of the matrix $X_{n\times p}$ [91].

1.5. AIM OF THE THESIS

Currently, the bottleneck of enzyme production is to improve fermentation performance, consequently the input parameters of the process. Besides, a significant challenge facing the market remains the concomitant production of high-value enzyme cocktails. Despite the organisms that are capable of producing multiple enzymes as single cultures, a microbial consortium is a promising approach due to higher productivity and elimination of challenges rather than single organisms (e.g. glucose repression).

This thesis aims to design an artificial microbial consortium for improving the fermentation performance of the enzyme cocktail containing protease, amylase, and lipase for various industrial applications. Besides, the optimization of process conditions was carried out by the Response Surface Methodology for the controlled production of enzyme cocktail by using an artificial bacteria-yeast consortium.

2. MATERIALS

2.1. MICROBIAL STRAINS

Bacillus amyloliquefaciens 7ys48

Candida rugosa ATCC 14830

Yarrowia lipolytica ATCC 20460

Saccharomyces cerevisiae CEN.PK113-7D

2.2. MEDIA AND CHEMICALS

Agar (#05040, Sigma Aldrich), α-amylase from *B.amyloliquefaciens* (#A7595, Sigma Aldrich), Calcium chloride dihydrate (#12022, Sigma Aldrich), Casein from bovine milk (#C7078, Sigma Aldrich), D(+)-Glucose monohydrate (#16301, Sigma Aldrich), 3,5-Dinitrosalicylic acid (#D0550, Sigma Aldrich), Folin & ciucateu's phenol reagent (#47641, Sigma Aldrich), Glycerol (#927.023.1000, IsoLab), Hydrochloric acid(#07102-2, Sigma Aldrich), Iodine (#207772, Sigma Aldrich), 2-propanol (#34863-2, Sigma Aldrich), Lipase from *Aspergillus sp.* (LP50 Lipaz enzimi, Tito), L-tyrosine (#T3754, Sigma Aldrich) Magnesium sulfate heptahydrate (#230391, Sigma Aldrich), 4-nitrophenyl palmitate (#N2752, Sigma Aldrich), 4-nitrophenol (#1048, Sigma Aldrich), Olive oil (Komili), Peptone from meat, bacteriological (#91249, Fluka), Potassium iodide (#03124, Sigma Aldrich), Potassium phosphate dibasic trihydrate (#471767, Sigma Aldrich), Potassium phosphate monobasic (#04243, Sigma Aldrich), Potassium sodium tartrate tetrahydrate (#S2377, Sigma Aldrich), Protease from *Bacillus sp.*(#P4860, Sigma Aldrich), Skim milk powder (#70166, Sigma Aldrich), Sodium carbonate (#795445, Sigma Aldrich), Sodium chloride (#31434, Sigma Aldrich), Sodium hydroxide (#06203, Sigma Aldrich, Starch soluble (#S9765, Sigma Aldrich), Trichloroacetic acid (#27242, Sigma Aldrich), Triton X-100 (#T8787, Sigma Aldrich), Trizma® base (#T1503, Sigma Aldrich), Tryptic soy agar, Tryptic soy broth (#4021552, Biolife), Yeast extract (#4122204, Biolife).

2.3. LABORATORY EQUIPMENT AND DEVICES

Cryo tube; mL: 2 (Isolab), Eppendorf tube; mL: 1,5, 2 (Isolab), Erlenmeyer Flask; mL: 250, 500, 1000 (Isolab), Falcon tube; mL: 15, 50 (Isolab), Graduated cylinder; mL: 10, 50, 100, 500, 1000 (Isolab), Glass bottle; mL: 50, 100, 250, 500, 1000 (Isolab), Inoculation loop (Isolab), Micropipette; µL: 1-10, 10-100, 20-200, 100-1000 (Eppendorf), Micropipette tip; µL: 10, 200, 1000 (Eppendorf, Thermo Scientific), Petri dish; 120 x 17 mm (Isolab), Serological pipette ;mL: 5, 10, 25 (SLP Life Sciences), Rubber suction bulb (Isolab), Syringe; mL: 20, 50 (Set®inject).

Autoclave (Daihan Scientific), Balance Scale (Shimadzu), Centrifuge (Eppendorf 5810R, Gyrozen), Drying oven (Binder), Fume hood (Greenlife), Incubator (WiseCube, Memmert), Microair Flow Cabinet MGK120, Magnetic stirrer (Daihan Scientific), Microcentrifuge (Eppendorf^{TM} 5424), pH meter (Mettler Toledo), Refrigerator; 4^{o}C, -20^{o}C (Arçelik), Refrigerator; -80^{o}C (Thermo Scientific Forma 88000 Series), Shaking incubator (New Brunswick Innova® 44), Spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific), Ultrapure Water (Milli-Q, Millipore), Vortex (Scilogex MX-S), Water bath (Grant SUB Aqua 12 Plus, Memmert)

2.4. SOFTWARE

MATLAB version: 9.4.0.813654 (R2018a) License Number: 40574562

LibreOffice 7.1.5

3. METHODS

3.1. MICROBIOLOGICAL PART

Selection of Microbial Consortia

Fifty different microorganisms obtained from the Culture Collection of Yeditepe University consisting of bacteria and yeast families were tested to their proteolytic, lipolytic, and amylolytic activities to design of microbial consortium. Selected microorganisms used in this work were specified in section 2.1., which are substrate-specific extracellular enzyme-producing organisms used in several experimental studies.

Skim milk agar was used for proteolytic activity screening. 50 gL^{-1} skim milk powder and 15 gL^{-1} agar prepared, separately and autoclaved at 121°C for 3 mins and 15 mins, respectively were mixed with a 1:1 ratio (v/v). Starch agar was used for amylolytic activity screening. 10 gL^{-1} soluble starch and 15 gL^{-1} agar were prepared and autoclaved at 121°C for 15 mins. Lipolytic activity was determined by phenol red agar. 1 gL^{-1} $CaCl_2$, 10 gL^{-1} olive oil, 15 gL^{-1} agar, 0.1 gL^{-1} phenol red were prepared with adjusting the pH to 7.4. Agar plates were incubated at 30°C for 48 h and clear zones were observed. In the starch agar test, clear zones were observed when they were treated with iodine solution into the blue-color complex. Skim milk agar is based on the demonstration of hydrolysis of casein, phenol red agar is based on decreasing the endpoint of pH-dependent on hydrolysis of olive oil, and starch agar test is based on the hydrolysis of amylose in the soluble starch. Enzymatic activities were evaluated with a clear zone (for proteolytic and amylolytic activities), and a yellow zone (for lipolytic activities) appeared on the agar at the end of 48 h incubation.

3.2. FERMENTATION CONDITIONS

3.2.1. Microorganism Storage

Microorganisms were grown into a Tryptic Soy Broth (TSB) medium in 0.25 L of Erlenmeyer flasks with a 1:5 ratio of working to total volume and incubated at 30°C and 150 rpm for 24 h. Cultures were mixed with the final 30 percent glycerol with a 1:1 ratio (v/v) and kept at -80°C for long-term storage.

3.2.2. Inoculum Preparation

Microorganisms stored in -80°C glycerol cultures were grown on Tryptic Soy Agar (TSA) at 37°C for 48 h. One loop of grown cells from TSA was inoculated into a TSB medium in Erlenmeyer flasks with a 1:5 ratio (wvol) and incubated for 24 h at 30°C and 150 rpm to run both single- and co-cultures.

3.2.3. Batch Fermentation for Both Single- and Co-Cultures

Both single- and co-cultures were performed under aerobic conditions in 1 L of Erlenmeyer flasks, with a 0.2 L (wvol) at 30ºC, 150 rpm, and initial pH 6.8 (without pH control). Co-cultures were performed as two different designs; BCY mixture is composed of *B.amyloliquefaciens*, *C.rugosa*, and *Y.liplolytica*; while BCS is composed of *B.amyloliquefaciens*, *C.rugosa*, and *S.cerevisiae*. For both single- and co-cultures, the fermentation was inoculated with a 3 percent (v/v) inoculum ratio, and initial inoculum composition (Bacterium/Yeasts) of co-cultures were 5, 2, and, 0.5 (mL) for three species, respectively. For evaluating co-culture effects on enzyme cocktail production, the medium was composed of 10 gL^{-1} soluble starch and 5 gL^{-1} olive oil as carbon sources, supplemented with nitrogen sources as 10 gL^{-1} peptone, 15 gL^{-1} yeast extract; cofactors as 1 gL^{-1} NaCl, 0.2 gL^{-1} $CaCl_2.2H_2O$, and 0.5 gL^{-1} $MgSO_4.7H_2O$. All media components were sterilized for 15 min at 121ºC, 1.5 atm. Olive oil was prepared separately and transferred into each Erlenmeyer flask before fermentation was initiated.

The degree of enzymatic synergism was calculated by dividing the observed enzymatic activity from each consortia by the minimum activity of the corresponding secretome from the respective single cultures. Synergistic growth was defined as having occurred when the total biomass developed in the consortium was higher than the biomass achieved from any corresponding single culture [71].

3.3. STATISTICAL OPTIMIZATION

3.3.1. Experimental design and Model Equation

The combined effect of carbon sources and inoculum ratio and their optimal levels on enzyme cocktail production (response functions) was evaluated by CCD. Table 3.1. indicates that the layout of the experimental domain for the three chosen independent variables that were carbon sources as starch, X_1 (gL^{-1}); olive oil, X_2 (gL^{-1}); and Initial inoculum of bacterium (*B.amyloliquefaciens*) to total yeasts (*C.rugosa*+*Y.lipolytica*, and *C.rugosa*+*S.cerevisiae*) in terms of volume/volume, X_3 (Bacterium/Yeasts (v/v)), with the levels of other solutions keeping constant. The independent variables were coded in compliance with the following equation [86,95].

$$X_i = \frac{x_i - x_o}{\Delta x} \qquad i = 1, 2, \ldots, k \tag{2.1}$$

where; X_i represents a coded independent variable, x_i represents the nominal independent variable, x_o represents the nominal value of the independent variable at the central point, Δx represents the step-change.

Table 3.1. Independent variables and levels of the design of the experiment

Independent Variables	**Symbols**	**Experimental Domain and Levels**				
		-1.4 (axial point)	-1 (lower level)	0 (central point)	1 (upper level)	1.4 (axial point)
Starch	X_1	3	5	10	15	17
Olive Oil	X_2	0.2	1	3	5	5.8
Inoculum Ratio (B/Y)	X_3	0	0.5	2	5	6

The experimental design included a total of 20 runs comprised of 2^3 factorial design (levels of -1 and 1), 6 axial points (levels of -1.4 and 1.4), and 6 central points (zero levels). Table 3.**2**. shows the experimental setup for factors of coded form. The second-order polynomial quadratic equation was employed for the following model of correlate dependent and independent variables because of sufficient for the optimum region.

$$y = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2.2)$$

The model indicates that the individual and combined effects of each variable where; y represents the response function (for each enzyme found in the enzyme cocktail), the coefficients $\beta_1, \beta_2, \beta_3$ represent the linear; $\beta_{11}, \beta_{22}, \beta_{33}$ represent the quadratic and, $\beta_{12}, \beta_{13}, \beta_{23}$ represent the interaction terms respectively, X_i and X_j represent the independent variables.

Table 3.2. Experimental setup and conditions, variables are given in coded forms

Run Order	Coded levels of Variables		
	X_1	X_2	X_3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	0	0	0
14	0	0	0
15	-1.4	0	0
16	1.4	0	0
17	0	-1.4	0
18	0	1.4	0
19	0	0	-1.4
20	0	0	1.4

3.3.2. Optimization of Responses and Validation Experiments

After the CCD design, the quadratic model is parameterized using experimental data and linear regression, and the optimization method of Nelder–Mead (N-M) simplex was used to find the optimum concentrations of starch, oil, and initial inoculum ratio (Bacterium/Yeasts). The method of N-M simplex optimization is based on a regular simplex of n+1 sides, where n is the number of variables [97].

Validation experiments were conducted as four new sets depend on maximizing each enzyme; protease (P_{Max}), amylase (A_{Max}), and lipase (L_{Max}), and maximizing enzyme cocktail (C_{Opt}). Fermentations were performed at 30°C, 150 rpm, 0.2 L of working volume with 3 percent (v/v) total inoculum ratio, initial pH of 6.8 (without pH control).

3.4. ANALYTICAL MEASUREMENTS

3.4.1. Determination of Biomass Concentration

For estimation of biomass (gL^{-1}), 10 mL of each aliquot of culture sample collected in 12 h intervals were centrifuged at 4000 rpm for 20 mins 4°C and separated from the supernatant. The pellet was resuspended twice with dH_2O and transferred into the 2 mL of pre-weighed Eppendorf tubes, and left at 70°C for drying until reach constant weight (for 48h) and weighed again.

3.4.2. Determination of Reducing Sugar Concentration

Reducing sugar determination was performed using the DNS method [98]. DNS reagent was prepared by mixing 1 g of 3,5-dinitrosalicylic acid, 40 g of potassium sodium tartrate tetrahydrate, and 20 mL of 2 N sodium hydroxide in a final volume of 100 mL which is completed with dH_2O. 25 µl sample or standard and 475 µl dH_2O were mixed with 500 µl DNS reagent and incubated at 100°C for 10 mins and cooled 5 min at 4°C and readings were done at 540 nm with a UV-Vis spectrophotometer. The unknown concentration of

reducing sugar produced was concluded from the glucose calibration curve in a concentration ranging between 0.625 to 7.5 gL^{-1} used as a standard.

3.4.3. Determination of Individual Activities of Enzyme Cocktail

Protease activity was determined using casein as a substrate [99]. Initially, 0.025 mL of sample was incubated at 37°C for exactly 10 mins with 6.5 gL^{-1} casein (for each sample, 0.025 mL of heat-treated (at 100°C for 15 mins) cell-free supernatant was used instead of sample for enzyme blank). After incubation, 0.13 mL of 110 mM Trichloroacetic acid (TCA) solution was added, incubated at 37°C for 20 mins, and centrifuged for 5 mins at 10000 rpm. 0.25 mL of supernatant was transferred into a new tube, 0.625 mL of sodium carbonate solution (Na_2CO_3) and 0.125 mL of Folin & Ciocalteu's Phenol (F-C) Reagent (1:3 dilution) were mixed and incubated 37°C for 30 mins. The optical density of a mixture was measured at 660 nm. The unknown concentration of tyrosine produced was concluded from tyrosine standard calibration in a concentration ranging between 0.06-0.01 µmole used as a standard.

The enzymatic activity is referred to in U/mL ($^{\mu mole}/_{mL \times min}$) that is defined as the 1 µmole of tyrosine from casein hydrolysis per min per mL under the defined assay conditions. The activity was determined according to the following equation.

$$^{Units}/_{mL} = \frac{tyrosine\ released\ (\mu mole) \times\ total\ volume\ of\ assay(mL)}{volume\ of\ sample(mL) \times time\ of\ assay(min) \times volume\ for\ colorimetric\ determination(mL)} \quad (2.3)$$

Lipase activity was determined using 4-nitrophenyl palmitate (pNPP) as a substrate [63]. 16.5 mM of pNPP was dissolved in 1 mL of isopropanol (solution 1) with a vortex for nearly 5 mins at room temperature. 40 µl of Triton X-100 and 10 µg of gum arabic were dissolved in 9 mL of 50 mM Tris-HCl buffer (pH 8) (solution 2). Solution 1 was added to solution 2 and the mixed emulsion was used as a substrate solution. 1 mL of the substrate was mixed with 100 µl of sample and incubated at 37°C for 20 min. The reaction was stopped by incubating at 100°C for 5 mins. A heat-treated (at 100°C for 15 mins) sample was used as a blank instead of a crude enzyme. The optical density of produced p-

nitrophenol (pNP) was measured at 410 nm. The unknown concentration of pNP produced was concluded from a pNP standard calibration curve in the 1.5 mM to 15 mM range.

The enzymatic activity is referred to in U/mL $\left({}^{\mu mole}/_{mL \times min}\right)$ that is defined as one unit (U) of lipolytic activity was defined as the enzyme amount required to generate 1 µmole of p-nitrophenol from pNPP hydrolysis per min per mL under the assay conditions. The activity was determined according to the following equation.

$$Units/_{mL} = \frac{p-nitrophenol\ released\ (mM) \times total\ volume\ of\ assay(mL)}{time\ of\ assay\ (min) \times volume\ of\ sample\ (\mathrm{mL})} \qquad (2.4)$$

Amylase activity was determined using a 10 gL^{-1} of soluble potato starch as a substrate [100]. Initially, 0.2 mL of sample was incubated at 37°C for exactly 10 mins with 0.2 mL of gelatinized starch (with tap water). Besides, 0.2 mL of heat-treated (at 100ºC for 15 mins) sample was incubated for enzyme blank, at the same time. The hydrolysis reaction was stopped by the addition of 0.4 mL of DNS reagent and samples were incubated at 100ºC for 15 mins then cooled at 4°C for 10 mins. The optical density of the mixture was measured at 540 nm. Maltose in a concentration ranging between 0.3 to 5 gL^{-1} was used as a standard.

The enzymatic activity is referred to in U/mL $\left({}^{\mu mole}/_{mL \times min}\right)$ that is defined as the 1 µmole of maltose from starch hydrolysis per min per mL under the defined assay conditions. The activity was determined according to the following equation.

$$Units/_{mL} = \frac{maltose\ released\ (mM) \times total\ volume\ of\ assay\ (mL)}{time\ of\ assay\ (\mathrm{min}) \times volume\ of\ sample\ (\mathrm{mL})} \qquad (2.5)$$

4. RESULTS AND DISCUSSION

4.1. SELECTION OF MICROBIAL CONSORTIA

Enzyme activities were first determined qualitatively from the fifty organisms to set a base for the designing of the artificial microbial consortium, given in Table 4.1. Among the screened strains, *Bacillus amyloliquefaciens* 7YS48 acting as the extracellular protease, amylase, and lipase producer; *Candida rugosa* ATCC 14830 acting as the lipase producer; *Yarrowia lipolytica* ATCC 20460 acting as the lipase producer; *Saccharomyces cerevisiae* CEN.PK113-7D acting as "cleaner", were chosen for the design of microbial consortium (Figure 4.1.).

Table 4.1. Organism screening to determine their qualitative protease, amylase, and lipase activities

Organisms	**Systematic name**	**Protease**	**Amylase**	**Lipase**
Bacillus subtilis	1ys8	+	+	+
Bacillus thuringiensis	1ys20	+	-	-
-	1ys32	-	-	+
Bacillus methylotrophicus	1ys37	+	+	+
Bacillus mojavensis	1ys48	-	-	-
Bacillus megaterium	1ys81	+	-	+
-	2ys18	+	+	+
-	2ys53	+	-	+
-	2ys68	-	-	+
Paenibacillus polymyxa	2ys79	+	-	+
Bacillus polymyxa	2ys75	+	-	+
Bacillus subtilis	3ys4	+	+	+
Staphylococcus hominis	3ys49	-	-	+
Rhodococcus corynebacterioides	3ys50	-	-	+
Nocardia cyriacigeorgica	3ys55	-	-	-
Pseudomonas mosselii	3ys62	+	-	-
Chryseobacterium shadongense	4ys2		-	-
Chryseobacterium shadongense	4ys3	+	+	+
Chryseobacterium shadongense	4ys13	+	-	+
Acinetobacter pittii	4ys17	+	-	-
Chryseobacterium shadongense	4ys26	+	-	+
Bacillus megaterium	4ys30	+	-	+
Bacillus subtilis	4ys31	+	+	+
Bacillus cereus	5ys5	-	+	+
Enterobacter cloacae	5ys56	-	-	+
-	5ys79	+	-	+
Bacillus cereus	6ys2	+	-	+

-	6ys7	-	-	+
Burkholderia cenocepacia	6ys19	+	-	+
Staphyllococcus pasteuri	6ys52	+	-	-
Chryseobacterium gleum	6ys59	+	+	+
Bacillus megaterium	6ys71	+	-	+
-	7ys24	+	+	+
-	7ys27	+	-	+
Bacillus thuringiensis	7ys31	+	-	-
Bacillus cereus	7ys32	+	-	-
-	7ys33	+	-	+
-	7ys34	-	-	+
-	7ys37	+	-	+
Klebsiella pneumoniae	7ys39	-	-	+
-	7ys47	+	-	+
-	7ys52	+	-	+
-	7ys54	+	-	-
-	7ys57	+	-	+
Bacillus amyloliquefaciens	7ys48	+	+	+
Bacillus cereus	-	+	-	+
Bacillus pumilus	-	+	-	+
Yarrowia lipolytica ATCC 20460	-	-	-	+
Saccharomyces cerevisiae CEN.PK 113-7D	-	-	-	-
Candida rugosa ATCC 14830	-	-	-	+

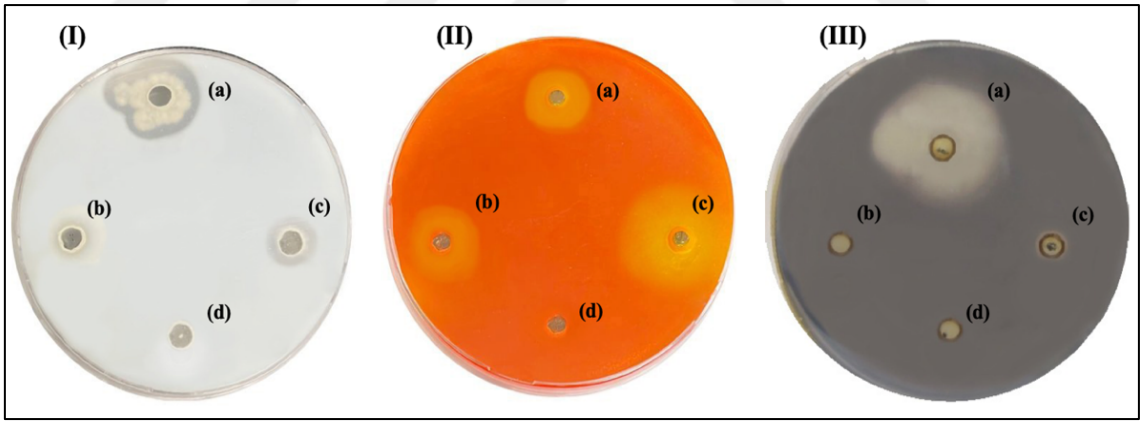

Figure 4.1. Qualitative extracellular enzymatic activities on agar plates for designing artificial microbial consortia. (I): protease; skim milk agar, (II): lipase; phenol red agar, (III): amylase; starch agar with an iodine test (a): *Bacillus amyloliquefaciens*, (b): *Yarrowia lipolytica* ATCC 20460, (c) *Candida rugosa* ATCC 14830, and (d) *Saccharomyces cerevisiae* CEN.PK113-7D

4.2. EFFECT OF MICROBIAL INTERACTIONS ON ENZYME COCKTAIL PRODUCTION

4.2.1. Fermentation Characterization of Single Cultures

Fermentations were conducted in a complex medium supplemented with starch and olive oil as carbon sources. According to studies in literature, the most effective inducers of amylase and lipase are soluble starch and olive oil, specifically oleic acid respectively [101,102]. The nitrogen source serves as a secondary energy source for growth and enzyme secretion in any organism. Among the various nitrogen sources used in the literature, peptone serves significant enzyme activity for protease. Furthermore, both peptone and yeast extract are the optimal nitrogen sources for amylase and lipase [63].

Growth profiles were evaluated in terms of cell dry weight ($gDWL^{-1}$). It was hypothesized that the spectral background noise of the medium during fermentation, due to the production of sophorolipid biosurfactant (dirty-white crystals) from olive oil, would cause the noise for optical cell density [103]. The unconsumed starch from nonproducing amylase fermentations was removed by the pellet washing. Roychoudhury *et.al.* reported that the final biomass concentration of *B.amyloliquefaciens* is 2.5 gL^{-1} with a biomass yield $Y_{x/s}$ 0.33 gg^{-1} in the presence of 10 gL^{-1} glucose [104]. In contrast, the final biomass level of *B.amyloliquefaciens* was determined to be in this study 3.7 gL^{-1} with biomass yield $Y_{x/s}$ 0.24 gg^{-1} (Figure 4.2. (a)). The increase in biomass was from olive oil as a second carbon source. The final biomass concentrations of *C.rugosa* and *Y.lipolytica* fermentations were determined to be 3.212 gL^{-1} and 6.285 gL^{-1}, respectively, with a biomass yield on oil consumed of 0.99 and 1.20 gg^{-1} $Y_{x/s}$, respectively (assume that olive oil was completely consumed). The final biomass concentration was found to be 3 gL^{-1} in a study for the production of *C.rugosa* lipase using 5 gL^{-1} of olive oil reported by Del Rio *et. al.* [105]. Additionally, Noor *et. al*. reported that in the *C.rugosa* lipase production using 10 gL^{-1} olive oil as an only carbon source, the biomass yield to the substrate was $Y_{x/s}$ 2.86 gg^{-1} with 5.17 gL^{-1} biomass concentration [106]. The final biomass concentration of *Y.lipolytica* was stated between 10 gL^{-1}- 30 gL^{-1} between values of 1.1-1.5 gg^{-1} $Y_{x/s}$ related to the production of lipase is a primary anabolic product in the presence of olive oil as a carbon source, respectively [107,108]. Najjar et. al. reported that *Y. lipolytica* can utilize fatty

acids selectively. However, hydrolysis of triglycerides to form glycerol and fatty acid occurs in the lipase production in the presence of olive oil. Ideally, yeasts would be using glycerol, which would inhibit the use of fatty acids. As a result, increased lipase production is associated with fatty acid consumption [105]. It is known that when glycerol is present, *Y.lipolytica* produces organic acids such as citric and 2-ketoglutaric acid. Additionally, it was reported that using glucose as a carbon source inhibits the production of *Y.lipolytica* lipase [109]. Additionally, the fermentation of *S.cerevisiae*, whose final biomass concentration was determined to be 3.88 gL^{-1} in this study, used only nitrogen sources and cofactors from the medium. Additionally, it is reported that when high concentrations of fermentable sugars (>20 gL glucose) are present or converted, *S.cerevisiae* has a high capacity for ethanol production via glycolysis [110].

B.amyloliquefaciens consumed reducing sugar derived from hydrolysis of starch, while *C.rugosa*, *Y.lipolytica*, *and S.cerevisiae* could not contribute to hydrolyzing of starch, so there was no amylase secretion in these fermentations (Figure 4.2. (b) and (d)). Furthermore, the maximum level of reducing sugar of *B.amyloliquefaciens* was 3.764 gL^{-1} at 24^{th}, and the final reducing sugar concentration was determined as 0.335 gL^{-1} at the 72 h of the fermentation period. Syu *et. al.* depicted that, the reducing sugar concentration from 10 gL^{-1} of starch hydrolysis increased within the first 12 h due to the hydrolysis of starch as a sole carbon source with a maximum concentration of 3.5 gL^{-1} [111]. The time lag of 12 h between the maximum reducing sugar level and the observed sugar level may be due to the consumption of olive oil as the second carbon source in this study. At this stage, the starch is hydrolyzed to produce glucose as an end product. The formation of glucose provides cell growth via glycolysis and, consequently, amylase secretion. The glycolytic pathway serves as a model for the energy-generating mechanisms found in cells.

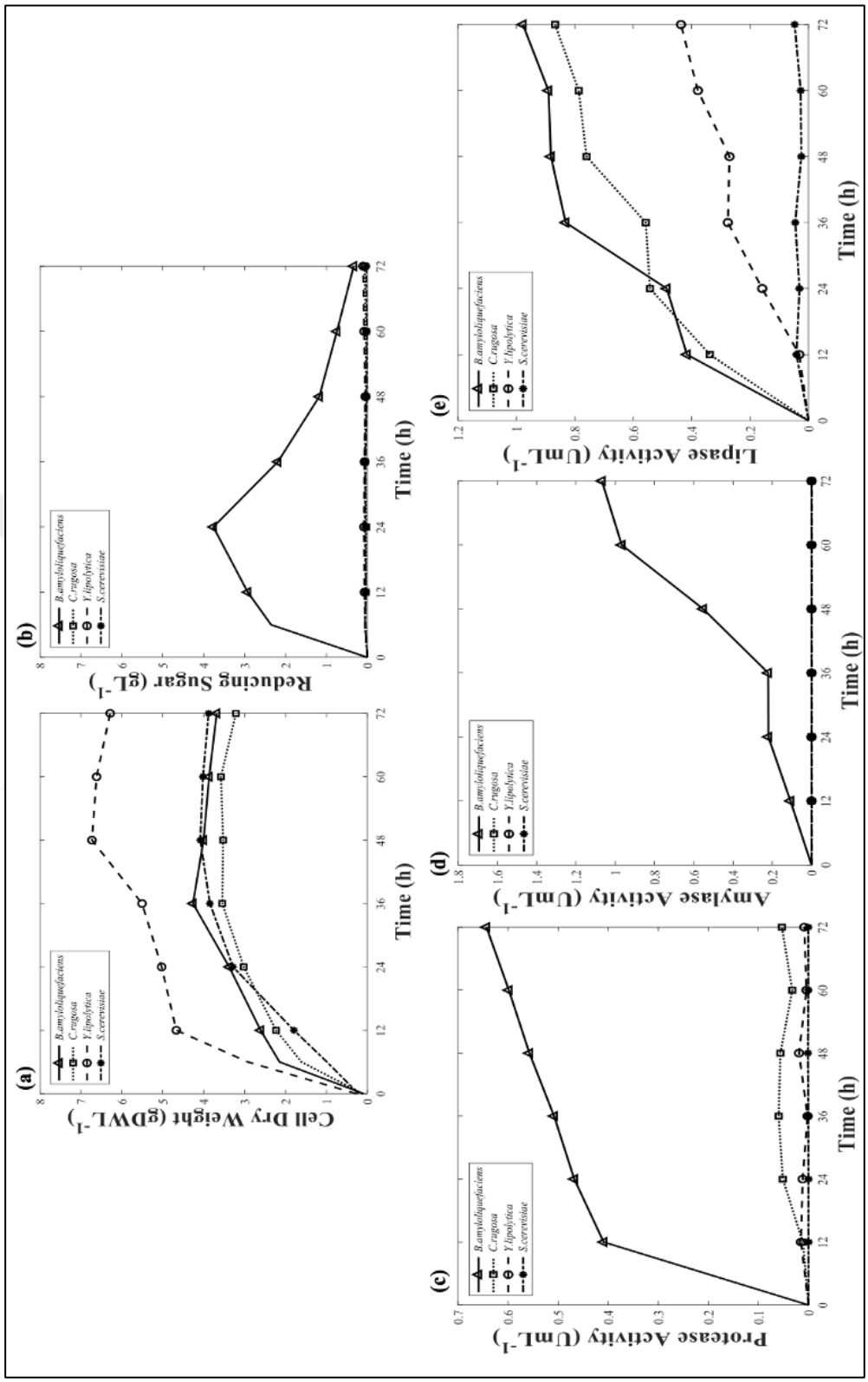

Figure 4.2. Comparative results of single cultures batch profiles in terms of (a) Biomass Concentrations ($gDWL^{-1}$), (b) Reducing sugar (gL^{-1}), (c) Protease Activity (UmL^{-1}), (d) Amylase Activity (UmL^{-1}), (e) Lipase Activity (UmL^{-1})

Although this process varies considerably between organisms, many of the core glycolysis enzymes appear to be conserved in the vast majority of organisms whose genomes have been sequenced. Glycolysis is responsible for the breakdown of carbohydrates, particularly glucose, and the fact that this process is conserved indicates that glucose is an important source of energy for a large number of organisms [112]. Continued amylase formation can further degrade the α-1,4 bonds of the various saccharides, stimulating both cell growth and amylase secretion. When the cells reached a certain population density to utilize more reducing sugars and the breakdown of sugars slowed down gradually, the reducing sugar concentration gradually decreased. Nonetheless, the reducing sugar profile of single cultures, in particular, demonstrates that sugar concentration initially increased due to starch hydrolysis and then decreased due to increased sugar consumption and slower or even no hydrolysis of saccharides [111].

In addition, *C.rugosa* and *Y.lipolytica* produced lipase in the presence of olive oil but, protease was not produced from peptone (Figure 4.2. (c) and (e)). While *B.amyloliquefaciens* produced all three enzymes [113], *S.cerevisiae* did not take part in the production of the enzyme cocktail. These results support the qualitative enzyme activity results set out in Figure 4.1. In conclusion, the final activities of each enzyme comprised in a cocktail of single cultures were determined as follows; protease and amylase for *B.amyloliquefaciens* were 0.643 Uml^{-1} and 1.068 Uml^{-1}, respectively; and lipases were 0.978 Uml^{-1}, 0.867 Uml^{-1}, and 0.437 Uml^{-1} for *B.amyloliquefaciens*, *C.rugosa*, and *Y.lipolytica*, respectively. As can be seen in Figure 4.2. (d), the amylase activity in *B.amyloliquefaciens* fermentation remained constant between 24-36 h, and then the activity continued to increase until reducing sugar was consumed. This may be due to excess intermediate saccharides repressing amylase secretion. Lipase activity profiles (Figure 4.2. (e)) seem to proceed via two stages which suggest the possibility that the three strains utilize the components (fatty acids and glycerol) of olive oil, sequentially. The activity of lipase was found to increase linearly with the chain length of the fatty acid. It was proposed that long-chain fatty acids are involved in the expression of lipase genes and that induction occurs at the transcriptional level [105,106]. In literature, numerous methods for determining enzyme activity have been reported. Additionally, despite the enzyme activities being typically determined in terms of the amount of substrate converted under

their defined conditions, evaluation of quantitatively enzymatic activities does not give an effective result [101,114–118].

4.2.2. Fermentation Characterization of Co-Cultures

The key criteria that need to be considered for the establishment of microbial consortium are described in Section 1.3. Strain combinations were formed based on the abundance values of the respective three strains relative to the initial inoculum (B/Y: initial bacterium-to-total yeasts (v/v)) in the microbial consortia, and the performance of strains in the current profiles of total growth and enzymatic activity on complex medium (Table 4.2.). Considering that bacteria, along with yeasts [71], comprise the majority of microbial consortia, this study focused on the synergistic and competitive effects arising from concomitant hydrolysis of starch and olive oil as two carbon sources. Both consortia thus included an inoculum range of bacterium with varying degrees of abundance alongside dominant yeast strains.

Table 4.2. Compositions of microbial consortia

Microbial Consortia	Composition
BCY	*Bacillus amyloliquefaciens* *Candida rugosa* ATCC 14830 *Yarrowia lipolytica* ATCC 20460
BCS	*Bacillus amyloliquefaciens* *Candida rugosa* ATCC 14830 *Saccharomyces cerevisiae* CEN.PK113-7D

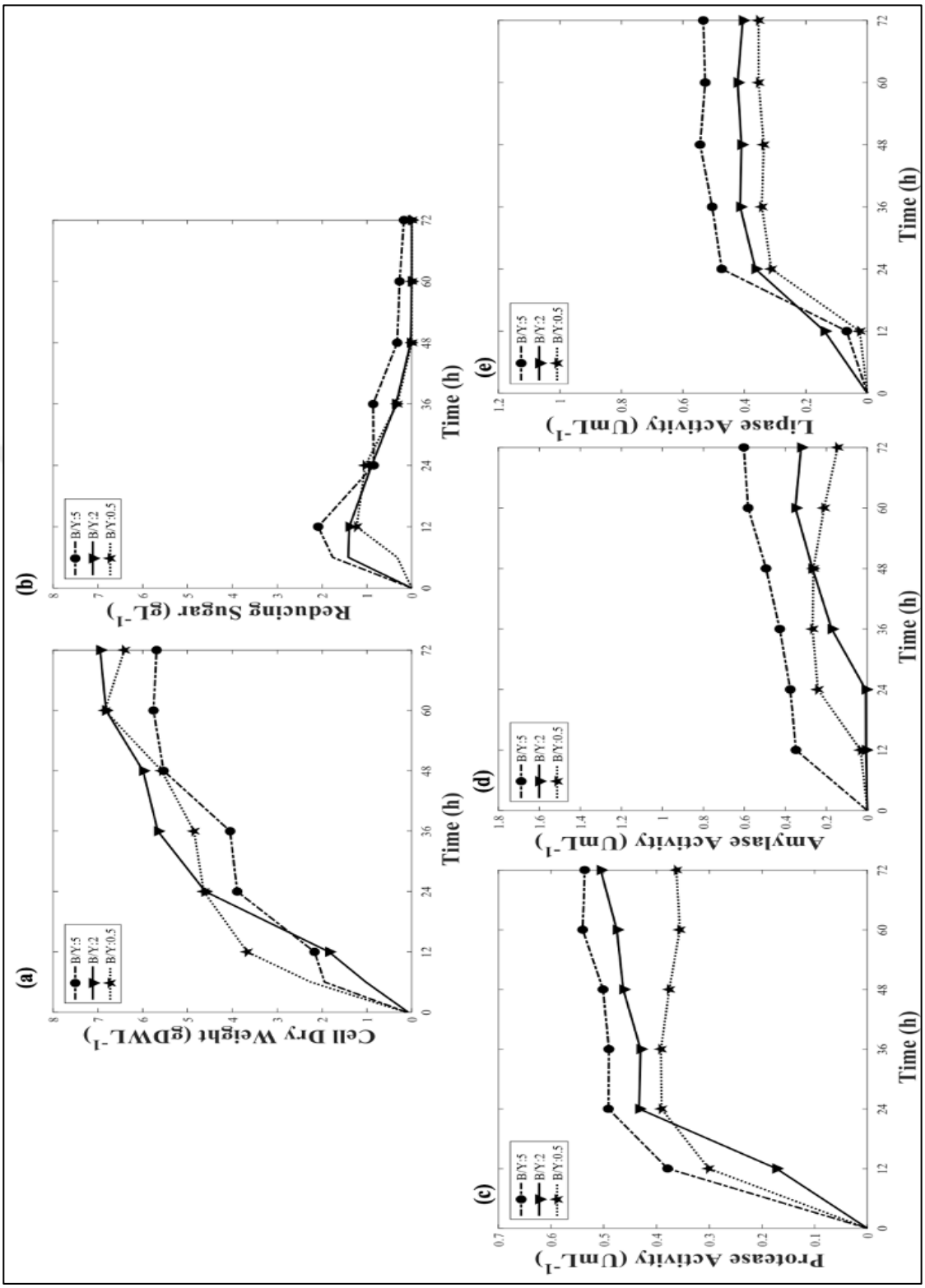

Figure 4.3. Effect of initial bacterium-to-total yeasts (B/Y) inoculum ratio of co-culture BCY over enzyme cocktail production in terms of (a) Biomass Concentrations ($gDWL^{-1}$), (b) Reducing sugar (gL^{-1}), (c) Protease Activity (UmL^{-1}), (d) Amylase Activity (UmL^{-1}), (e) Lipase Activity (UmL^{-1})

Co-cultures of BCY (Figure 4.3.) were performed under identical conditions as single-cultures. The biomass concentrations of co-culture BCY was higher than single cultures, however, biomass yield on starch and olive oil consumption $Y_{x/s}$ (0.4, 0.5, and 0.4 gg^{-1} for B/Y 5, 2, and 0.5, respectively) was higher from the only single culture of *B.amyloliquefaciens* (Figure 4.3. (a)). One possible explanation is that when the primary nutrients are depleted during exponential growth, strains in BCY can continue to grow by utilizing metabolites generated by others. To detail the growth profile in terms of strain interactions on complex medium, it is necessary to examine their individual growth profiles in the consortium. When these strains were associated with each other through different initial B/Y (5, 2, 0.5) ratios, the consumption of reducing sugar increased gradually in connection with increasing the yeasts ratio in the consortium during the fermentation period (Figure 4.3. (b)). The maximum level of reducing sugars was regressed at 12 h compared to the single culture of *B.amyloliquefaciens* (Figure 4.2. (b)), however, the maximum level of reducing sugar decreased gradually as the total yeast content increased, indicating that there was no oligosaccharides accumulation (assuming that starch was consumed). In addition, the final reducing sugars were consumed 48h for B/Y 5, 36 h for B/Y 2, and 0.5. Reducing sugar released from starch hydrolysis was depleted at 72 h in the single culture of *B. amyloliquefaciens*.

The final (72 h) enzyme activities in the cocktail (Figure 4.3. (c), (d), and (e) showed that despite the increasing reducing sugar consumption, there was a significant decrease in amylase activity compared to the single culture of *B.amyloliquefaciens* (decrease in 1.77-fold for B/Y:5; 3.30-fold for B/Y:2; and 4.33-fold for B/Y:0.5). Lipase activities decreased in BCY (in 1.63-fold for B/Y:5, 2.14-fold for B/Y:2, and 2.45-fold for B/Y:0.5) when compared to the single culture of *C.rugosa*, which is consistent with the hypothesis that increases in total yeasts ratio results substrate competition. Besides, since all of the consortium members are lipase producers, different inoculum ratios could not eliminate substrate competition. As the concentration of the total yeast increased, protease activities in co-cultures decreased (1.20-fold for B/Y:5, 1.27-fold for B/Y: 2 1.78-fold B/Y:0.5). This was due to sharing utilization of peptone from *B.amyloliquefaciens* and other organisms that consume peptone but do not produce protease.

In addition, while the increase in protease and lipase activities in single cultures continued until the end of fermentation (72 h), there were remained constant for all three co-cultures

from 24 h of fermentation. The initial inoculum ratio affected the microbial dynamics [119], resulting in a decrease in the activity of these three enzymes for three different inoculum ratios.

Furthermore, studies demonstrated that mixed culture was could not grow synergistically with utilizing glucose. Cooperation is unlikely to be beneficial in the presence of a carbon source such as glucose, and thus competition or neutralism are expected cases. Numerous published studies support that substrate complexity influences the type of microbial interaction. [120,121]. In this case, because unfavorable substrate competitions among microorganisms reduced three enzyme activities in the cocktail for BCY, the focus was on obtaining synergistic effects of microorganisms to create a more robust and stable co-culture system. For this reason, *S.cerevisiae* was chosen, which acts as just a 'cleaner' for intermediate products in the community, over *Y.lipolytica* to reduce substrate competition that occurs from olive oil consumption.

Co-cultures of BCS (Figure 4.4.) were evaluated under the same conditions BCY and single fermentations as control. The maximum biomass concentration was achieved initial bacterium-to yeasts inoculum (v/v) 2. However, there were no significant differences among the biomass yields on starch and olive oil consumption $Y_{x/s}$ (0.26,0.34, and 0.30 gg^{-1} for B/Y 5, 2, and 0.5, respectively) of three fermentations and lower than the yields of BCY and single cultures of yeasts. In addition, the decrease in biomass concentrations was observed after 36 h in the fermentation period that may be due to consumption of limited carbon and nitrogen sources [122].

When hydrolytic effectiveness of the BCS was evaluated, the maximum levels of the concentration of the reducing sugar resulting from the starch hydrolysis (Figure 4.4. (b)) were higher than that of the corresponding to the fermentations of BCY and *B.amyloliquefaciens* [123]. The reducing sugar concentration for each set of fermentation reached its maximum value within the first 12 h (as similar to BCY) and depleted at the 48h into the fermentation period. As a result, the starch hydrolysis and for each corresponding experimental setup (B/Y 5, 2, and 0.5) of BCS were found to be approximately 2-fold higher than BCY (Figure 4.3. (b)). Since microorganisms require sugar as a carbon source to grow [111], the physiology of members in co-culture was improved by the hydrolysis of starch.

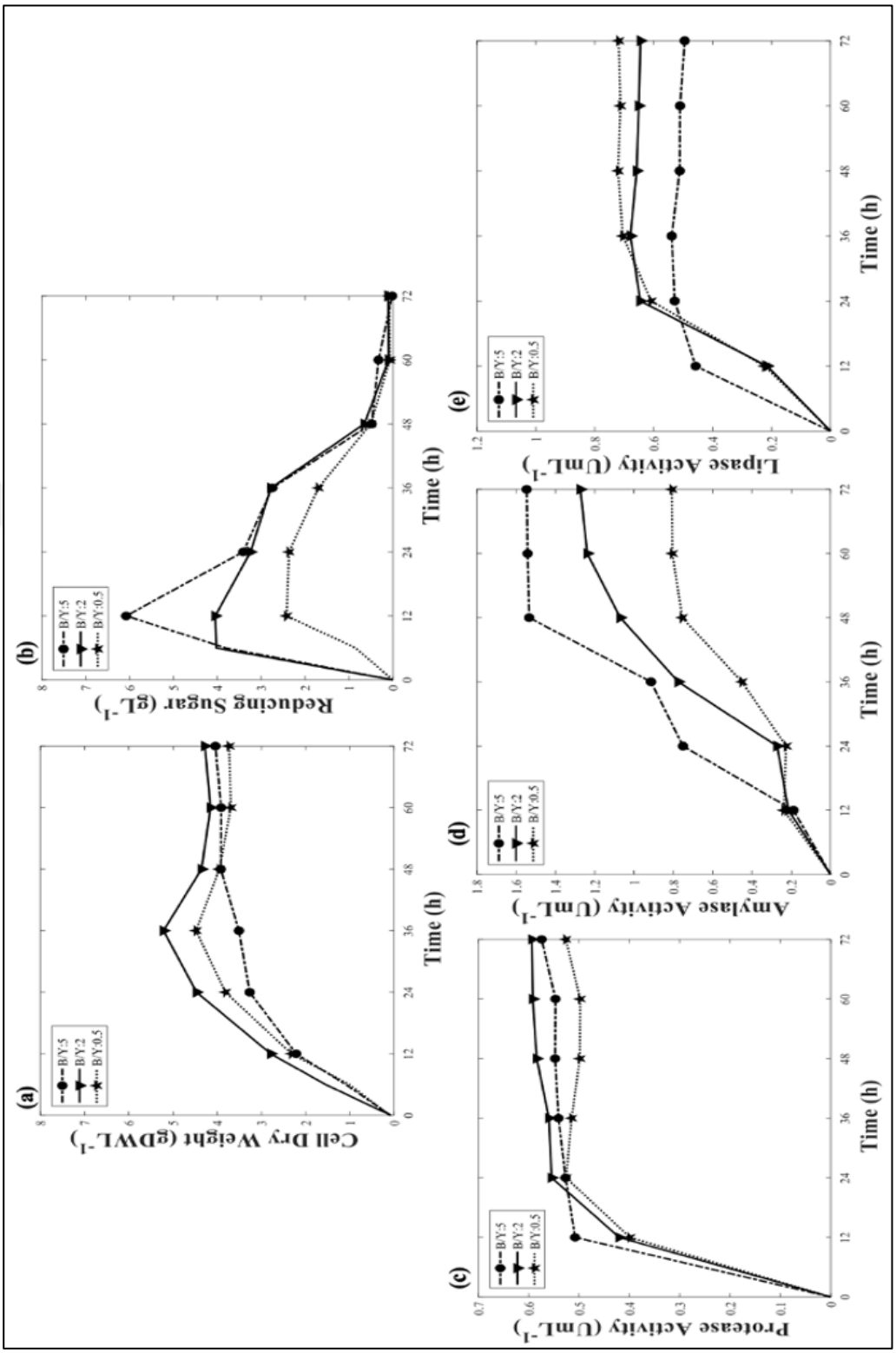

Figure 4.4. Effect of initial bacterium-to-total yeasts (B/Y) inoculum ratio of co-culture BCS over enzyme cocktail production in terms of (a) Biomass Concentrations ($gDWL^{-1}$), (b) Reducing sugar (gL1), (c) Protease Activity (UmL^{-1}), (d) Amylase Activity (UmL^{-1}), (e) Lipase Activity (UmL^{-1})

Thus, the growing organisms consumed the intermediate glucose produced by starch hydrolysis. However, in the present study, consuming the portion of excess intermediate glucose, which repressed amylase secretion [102,111], was improved by *S.cerevisiae* which is not contribute to any enzyme secretion. As a matter of fact that the presence of *S.cerevisiae*, which acts as the consortium's cleaner, stimulated the enzyme synthesis which is unattainable in single cultures.

In the 48h of fermentation, the intermediate glucose consumption by *S.cerevisiae* and redesigning of consortium members creating substrate competition resulted in a 3.10-fold for B/Y:5, 4.00-fold for B/Y:2 and 2.87 B/Y:0.5 increase in amylase activity (Figure 4.4. (d)), and approximately 1.61-fold for B/Y:2 and 2.14-fold for B/Y:0.5 increase in lipase activity of BCY shown in Figure 4.3. (e). However, lipase activity still decrease in 1.48-fold for B/Y:5; 1.15-fold for B/Y:2, and 1.05-fold for B/Y: 0.5 compared to the single culture of *C.rugosa* (Figure 4.2. (e)), and increasing amylase activities related to fermentation of *B.amyloliquefaciens* (Figure 4.2. (d)) were found to be 2.78-fold and 1.94 fold for B/Y 5 and 2, and 1.37-fold for B/Y 0.5. As seen in Figures 4.2. (d), 4.3 (d), and 4.4 (d), there was no significant difference in protease activity between BCY and BCS fermentations, except a 1.45-fold increase of B/Y 0.5. This may be due to the low bacterial inoculum ratio in BCY which is defined as non-synergistic interaction and nitrogen sources may not have negative effects on microbial interactions in this consortium. Furthermore, because environmental parameters are important to better understand their effect on the total microbial growth and enzyme production of the consortium, optimizing the levels of nutrient requirements and balancing the initial inoculum ratio are key steps in co-culture establishment to enhance enzyme production.

4.2.3. Synergism of Growth and Enzyme Production in Co-Cultures

Synergistic growth of both co-cultures was common in complex medium (Figure 4.5. (a), (b)). In other words, co-cultures of BCY followed to BCS exhibited significantly greater growth when compared to their related single cultures at the 36 h of the fermentation period.

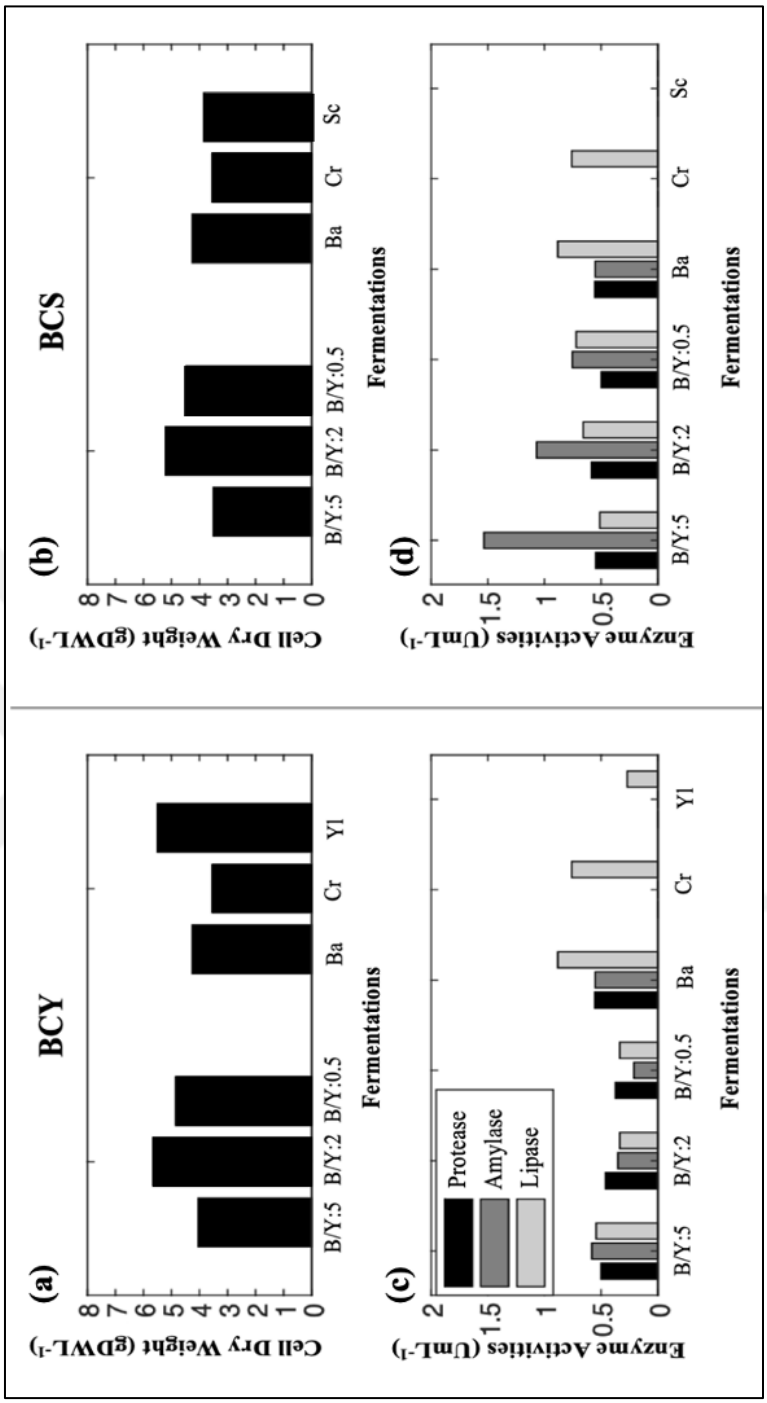

Figure 4.5. Characterization of synergistic consortia. Biomass concentrations ($gDWL^{-1}$) of (a) BCY, (b) BCS and their corresponding single cultures at the 36 h of the fermentation period. Synergistic and non-synergistic enzymatic activities of co-cultures (c) BCY, (d) BCS and their corresponding single cultures at the 48 h of the fermentation period. Explanation: B/Y:5, B/Y:2, B/Y0.5 initial inoculum ratios (bacterium-to-total yeasts**,** v/v), Ba: *B.amyloliquefaciens*, Cr: *C.rugosa*, Yl: *Y.lipolytica*, Sc: *S.cerevisiae*

It has been reported by Deng et. al., the concentration of biomass in consortia having non-synergistic interaction for metabolite secretion could be higher than in single cultures [121]. Besides, fermentations of B/Y:2 and B/Y:0.5 of the BCS reached higher biomass concentrations than its corresponding single cultures. However, despite the production of three enzymes in the cocktail being stimulated in a time-dependent manner in both consortia, BCY did not show any synergistic enzymatic activity (Figure 4.5. (c)). In contrast, the BCS displayed higher amylase activity at the 48 h of the fermentation period (Figure 4.5. (d)). The increased amylase activity was attributed to the presence of *S.cerevisia* which acts as the "cleaner" in the system. However, protease activities of BCS did not change than their corresponding single culture (*B.amyloliquefacens*), lipase activities of three co-cultures of BCS were lower than their corresponding single cultures. Theoretical works showed that negative interactions have been shown to drive networks toward growth and stability, whereas positive interactions have been linked to further increased metabolic activity [124,125]. Therefore, while the system of BCY was only growth-synergistic, BCS was defined as amylase-synergistic next to a growth-synergistic system.

To better compare of two consortia with their related single culture, volumetric starch production/consumption rates were plotted (Figure 4.6.). The starch hydrolysis rate of BCS (Figure 4.6. (a)) was found to be higher than BCY (Figure 4.6. (b)). One possible explanation is the absence of oligosaccharide accumulation from starch hydrolysis in BCY. In addition, starch (amylose) was hydrolyzed to reducing sugars at the 18 h of fermentation period in both consortia, while in the related single culture (*B.amyloliquefaciens*) (Figure 4.6. (c)), starch hydrolyzing was done at the 22h.

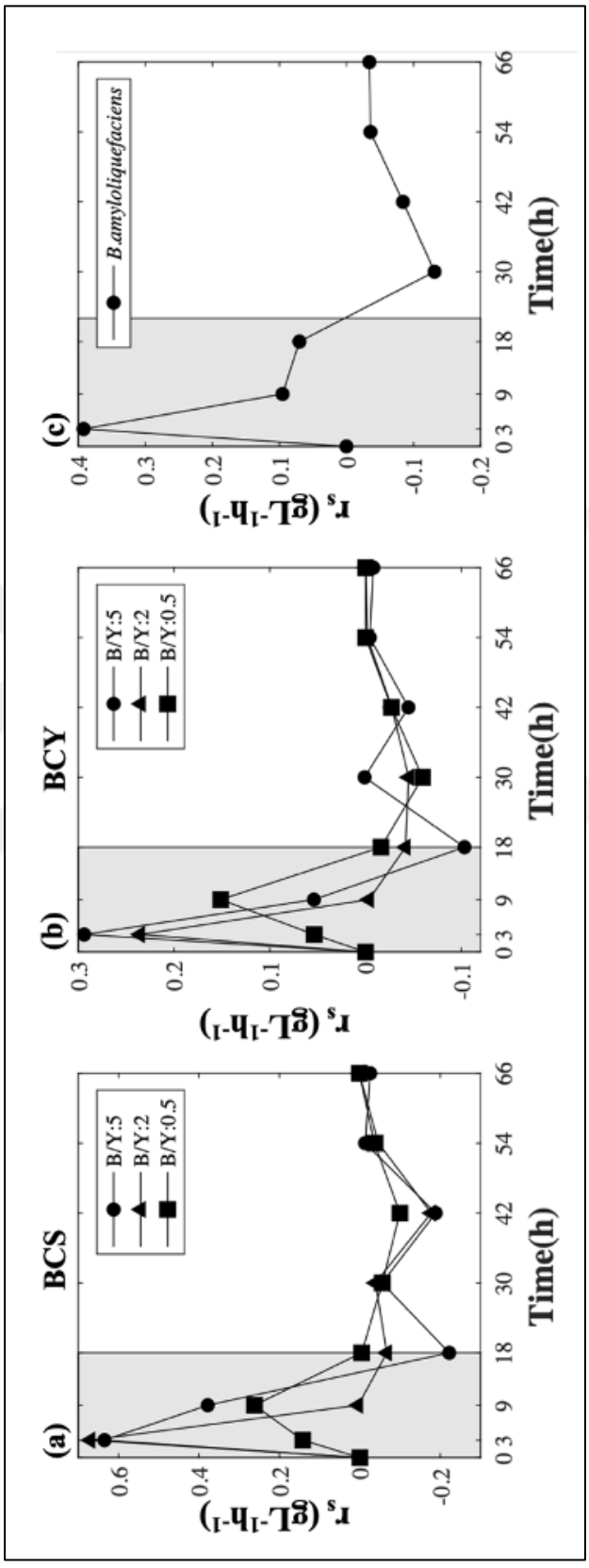

Figure 4.6. Comparison of volumetric starch production/consumption rates r_s ($gL^{-1}h^{-1}$)
(a) BCS, (b) BCY, (c) their corresponding single culture of *B.amyloliquefaciens*

4.3. STATISTICAL OPTIMIZATION

4.3.1. Optimization of The Extracellular Enzyme Cocktail Production In Co-culture

The experimental results of the CCD design including twenty different compositions of the co-culture setups of BCS were fitted by use of second-order polynomial equation using with analysis of multiple regression. Table 4.3. indicates the experimental and predicted enzyme activities in the cocktail obtained from the analyses conducted during these intervals.

The maximum and minimum enzyme activities were determined as in the 8^{th} and 19^{th} experiments for protease; in the 17^{th} and 19th experiments for amylase; in the 20^{th} and 5^{th} experiments for lipase, respectively. However, the predicted minimum activity for amylase was in the 3rd experiment. Because of the factor of B/Y (X_3) being set to the axial point (-1.4), which means that the initial bacterium ratio act as amylase producer is zero in experiment 19^{th}. In addition, the model was unable to predict lipase activity in the 17^{th} and 18^{th} experiments because the olive oil concentration (X_2) was at the axial points.

In regression statistics, analysis of variance (ANOVA) with full quadratic polynomial models of each enzyme are given in Tables 4.4., 4.5., and 4.6. Multiple determination coefficients (R^2) for three responses (protease, amylase, and lipase) were found as 0.665, 0.583, and 0.752, respectively. In other words, values of R^2 indicate that the model was unable to explain 33.5 percent for protease, 41.7 percent for amylase, and 24.8 percent for lipase of the total variation in the system. Additionally, adj-R^2 values were obtained as 0.363 for protease, 0.208 for amylase, and 0.530 for lipase confirmed the significance of the models. The higher adj-R^2 values show that the model was generated with sufficient data, and further R^2 and adj-R^2 values should be close to one another for a decent model [119]. As a matter of fact that multiple correlation constants were found to be 0.815 for protease, 0.764 for amylase, and 0.867 for lipase indicates that the second-order model equation is adequate for this system under the given experimental domain. The standard errors of the system were further found as 0.075 for protease, 0.864 for amylase, 0.351 for lipase. Hence, noise levels inflicted by enzyme activity measurements are considered for this system.

Table 4.3. Experimental design and results with the three-factor, five-parameter system for production of enzyme cocktail in co-culture, nominal values are described in parenthesis (Prt: Protease, Amy: Amylase, Lip: Lipase)

Run Order	**Levels of Variables**			**Observed Response (Uml^{-1})**			**Predicted Response (Uml^{-1})**		
	X_1	X_2	X_3	***Prt***	***Amy***	***Lip***	***Prt***	***Amy***	***Lip***
1	-1 (5)	-1 (1)	-1 (0.5)	0.584	1.685	0.655	0.542	1.365	0.815
2	1 (15)	-1 (1)	-1 (0.5)	0.648	1.716	0.601	0.602	1.595	0.594
3	-1 (5)	1 (5)	-1 (0.5)	0.572	0.704	0.474	0.506	0.457	0.646
4	1 (15)	1 (5)	-1 (0.5)	0.611	1.598	0.485	0.569	1.477	0.650
5	-1 (5)	-1 (1)	1 (5)	0.599	1.519	0.402	0.595	1.660	0.570
6	1 (15)	-1 (1)	1 (5)	0.637	1.226	0.560	0.647	1.320	0.721
7	-1 (5)	1 (5)	1 (5)	0.620	1.351	0.586	0.626	1.457	0.675
8	1 (15)	1 (5)	1 (5)	0.680	1.080	0.918	0.676	1.550	0.984
9	0 (10)	0 (3)	0 (2)	0.530	1.632	0.618	0.493	1.686	0.733
10	0 (10)	0 (3)	0 (2)	0.533	1.582	0.662	0.493	1.686	0.733
11	0 (10)	0 (3)	0 (2)	0.516	1.893	0.707	0.493	1.686	0.733
12	0 (10)	0 (3)	0 (2)	0.491	1.803	0.653	0.493	1.686	0.733
13	0 (10)	0 (3)	0 (2)	0.505	1.721	0.560	0.493	1.686	0.733
14	0 (10)	0 (3)	0 (2)	0.528	1.649	0.668	0.493	1.686	0.733
15	-1.4 (3)	0 (3)	0 (2)	0.538	0.884	0.814	0.592	1.059	0.417
16	1.4 (17)	0 (3)	0 (2)	0.628	1.541	0.647	0.666	1.479	0.443
17	0 (10)	-1.4 (0.2)	0 (2)	0.518	1.766	0.218	0.562	1.892	$0.475i$
18	0 (10)	1.4 (5.8)	0 (2)	0.484	1.718	0.515	0.546	1.626	$0.415i$
19	0 (10)	0 (3)	-1.4 (0)	0.142	0.019	1.231	0.423	1.355	1.138
20	0 (10)	0 (3)	1.4 (6)	0.558	1.744	1.592	0.547	1.500	1.333

Also, the effect of the initial inoculum ratio of each strain of the consortium was mentioned on enzyme secretion in this study. However, since the individual growth profiles of partners as a result of microbial interaction are unknown, considering that B/Y affects the system's total variation may be another explanation of the low value of the multiple correlation coefficient.

Table 4.4. ANOVA from regression for protease production obtained using CCD

	df	**SS**	**MS**	**F**	**Significance F**
Regression	9	0,112	0,012	2,205	0,117
Residual	10	0,057	0,006		
Total	19	0,169			

Table 4.5. ANOVA from regression for amylase production obtained using CCD

	df	**SS**	**MS**	**F**	**Significance F**
Regression	9	10.453	1.161	1.555	0.250
Residual	10	7.468	0.747		
Total	19	17.921			

Table 4.6. ANOVA from regression for lipase production obtained using CCD

	df	**SS**	**MS**	**F**	**Significance F**
Regression	9	3.754	0.417	3.376	0.036
Residual	10	1.235	0.124		
Total	19	4.990			

The regression coefficients and their significance levels for each enzyme of full quadratic polynomial models are given in Table 4.7., 4.8., and 4.9. The statistical significance of equation terms was evaluated based on t-values and P-values. The greater t-value and the smaller the P-value magnitudes mean that the more significant the corresponding coefficient [126].

Table 4.7. Parameters and significance levels found for the production of protease (y_1) in enzyme cocktail in co-culture

	Parameter Estimate	**P-value**	**Std Error**	**t Stat**
$\boldsymbol{\beta_o}$	0.534	0.007	0.158	3.376
$\boldsymbol{\beta_1}$	-0.056	0.038	0.023	-2.398
$\boldsymbol{\beta_2}$	-0.061	0.258	0.051	-1.199
$\boldsymbol{\beta_3}$	0.026	0.597	0.048	0.546
$\boldsymbol{\beta_{11}}$	0.003	0.013	0.001	3.033
$\boldsymbol{\beta_{22}}$	0.008	0.236	0.007	1.261
$\boldsymbol{\beta_{33}}$	-0.003	0.633	0.006	-0.492
$\boldsymbol{\beta_{12}}$	-0.000	0.994	0.003	-0.007
$\boldsymbol{\beta_{13}}$	-0.000	0.977	0.002	-0.029
$\boldsymbol{\beta_{23}}$	0.004	0.484	0.006	0.728

Table 4.8. Parameters and significance levels found for the production of amylase (y_2) in enzyme cocktail in co-culture

	Parameter Estimate	**P-value**	**Std Error**	**t Stat**
$\boldsymbol{\beta_o}$	0.126	0.946	1.816	0.070
$\boldsymbol{\beta_1}$	0.515	0.085	0.269	1.914
$\boldsymbol{\beta_2}$	-0.856	0.171	0.581	-1.474
$\boldsymbol{\beta_3}$	0.992	0.101	0.549	1.807
$\boldsymbol{\beta_{11}}$	-0.023	0.083	0.012	-1.927
$\boldsymbol{\beta_{22}}$	0.042	0.585	0.075	0.564
$\boldsymbol{\beta_{33}}$	-0.123	0.111	0.070	-1.749
$\boldsymbol{\beta_{12}}$	0.032	0.316	0.031	1.057
$\boldsymbol{\beta_{13}}$	-0.038	0.189	0.027	-1.411
$\boldsymbol{\beta_{23}}$	0.057	0.415	0.067	0.851

Table 4.9. Parameters and significance levels found for the production of lipase (y_3) in enzyme cocktail in co-culture

	Parameter Estimate	P-value	Std Error	t Stat
$\boldsymbol{\beta_o}$	0.275	0.718	0.739	0.372
$\boldsymbol{\beta_1}$	0.099	0.386	0.109	0.907
$\boldsymbol{\beta_2}$	0.452	0.085	0.236	1.914
$\boldsymbol{\beta_3}$	-0.784	0.006	0.223	-3.511
$\boldsymbol{\beta_{11}}$	-0.007	0.169	0.005	-1.482
$\boldsymbol{\beta_{22}}$	-0.094	0.011	0.030	-3.099
$\boldsymbol{\beta_{33}}$	0.115	0.003	0.029	4.013
$\boldsymbol{\beta_{12}}$	0.008	0.537	0.012	0.639
$\boldsymbol{\beta_{13}}$	0.011	0.325	0.011	1.036
$\boldsymbol{\beta_{23}}$	0.021	0.457	0.027	0.774

The empirical relationship between responses (protease, y_1; amylase, y_2; and lipase y_3) and the variables investigated using the following equation:

$$y = \beta_o + \beta_1 * Starch + \beta_2 * Oil + \beta_3 * InoculumRatio + \beta_{11} * Starch^2 + \beta_{22} * Oil^2 + \beta_{33} * InoculumRatio^2 + \beta_{12} * Starch * Oil + \beta_{13} * Starch * InoculumRatio + \beta_{23} * Oil * InoculumRatio \quad (4.1)$$

Where y is the response, $\beta_1, \beta_2, \beta_3$ linear terms; $\beta_{11}, \beta_{22}, \beta_{33}$ quadratic terms; $\beta_{12}, \beta_{13}, \beta_{23}$ interaction terms.

Generally, when P-values are less than 0.050, the model terms are significant, whereas values more than 0.100 suggest that the model terms are insignificant [119]. Out of three responses that were examined; intercept (constant term) effect, linear effect of starch (P = 0.037) and quadratic effect of starch (P = 0.012) are significant for protease secretion (Table 4..), linear effect of starch (P = 0.085) and quadratic effect of starch (P = 0.083) are significant for amylase secretion (Table 4.8. .) with a CI: 90 percent, and linear of oil (P = 0.085), the quadratic effect of oil (P = 0.011) with a CI: 90 percent, and the linear main effect of B/Y initial inoculum ratio of bacterium-to-total yeasts (P = 0.006), the quadratic main effect of B/Y (P = 0.002) with a CI: 95 percent are found to be significant for lipase secretion (Table 4.9. . Certain terms in the second-order model may fail to describe the system due to random errors, as their high P-values imply their statistical insignificance.

Hence, to develop the present model in terms of evaluating the certain term effects, the number of factors that affect the corresponding enzyme cocktail production in certain conditions can be expanded. In addition, numerous publications exist on optimizing the medium components and physical parameters of growing conditions for the synthesis of protease, amylase, and lipase enzymes both separately and together [45,98,109,126–128]. However, studies are very scanty in the literature on the optimal concomitant production of these enzymes in a single medium by a microbial consortium.

Response surfaces are three-dimensional (3-D) representations of the independent factor effects on dependent responses (response values on gradual color from blue to yellow). Figure 4.7. illustrates the plots of response surfaces for enzyme cocktail secretion in BCS.

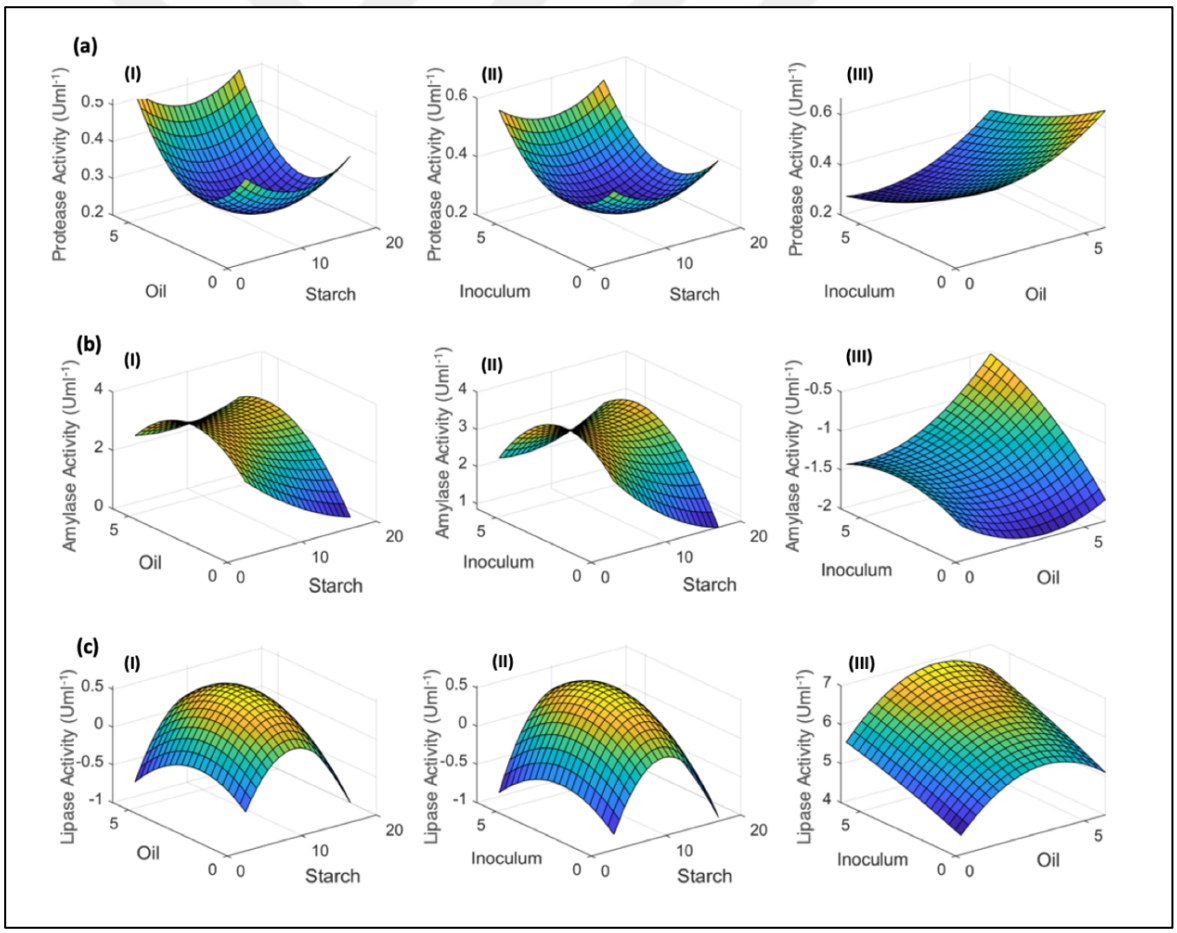

Figure 4.7. Response surface plots for enzyme activities (UmL^{-1}), (a) Protease Activity, (b) Amylase Activity, (c) Lipase Activity in the cocktail as functions of starch and oil concentration, and initial Bacterium-to-total Yeasts ratio (B/Y) in a co-culture of BCS

Figure 4.7. (a) demonstrates, surface plots of protease activity responding to oil and starch concentration (I), initial inoculum ratio and starch concentration (II) had a convex shape, which is a bowl-shaped, and initial inoculum ratio and oil concentration (II) showed an inverse relationship. As obvious in Figure 4.7. (b), the surfaces were saddle, which as the oil concentration (I) and initial bacterium ratio (II) increased, the amylase activity is first increased then began to decrease when the starch concentration is kept constant. On the other hand, the plot of inoculum ratio and oil concentration (III) did not demonstrate any tendency, and the surface plot indicated a negative curvature. As illustrated in Figure 4.7. (c), the responses obtained were concave (I, II) which is dome-shaped, implying that well-defined optimum operating conditions existed for lipase activity in determined manipulated factors.

4.3.2. Optimization of Responses and Model Validation

Enzyme cocktail were optimized with parameters of second-order polynomials using the method of the Nelder Mead Simplex [86,97], which is based on a black-box optimization method with real parameters that work well with irregular goal functions, with the nominal design in the predetermined experimental factor range (Table 4.3.). The optimum conditions (

Table 4..) were determined by three sets for maximizing each enzyme; protease (P_{Max}), amylase (A_{Max}), and lipase (L_{Max}). Furthermore, to maximize the cocktail based on each enzyme activity (C_{Opt}), optimum conditions were determined based on the level of the factors of CCD with the following equation that was obtained from the ratio of enzyme activities within the experimental design for maximizing each enzyme in the cocktail:

$$y_{Opt} = 3.43*Protease + 1.00*Amylase + 2.43*Lipase \quad (4.2)$$

Table 4.10. Optimum conditions and results for enzyme cocktail production (P_{Max}: Maximizing of protease, A_{Max}: Maximizing of Amylase, L_{Max}: Maximizing of Lipase in the cocktail, C_{Opt}: Enzyme cocktail, Prt: Protease, Amy: Amylase, Lip: Lipase)

	Factors			**Predicted Activities (UmL^{-1})**			**Observed Activities (UmL^{-1})**		
	Starch (gL^{-1})	***Oil (gL^{-1})***	***(B/Y)***	***Prt***	***Amy***	***Lip***	***Prt***	***Amy***	***Lip***
P_{Max}	17.00	0.05	4.20	0.74	1.34	0.00	0.64	1.29	0.00
A_{Max}	9.03	0.05	2.71	0.57	1.92	0.00	0.59	1.91	0.00
L_{Max}	8.42	2.76	0.05	0.43	1.29	1.13	0.11	0.00	1.16
C_{Opt}	17.00	3.00	3.57	0.70	1.40	0.61	0.62	1.57	0.58

All models proposed different amounts of starch, oil, and initial inoculum ratio in different enzyme cocktail strategies. The amount of oil, which is a lipase inducer, was kept to a minimum for P_{Max} and A_{Max}; while lipase was maximized (L_{Max}), *B.amyloliquefaciens*, which is the producer of all three enzymes, was kept to a minimum in the initial inoculum (B/Y) ratio.

To check the capability of model estimation and to verify the enzyme cocktail activities, four sets of validation experiments (P_{Max}, L_{Max}, A_{Max}, C_{Opt}) were carried out at conditions given in Table 4.9. Enzyme activities obtained at 72 h of fermentation were compared with the model activities. As obvious, the four models have a strong predictive capability. Despite the presence of noise, four models could frequently estimate the optimum medium composition for their associated enzyme activities. The observed activities of protease and amylase in the L_{Max} were lower than their predicted activities. This limitation was because of the low initial bacterium ratio which acts as the protease and amylase producer in the consortium. The noise of other enzyme activities between observed and model prediction values is contained within the experimental error range determined from the experimental design (Table 4.2.).

In Figure 4.8. (a), maximum biomass concentration was obtained in L_{Max} with a yield of biomass to total starch and olive oil consumption Yx/s 0.34 gg^{-1} and followed by AMax (0.24 gg^{-1}) and P_{Max} (0.21 gg^{-1}). Additionally, P_{Max} had the highest reducing sugar level,

followed by A_{Max} (Figure 4.8. (b)). Also, *B.amyloliquefaciens* was unable to hydrolyze starch due to its low initial inoculum ratio at L_{Max}, and parallel to this, both amylase and protease activities (Figure 4.8. (c) and (d)) could not be obtained concerning their predicted activities. In addition, the duration for maximum amylase and lipase (Figure 4.8. (e)) production (48 h) was regressed to 24 h. In addition to the validation experiment of C_{Opt}, single cultures of the consortium members were performed under optimum conditions of C_{Opt} (Figure 4.9.). The biomass concentration of *B.amyloliquefaciens* (Figure 4.8. (a)) with a yield of biomass to total starch and olive oil consumption $Y_{x/s}$ 0.31 gg^{-1} was obtained higher than its single-fermentation given in Figure 4.2. Furthermore, the yields of biomass to total starch and olive oil consumption for C_{Opt} and *C.rugosa* were found to be $Y_{x/s}$ 0.17 gg^{-1} and 1.00 gg^{-1}, respectively. However, there was no significant increase in amylase secretion.

Hence, hydrolysis of a higher amount of starch is caused by the presence of amylase [129]. During balanced microbial growth, a rise in components such as DNA, RNA, and proteins are generated by the cells based on the same rate as cell number. As a result, it becomes critical to quantify any of these components and to ascertain the growth profile, which is reliant on the original population of inoculation. A cell produces an enzyme at a steady rate that is directly proportionate to the cell count and growth rate during microbial growth. However, integration of the inducers in the media under the optimum conditions leads to the secretion of the enzymes by each of the associated strains in the consortium. As a result, the amount of enzyme secreted per cell increases and then stabilizes at a value specified by growth. However, because the initial inoculum ratios varied among strains, the microbial growth could not be compared in terms of improved enzyme secretion [130]. Additionally to the effect of co-culture on the profile of reducing sugar consumption (Figure 4.9. (b)), this observation is proof of the existence of synergism between *S. cerevisiae* and other populations present in the consortium. It can be concluded that according to co-culture and its single cultures which carried out under their optimum conditions; 1.14-fold increase in protease activity and 3.46-fold increasing in amylase activity was observed, and 1.79-fold and 1.34-fold decreasing was observed in lipase activity compared to the total enzyme activity of single cultures (*B.amyloliquefaciens* and *C.rugosa*) due to substrate competition, respectively.

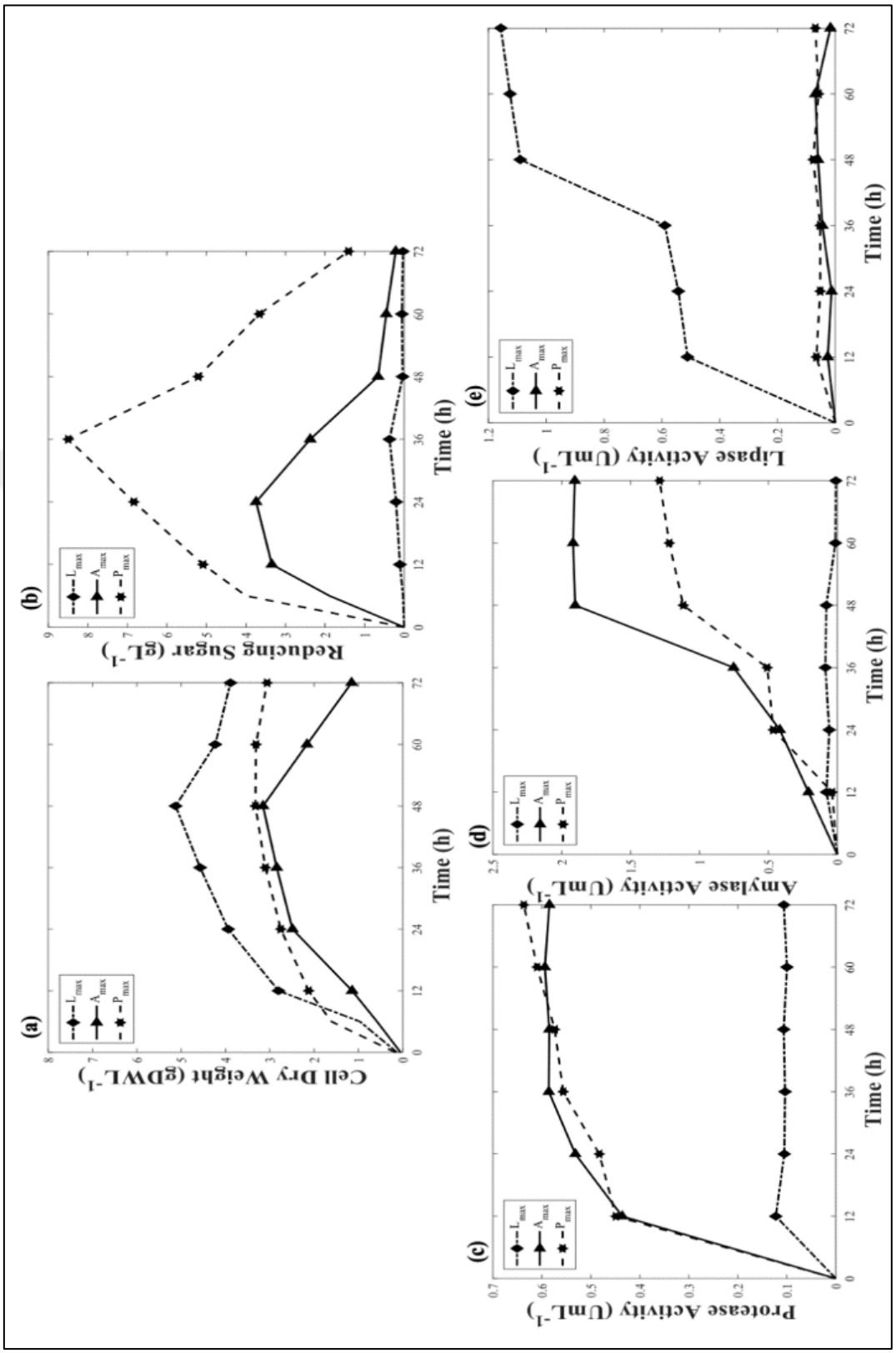

Figure 4.8. BCS batch profiles of validation experiments given in optimum conditions. (a) Biomass Concentrations ($gDWL^{-1}$), (b) Reducing sugar (gL^{1}), (c) Protease Activity (UmL^{-1}), (d) Amylase Activity (UmL^{-1}), (e) Lipase Activity (UmL^{-1})

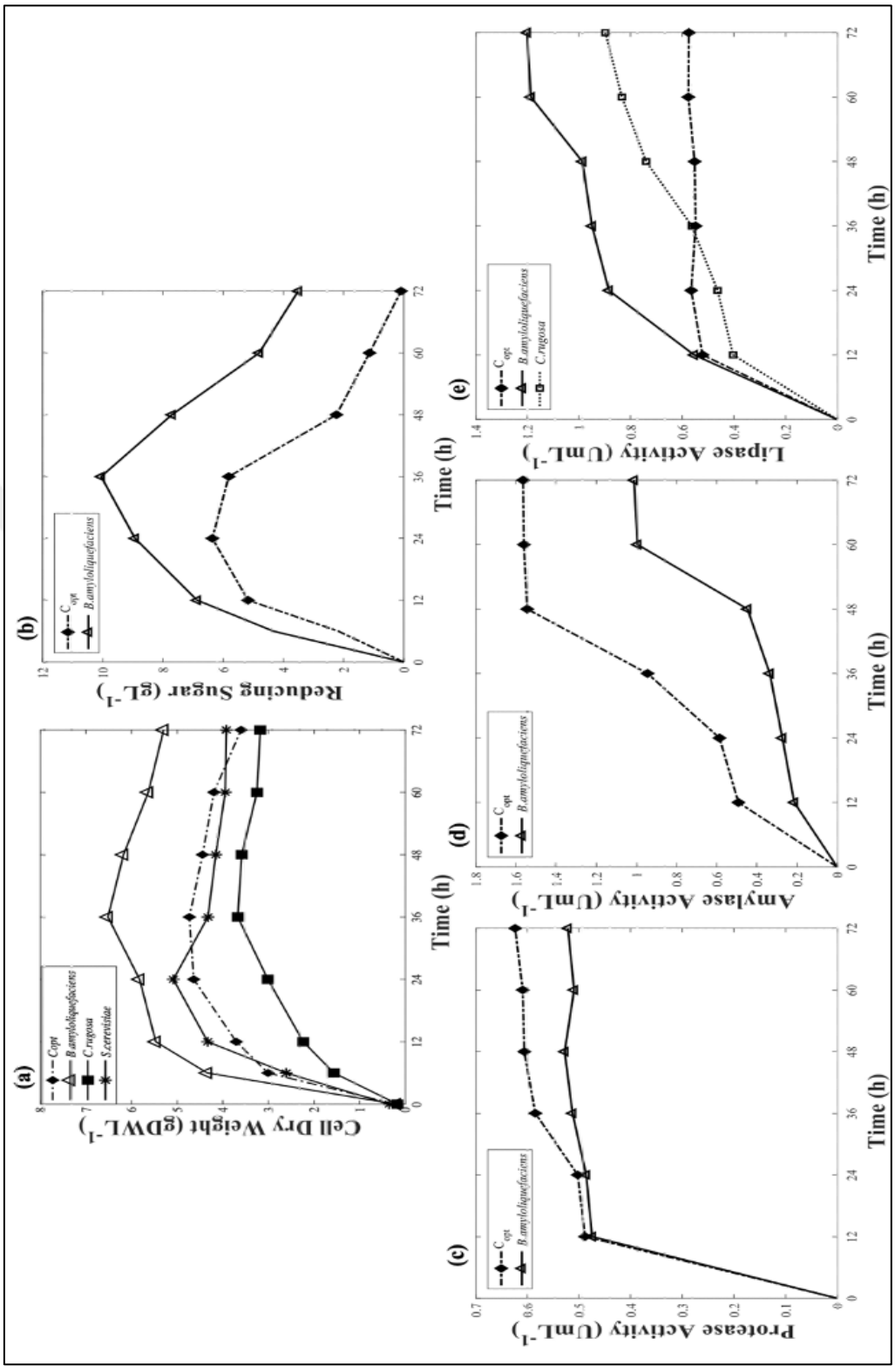

Figure 4.9. BCS batch profiles of validation experiment C_{Opt} and its related single cultures given in optimum conditions. (a) Biomass Concentrations ($gDWL^{-1}$), (b) Reducing sugar (gL^{1}), (c) Protease Activity (UmL^{-1}), (d) Amylase Activity (UmL^{-1}), (e) Lipase Activity (UmL^{-1})

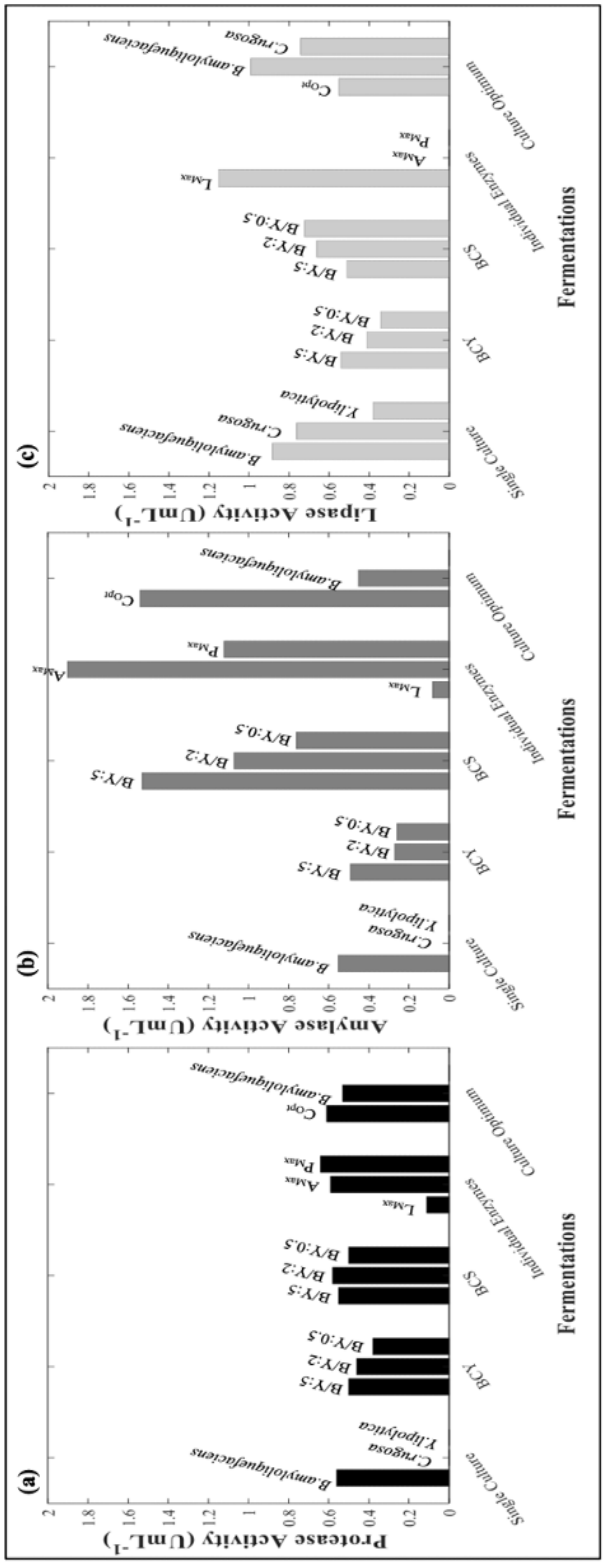

Figure 4.10. Comparison of enzyme activities of single cultures, co-cultures, and validation experiments at 48 h of fermentation. (a) Protease Activity (UmL^{-1}), (b) Amylase Activity (UmL^{-1}), (c) Lipase Activity (UmL^{-1})

Figure 4.10. indicates a comparison of all enzyme activities at 48 h of fermentation. BCS did not compromise from protease activities in enzyme cocktail production (Figure 4.10. (a)), the maximum amylase activity was found in A_{Max} which was increased 3-45-fold, and increased 2.78-fold in BCS (B/Y:5) from the single culture of *B.amyloliquefaciens* (Figure 4.10. (b)), the maximum lipase activity was found in L_{Max} which was increased 1.51-fold from the single culture of *C.rugosa* despite the substrate competition.

5. CONCLUSION AND FUTURE STUDY

This thesis evaluates to a novel approach that is effects of two different artificial bacteria-yeast consortia, which were defined as BCY and BCS, on enzyme cocktail production including protease, amylase, and lipase. Furthermore, fermentation conditions were optimized by using Response Surface Methodology with Central Composite Design based on the second-order polynomial model for enzyme cocktail production by artificial microbial consortium. The design of an artificial microbial consortium is a key step for the overproduction of related enzyme cocktail. The consortium BCY was described as a growth-synergistic consortium, whereas BCS was both growth-synergistic and amylase-synergistic consortium. Due to the synergism of consortium BCS, the fermentation period was regressed to 24 h when related enzyme activities and the profile of reducing sugar consumption were considered. Also, amylase activity increased due to the synergism of BCS, substrate competition from olive oil in the consortium affected enzyme production, negatively, and protease activities did not decrease in the cocktail. Also, statistical optimization further improved fermentation performance (despite the substrate competition) in terms of amylase and lipase activities based on optimization of carbon sources (starch and olive oil) and initial inoculum ratio of consortium based on bacterium-to total yeast (B/Y).

To be extended in this thesis, metabolites, and by-product profiles such as ethanol, glycerol can be examined. The consumption profile of olive oil used as an inducer of lipase production can be determined. The parameters used in the experimental design can be evaluated in terms of full medium composition (nitrogen sources and cofactors). In addition, the individual profiles of consortium members can be evaluated to understand their interactions during the fermentation period. To eliminate substrate competition, a synthetic or semi-synthetic microbial consortium design can be resorted (deleting the lipase gene in bacterium).

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