PRODUCTION AND OPTIMIZATION OF POLY γ-GLUTAMIC ACID FROM *BACILLUS SUBTILIS* USING RICE HUSK BY SOLID STATE FERMENTATION

BY

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER DEGREE IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,
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APRIL, 2021

DECLARATION

I declare that the work in this dissertation entitled "Production and Optimization of Poly γ-Glutamic Acid from *Bacillus subtilis* using Rice Husk by Solid State Fermentation" has been performed by me in the Department of Biochemistry, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

STEPHEN DIO TITUS _____ _____

Name of Student Signature Date

CERTIFICATION

This dissertation entitled PRODUCTION AND OPTIMIZATION OF POLY γ-GLUTAMIC ACID FROM *BACILLUS SUBTILIS* USING RICE HUSK BY SOLID STATE FERMENTATION by Stephen Dio TITUS meets the regulations governing the award of the degree of Master of Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Poly-γ-glutamic acid (γ-PGA) is an anionic polyamino acid composed of L and D glutamic acid isomers linked via amide bond between the α-amino and γ-carboxylic groups of adjacent monomers largely synthesised by microorganisms of the Genus *Bacillus.* γ-PGA has been explored in the design of scaffolds, nanosystems, adhesives, hydrogels, thickeners and humectants because of its non-toxicity, non-immunogenicity, water solubility and biodegradability. This study was undertaken to optimize the production of γ-PGA from *Bacillus subtilis* using rice husk by solid state fermentation. Different *Bacillus subtilis* were isolated from water, soil and *Daddawa* samples collected in Samaru, Zaria, Nigeria and used for the production of γ-PGA. The isolates were characterised by biochemical and molecular techniques and then subjected to screening and production optimization by central composite design of response surface methodology. Among the 3 isolates (S1, S4 and S5), S5 has a significantly higher yield of 2.5 g/L of γ-PGA, hence was used in solid state fermentation for the optimization and production of γ-PGA using rice husk as the basal media. Exactly eleven (11) media components and process parameters were screened using definitive screening design (DSD) with citric acid, glycerol and 6-diazo-5-oxo-L-norleucine (DON) having the most significant influence on the yield which were optimized by response surface methodology using central composite design (CCD). A maximum production of γ-PGA of 19.55±5.75 mg/g and molecular weights within the range of 35 kDa to 96 kDa bands by SDS-PAGE were obtained when the concentrations of medium components were set at 25 μg/100g DON, 7.5% citric acid and 9.96% glycerol. Hence, *B. subtilis* isolate S5 is a promising strain for the production of low molecular weight γ-PGA from rice husk via solid state fermentation.

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ABBREVIATIONS, DEFINITIONS, GLOSSARY AND SYMBOLS

CFU: Colony forming unit.

CwlO: γ-glutamyl-hydrolase gene.

CwlS: Peptidoglycan hydrolase genes.

DON: 6-Diazo-5-oxo-L-norluecine.

DSD: Definitive screening design.

FCCD: Full central composite design.

FT-IR: Fourier transform-Infra-red spectroscopy.

GGT1: Gamma-glutamyl Transpeptidase 1.

glpFK: Cytoplasmic membrane transporter of glycerol.

Glr: Glutamate receptor 1 gene.

mg/gds: Milligram per gram of dry substrate.

MOPS: 3-morpholinopropane-1-sulfonic acid.

OU749: is a patented three-ring sulfonated benzene derivative that can competitively inhibit γ-glutamyl transpeptidase.

pgs: poly-γ-glutamic acid synthase gene with the subunits designated as pgsA, pgsB, pgsC and pgsE.

RacE: Glutamate racemase gene.

S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11 and S12 are assigned symbols for the isolates obtained from the sample of soil, water and *Daddawa*.

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

*yrp*C: Glutamate racemase2 gene.

γ-GTP: Gamma glutamyl transpeptidase.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

The search for better ways to improve the standard of living through development of new and safer technologies in the area of enzymes and industrial biopolymers in the production of useful medical and pharmaceuticals drivers such as hydrogels, drugs carriers, gene delivery vehicles and super absorbents is in the centre stage of modern day research (Das *et al*., 2018; Ramazan, 2019). Poly γ-glutamic acid (γ-PGA) is one of such biopolymers (Zhang *et al.*, 2018b). Poly γ-glutamic acid is a polyamide, anionic biopolymer composed mainly of D-and L-glutamic acid units connected via amide linkages between α-amino and γ-carboxylic acid groups of the monomers (Hsueh *et al*., 2017). γ-PGA is classified into; γ-L-PGA (only L-glutamic acid residues), γ-D-PGA (only D-glutamic acid residues), and γ-LD-PGA (both L- and D-glutamic acid residues) based on the enantiomers of glutamic acid residues (Luo *et al.*, 2016).

γ-PGA is water-soluble, nontoxic, non-immunogenic and edible biopolymer. It also has adhesive, film forming, and moisture retention properties making it an interesting material for drug delivery/release, bio-adhesive, cosmetics, food, agriculture, and sewage treatment (Lin *et al*., 2016). It also has the potential to be used for protein crystallization, as a soft tissue adhesive and a non-viral vector for safe gene delivery (Ogunleye *et al.*, 2015).

There are four methods of γ-PGA production: chemical synthesis, peptide synthesis, biotransformation, and microbial fermentation (Hara *et al.*, 2014). Microbial fermentation is the most cost-effective and has numerous advantages, including use of inexpensive raw materials, minimal environmental pollution, high natural product purity, and mild reaction

conditions (Luo *et al.,* 2016). The microbial biosynthesis is comprised of racemization, polymerization, regulation, and degradation (Cai *et al.,* 2017). The racemization is an important step in γ-PGA biosynthesis with D-isomer as either homo (D-Isomer only) or co-polymer (D and L isomers mixed) (Hara *et al.,* 2014).

γ-PGA microbial fermentation is exclusively of gram-positive bacteria (genus: Bacillus, class: Bacilli) except for recombinant *E. coli*. γ-PGA producing microorganisms are classified based on their glutamic acid requirement for γ-PGA biosynthesis; some require exogenous glutamic acid which must be supplied in the growth medium. Members of this class are termed glutamate-dependent, while the other class does not require the supply of glutamic acid in the growth medium due to their ability to synthesize glutamic acid using tricarboxylic acid (TCA) cycle intermediates (Lin *et al.,* 2016). The former predominates most of the known PGA producers, such as *Bacillus subtilis* chungkookjang, *B. licheniformis* ATCC9945A, *B. subtilis* (natto) IFO333 and *B. subtilis* RKY3. The glutamate independent γ-PGA producers include *B. subtilis* C1, *B. subtilis* TAM-4, *B. licheniformis* A35, *and B. licheniformis* SAB-26 (Songa *et al.,* 2010).

Unlike most proteinaceous materials, γ-PGA is synthesized in a ribosome-independent manner; thus, substances that inhibit protein translation have no inhibitory effects on the production of γ-PGA. Furthermore, due to the γ-linkage of its component glutamate residues, γ-PGA is resistant to proteases that cleave α-amino linkages (Luo *et al.,* 2016). The bulk of γ-PGA is synthesized at the onset of the stationary growth phase due to nutrient starvation/limitation during this phase (Kimura *et al.,* 2004b; Hsueh *et al.,* 2017).

Bacillus subtilis has the property of producing a great number of various industrial enzymes and biopolymers (Gu *et al.,* 2018).

There are five genes identified to be involved in γ-PGA synthesis in *B. subtilis*. i.e, *pgsB, pgsC, pgsA, pgsE* and *pgdS* (Bajaj and Singhal, 2011). *pgsBCA* operon was identified as the sole machinery handling the polymerizing γ-PGA at the active site of the synthase complex (*PgsBCA*) which is ATP-dependent (Kimura *et al.,* 2004). Two signal transduction systems have been implicated in the down and up regulation of *pgs* operon (ComP-ComA regulator and DegS-DegU, DegQ, and SwrA system). Alteration of degQ alters the synthesis of γ-PGA and also down-regulates the production of degradation enzymes. Exo-γ-glutamyl transpeptidase (γ-GTP) as well as endo-γ-glutamyl transpeptidase (α-GTP) are two enzymes identified to be responsible for degrading synthesized γ-PGA. The first enzyme was implicated in the exogenous breakdown of stored γ-PGA in *B. subtilis* so that it can be used as carbon and nitrogen source (Kobayashi, 2008) usually expressed during the stationary phase under the control of the ComQXPA quorum-sensing system (Mordini *et al.,* 2013). Inhibiting or knocking down γ-PGA hydrolase γ-GTP) can result in better yield with high molecular weight. It has been established that *B. subtilis* strains deficient in γ-GTP were unable to cleave γ-PGA and they sporulates earlier than wild-type strains (Kobayashi, 2008).

Optimization techniques, especially the response surface methodology are statistical techniques used in the design and simulation of experiments (Kimura and Fujimoto, 2014). A lot of biomass has been used such as Cassava peels (John *et al*., 2020), cow dung (Yong *et al*., 2011), Bagasse (Feng *et al*., 2014b), Sago (Mahanraj *et al*., 2019), Soy hull (Anju *et al*., 2018) among others in the production of γ-PGA using different production microorganism.

Rice is a staple food for more than half of the world's population. It is estimated globally that rice husk constitute more than 20% of the over 500 million tons of paddy produced (Runge *et al*., 2019). With Nigeria having an estimated production of 4.435 million tons annually and projected to increase at the rate 9.07% annually (Knoema, 2021) As a result of the volume of rice produced annually, husks generated from the milling of rice are eventually becoming a nuisance in major dumping sites (Chauhan *et al.,* 2017). Rice husk is a high potential substrate, which is amenable for value addition. It contains about 34-62% CHO, 15-20% fat, 11-15% Protein, 7-10% ash 7-11% fibre (Mohammed *et al.*, 2017; Song *et al*., 2019). Despite the enormous potential that could be tapped, most of the husk from rice milling is either burnt or dumped as waste in open fields and a small amount is used as fuel for boilers, electricity generation and bulking agents for composting of animal manure (Mohammed *et al.,* 2017).

1.2 Statement of Research Problem

Currently used materials for drug and gene delivery (viral particles and liposomes) have significant side effects (Thomas *et al.,* 2003; Hughes, 2017) and are also susceptible to degradation by enzymes (Gaber *et al.,* 2017; Wen *et al.,* 2017). γ-PGA on the other hand promises to be a more suitable candidate, but its application is limited by high cost of production from recombinant DNA technology as well as the raw materials required in the fermentation(Meerak *et al*., 2008). Although microbial production of γ-PGA has since been established, the cost of production, which ultimately affects the market price (i.e. ₦110,000.00 per 100mg high-purity sodium salt γ-PGA (Sigma Aldrich, 2019)) is still very high and this is one of the major limitations to the widespread application of this polymer. Based on this, most researches on microbial production of γ-PGA focused on the

optimization of growth conditions with the hope of obtaining a high yield, specific enantiomeric composition and desired molecular mass of γ-PGA at reduced cost (Ogunleye *et al.,* 2015; Ajayeoba *et al.,* 2019).

1.3 Justification

Considering the cost of fermentation of γ-PGA, there is need to employ the use of cheap and readily available biomass for its microbial fermentation which in a way will serve the dual purpose of bioremediation to minimize environmental pollution at the same time generate microbial products of commercial importance (Bankar *et al.,* 2018).

The knowledge of the enzymes and genes involved in γ-PGA production is important not only in increasing the production with respect to reducing cost, but also helps to understand the mechanism of γ-PGA applications (Ogunleye *et al.,* 2015).

Inhibiting the activity of γ-GTP during γ-PGA biosynthesis could improve the final yield consequent from reports that alteration of degQ gene results in low activity of γ-GTP (Stanley and Lazazzera, 2005). This could result in higher yields of γ-PGA with specific properties in contrast to the normal fermentation (Ogunleye *et al*., 2015). Also, optimization of γ-PGA with respect to cost of production, molecular weight and its conformational/enantiomeric properties is a major step in making its application practical Sharma *et al.,* 2017).

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study was to optimize the production of γ-PGA from *B. subtilis* via solid-state fermentation (SSF) using rice husk.

1.3.2 Specific Objectives of the Study

The specific objectives of the study were to:

i. Isolate and characterize *B. subtilis* from soil, water and *Daddawa*.

ii. Screen and select promising isolate for γ-PGA production.

iii. Dock 6-diazo-5-oxo-L-norluecine (DON) against γ-GTP and determine the minimal inhibitory concentration (MIC) of DON on *B. subtilis.*

iv. Optimize the medium and process parameters for the production of γ-PGA by response surface methodology.

v. Characterize the produced γ-PGA.

CHAPTER TWO

2.0 LITERATURE REVIEWS

2.1 Discovery and History of γ-PGA

Poly-γ-glutamic acid is a multifarious biopolymer, (Bajaj and Singhal, 2011) and one of the emerging biopolymers that is currently in the centre stage of active investigation. This is because of its desirable properties such as non-immunogenicity, non-toxicity, high retention and solubility in water as well as availability of functional groups for attachment of other bioactive species (Pacini *et al.*, 2012). So much has been done to understand the biogenesis and genes involved in its synthesis, regulation, degradation, industrial production and applications in various fields (Anju *et al.*, 2018), but more investigations need to be done to fully establish the mechanism of production, optimal conditions of production especially from cheap renewable sources before a substantial increase in its microbial productivity and full industrial exploitation can be achieved (Khalil *et al.*, 2016).

Ivanovics and Bruckner (1937), where the first to report the presence of γ-PGA in the capsule of *Bacillus anthracis* but they were only able to isolate and report its composition, though the research was a ground breaking one into an unending possibilities from γ-PGA (Mohkam *et al.*, 2016). Later work by Bovarnick (1942) gave more insight to the chemical nature of the polymer (Gamarra, 2018). Working with *B. subtilis*, strain 41259 grown in a simple medium, Bovarnick (1942) established that the peptide is composed solely of L or D or both L and D glutamic acids in γ-linkages at the ω position. He also provided evidence in favour of peptide linkage formation via the ω-carboxyl groups of the glutamic acid and that the glutamic acid molecule skeleton can be used by the microorganism as a substrate for the formation of this peptide. Moreover, the reaction mechanism involves α-iminoglutaric acid

or its derivatives (Pearson, 2015). The mucilage of *Natto* and *Chungkookjang* (traditional Japanese and Korean delicacies, respectively, prepared from soybeans fermented with *Bacillus subtilis*) contains a mixture of poly gamma glutamic acid and fructan (Hsueh *et al.*, 2017).

Since the report of Bovarnick (1942), more *Bacillus* species were also discovered which produced γ-PGA extracellularly into the fermentation broth (Shih, 2001). Ashiuchi and Misono (2005) also suggested that PGA production may be achieved using Gram-positive *Bacilli* such as *B. anthracis* and the extremely halophilic archae, *Natrialba aegyptiaca*. These microorganisms produce PGA polymers of 10 to more than 1000 kDa with specific D/L-ratios (Ashiuchi and Misono, 2002).

In its primary physiologic sense, γ-PGA serves a range of purposes. Though its function in some microorganisms is not completely understood and is suggested to depend on the environment of the organism in question and whether it is bound to peptidoglycan or released into the extracellular environment (Luo *et al.*, 2016). Peptidoglycan bound γ-PGA is believed to protect bacterial cells against phage infections and prevent antibodies from gaining access to the bacterium (Luo *et al.*, 2016). Kocianova *et al*. (2005) reported that *Staphylococcus epidermidis* synthesizes surface-associated γ-PGA to protect them against antimicrobial peptides and escape phagocytosis thereby contributing to their virulence nature. For organisms that releases γ-PGA into the environment, it helps in sequestering toxic metal ions, decrease salt concentration, provide a carbon source, and protect against adverse conditions (Yan *et al*., 2015). γ-PGA can also improve the formation of biofilms and assist absorption of essential nutrients from the environment (Hezayen *et al*., 2001; Yan *et al*., 2015). The nematocysts of cnidarians also contain some amount of γ-PGA.

Weber (1990) reported that, 2.80±0.20 mM of anions found/mg of isolated and purified dry nematocysts (capsular secretory products of stinging cells) from Hydra contains PGA which contributes in the generation and regulation of an internal osmotic pressure that amounts to 160 bar. The degree of polymerization is heterogeneous and dependent on the particular type of nematocyst *in situ* with intracapsular glutamic acid monomer concentration as high as 2 M (Weber, 1990). This is the first time γ-PGA, which are known to occur in some selected bacteria, were reported in eukaryotes. It is suggested that they may also be present as predominant components in nematocysts of other *cnidarian* species and thus might represent a class of compounds which is characteristic for a whole phylum of the animal kingdom (Weber, 1990).

2.2 Mechanism of Biosynthesis of γ-PGA

For the proper exploitation of γ-PGA, the mechanism of its biosynthesis is paramount. Studies have been engaged to unravel the genes and enzymes involved in γ-PGA synthesis so as to pave a way for metabolic manipulation of γ-PGA producing organisms. These studies have contributed immensely in the design of production systems (Candela and Fouet, 2006; Buescher and Margaritis, 2007 and Ogunleye *et al*., 2015).

The understanding of the pathway of γ-PGA biosynthesis in *B. subtilis* has been studied by a number of researchers. The pathway of γ-PGA biosynthesis was first proposed by Shih and Van (2001) to be majorly a transglutaminase reaction with glutamic acid as the limiting factor for high yield γ-PGA production in *Bacillus subtilis* (Shih and Van, 2001). A glutamyl compound acting as acceptor of the incoming glutamate resulting in the elongation of the glutamyl moiety by one glutamate after each step of the reaction via glutamine: α-ketoglutarate aminotransferase/glutamate synthase (GOGAT/GS) pathway

(Figure 2.1) generating the required glutamate or from exogenous sources for the biosynthesis of the γ-PGA (Guang-ming *et al*., 2014). The endogenous synthesis of glutamic acid endogenously actually can take place in two ways thus; in the absence of glutamine, L-glutamate is synthesized from α-ketoglutaric acid shunted from citric acid cycle and ammonium sulfate by glutamate dehydrogenase. The second alternative is the synthesis of the L-glutamine from α-ketoglutaric acid and L-glutamic acid by the action of glutamine synthetase pathway catalyzed by 2-oxoglutarate aminotransferase (Adebayo, 2018).

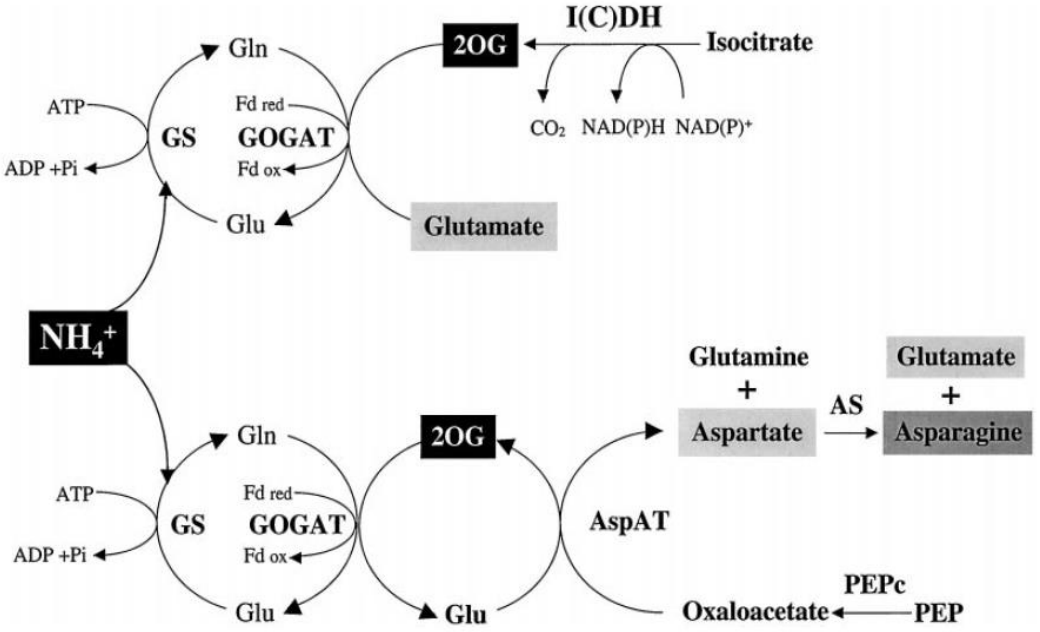

Figure 2.1: Mechanism of Glutamate Biosynthesis (Lancien *et al.*, 2000)

Shih and Van (2001) reported that glutamyl transamidase activity from crude extracellular extracts of *B. subtilis* 9945A. The glutamyl transamidase was believed to catalyze the transfer of glutamyl group from L-glutamine to glutamyl dipeptide or the optical isomer of L-glutamine (D-glutamine) resulting in the formation or elongation of the polymer chain (Ogawa *et al*., 1997; Shih and Van, 2001). Moreover a number of racemase enzymes were

identified in such extracellular fractions including alanine racemase and D- and L-glutamyl transaminases. On the basis of these evidences from various works, a mechanism was proposed for γ-PGA biosynthesis in *B. subtilis* that is ribosome independent but enzyme catalyzed (Feng *et al.*, 2007). The proposed pathway requires L or D or both L and D-glutamic acid units; depending on the strain, derived either exogenously or endogenously (using α-ketoglutaric acid as a direct precursor). It was divided into four stages for the purpose of clarity; racemization, polymerization, regulation, and degradation (Jiang *et al.*, 2019).

2.2.1 Racemization

The composition of γ-PGA varies in different strains of *B. subtilis*; those that incorporate both the L and D isomers of glutamate have to produce the racemates themselves to meet up with their physiologic needs in the cell extracellular environment (Ashiuchi *et al*., 1999; Ashiuchi *et al*., 2004; Wu *et al*., 2006); also, the incorporation of the D-isomer is dependent on the racemization process that converts L-glutamate to D-glutamate. Two homologues of the glutamate racemase gene (*rac*E/*glr* and *yrp*C) have been identified in *B. subtilis.* The *glr* is needed in converting L-glutamate into D-glutamate for the synthesis of γ-PGA (Shih and Van, 2001). It was also established that, *RacE* and *yrpC* are found in the cytosol and they have high affinity and selectivity for glutamate and a preference for the L-form, but are not directly responsible for the synthesis of γ-PGA. Little is known on the entire biological role they perform in the cell (Luo *et al*., 2016). Buescher and Magaritis (2007) proposed a trio-mechanism of racemization of L-glutamic acid: an indirect one by amino transferase, a direct one by glutamic acid racemase mediated by the enzymes; *Glr* or RacE and a direct one by glutamic acid racemase (Yrpc). They suggested that Glr/RacE is only

responsible for the provision of the glutamic acid monomers (Adebayo, 2018), while the indirect routes are unlikely to be taken *in vivo* due to likely decrease in aminotransferase activity during the production of γ-PGA (Adebayo, 2018). Glr is a cytosolic enzyme with high selectivity for glutamic acid has preference for L- glutamic acid (Ogunleye *et al*., 2015).

2.2.2 Polymerization

Polyglutamate synthase (*pgs*) is the sole enzyme complex responsible for the synthesis of γ-PGA (Kimura *et al.,* 2004a). The complexes' proteins are encoded by four genes (*pgsB, C, A*, and *E*) located on the same open reading frame *in B. subtilis* (Bajaj and Singhal, 2011). The sole machinery responsible for polymerization of γ-PGA is the ATP-dependent PgsBCA complex (Luo *et al*., 2016). *PgsB* and *pgsC* forms the complex's catalytic site, whereas, the elongated chain seems to be removed from the active site by *pgsA* so that the next monomer can be added and may also be involved in transporting γ-PGA across the membrane of the cell into the extracellular environment (Ashiuchi *et al*., 2001a; Ogunleye *et al*., 2015). The biological function of *PgsE* on the other hand, is believed to be not necessarily important in *B. subtilis* because at considerable high concentrations of *pgsB, pgsC,* and *pgsA, B. subtilis* were able to produce γ-PGA in an enriched media. Other reports have it that Zn^{2+} enhances the function of *pgsE* in *B. subtilis* and increases the total production yield of the microorganism (Luo *et al*., 2016). Due to the high instability and hydrophobic nature of *PgsBCA* complex, it is yet to be isolated in its active state (Adebayo, 2018). The biosynthesis of γ-PGA takes place in the membrane bound complex from enantiomers of glutamic acid and ATP. The cleavage of ATP molecule generates the energy needed for the polymerization reaction (Buescher and Magaritis, 2007). First, ATP donates its phosphoryl group to a terminal carboxyl group of elongated γ-PGA through

substrate-dependent ATP hydrolysis (Figure 2.2) which is then followed by a nucleophilic substitution of the phosphoryl group amino group of glutamic acid resulting in the formation of an amide linkage and elongation of the γ-PGA by one glutamate moiety (Sung *et al*., 2005). The activity of *PgsBCA* was found also to be dependent on the presence of Mg^{2+} and less compact cell membranes with shorter phospholipids aids the transportation of γ-PGA outside the cell (Buescher and Margaritis, 2007).

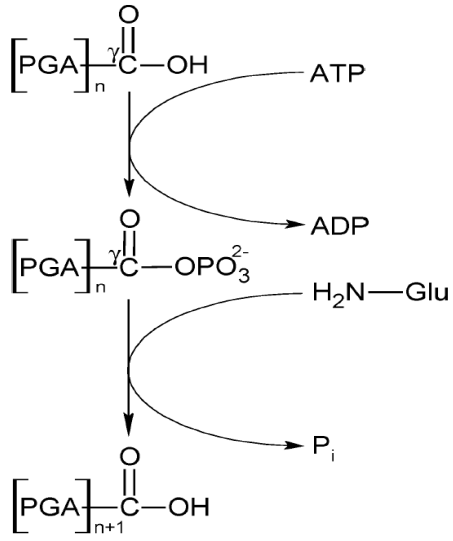

Figure 2.2: Mechanism for the polymerization reaction in *B. subtilis* (Buescher and Margaritis, 2007)

2.2.3 Regulation of γ-PGA

There is a two-way signal transduction system that regulates γ-PGA synthesis as reported by Kobayashi (2007); ComP-ComA regulator, and a two-part DegS-DegU, DegQ, and SwrA system (Luo *et al*., 2016) that involves the phosphorylation of a histidine residue of kinase molecule in the event of a stimulus. The phosphoryl group is then transferred to an aspartate residue of a cognate response regulator which usually acts as a transcription factor (Adebayo, 2018). DegSU, DegQ and ComPA have a quorum sensing transcriptional

regulatory effects and also, in response to phase variation signals and osmolality making SwrA appears to be post transcriptional (Stanley and Lazazzera, 2005). The presence of SwrA and phosphorylated DegU (DegU-P) is important in the activation of pgs operon thereby regulating γ-PGA production (Adebayo, 2018). Although, at a reasonable amount, DegU-P has been reported to directly activate pgs expression instead of SwrA and high levels of DegQ; however, SwrA was discovered to be necessary for γ-PGA production under some experimental conditions (Ogunleye *et al*., 2015). The role of DegQ in γ-PGA synthesis was identified by knocking out the DegQ gene in *B. subtilis* (*natto*) and replacing it with suppressor mutants that can produce γ-PGA in the absence of degQ (Adebayo, 2018). This alteration of *deg*Q prevents the synthesis of γ-PGA and effectively down regulates the production of degradation enzymes (Adebayo, 2018).

2.2.4 Degradation γ-PGA

Bacilli have two enzymes system that can degrade γ-PGA. They are endo-γ-glutamyl peptidase and exo-γ-glutamyl peptidase also known as γ-glutamyl transpeptidase (γ-GTP) as reported by Obst and Steinbuchel (2004). Endo-γ-glutamyl peptidase is released into the medium so as to cleave high molecular weight γ-PGA into fragments of about 1 kDa to 20 kDa (Luo *et al*., 2016). γ-GTP on the other hand, cleaves and subsequently transfers γ-PGA to an acceptor molecule or H_2O, resulting in transpeptidation or hydrolysis, respectively (Figure 2.3). γ-GTP display exohydrolase activity toward γ-PGA, releasing glutamate as a source of carbon and nitrogen during starvation and adverse conditions (Morelli *et al*., 2014). In humans, γ-GTP is a key enzyme in glutathione metabolism, and catalyzes the formation of γ-glutamic acid di- and tri-peptides *in vitro* (Luo *et al*., 2016).

The genes (*ywtD*, *dep*, or *pgdS*) coding for endo-γ-glutamyl peptidase are in the same orientation as, the *pgsBCA* operon and located directly downstream to it. Whereas γ-GTP is expressed during the stationary phase and controlled by ComQXPA quorum-sensing system located on the chromosome distant from the *pgsBCA* cluster (Luo *et al*., 2016). Goto and Kunioka (1992), proposed a schematic pathway of γ-PGA synthesis in *B. subtilis* IF03335. According to their report, in the setup where there was no additional carbon source in the medium, PGA was hardly produced. Citrate was the most preferred carbon source with the yield of 0.95g/100ml in the presence of ammonium sulphate as ammonia donor. α-ketoglutarate combine with ammonium ion to produce intracellular L-glutamic acid which enters the extracellular environment and get polymerized onto the chain of the existing γ-PGA Also, they reported the presence of impurities in the form of polysaccharides when glucose was the added carbon source (Goto and Kunioka, 1992).

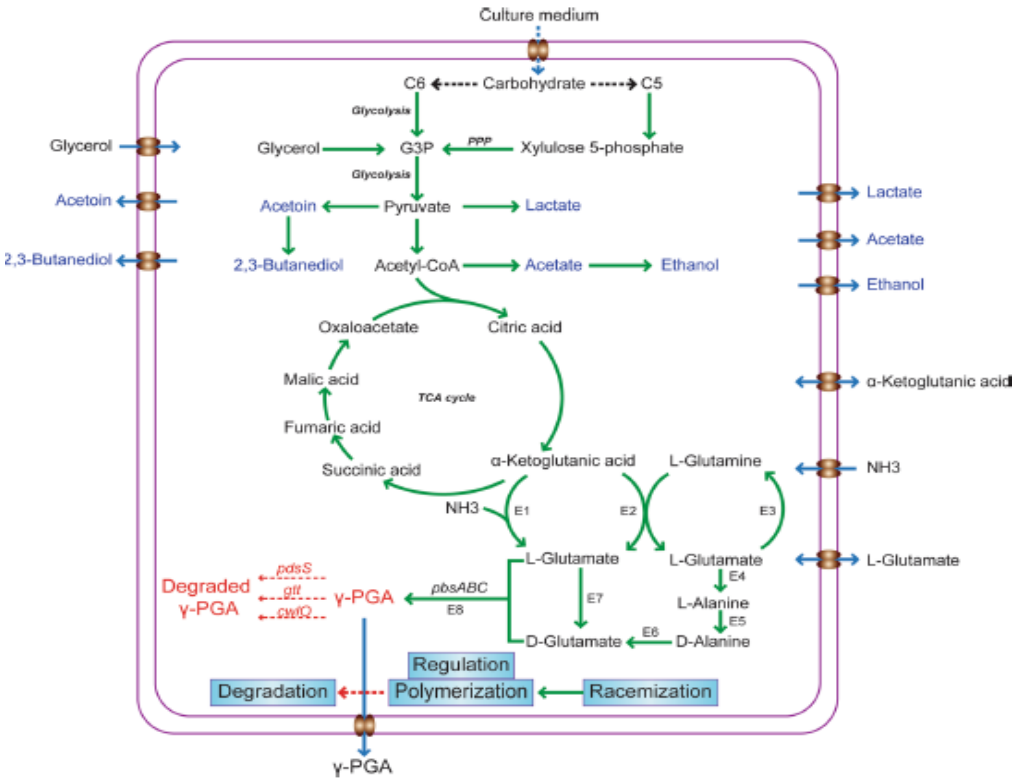

Figure 2.3: Schematic of γ-PGA biosynthesis in *B. subtilis* (Luo *et al.*, (2016)

2.3 Genes Involved in γ-PGA

Shih and Van (2001) reported that *B. subtilis* TAM-4 had no plasmid and the gene coding for the formation of γ-PGA lies on the genomic DNA. In *B. subtilis* (natto), the gene related to γ-PGA biosynthesis was suggested by Hara and Ueda (1982) to be borne on a plasmid, but Tran *et al*. (2000) reported their findings that the plasmid do not encode any gene product required for γ-PGA production, suggesting that, genes involved in γ-PGA biosynthesis are most likely located on the genomic DNA of the organism.

A number of genes responsible for γ-PGA biosynthesis have been identified by various investigators. Feng *et al*. (2014b) working with a mutant strain derived from *B. amyloliquefaciens* C06 with its epsA gene deleted was not able to produce biofilm and showed an increased γ-PGA production from 3.2 to 6.8g/L (Feng *et al*., 2014b). Though, a number of researches have been centered on knocking out genes coding for enzymes that degrade γ-PGA, certain peptidoglycan hydrolases, such as LytE, LytF, CwlS and CwlO, can degrade γ-PGA (Smith *et al*., 2000; Bisicchia *et al*., 2007). Feng et al. *et al*. (2015) investigated the effects of single deletions of the lytE, lytF, cwlS and cwlO genes on γ-PGA production in *B. subtilis* (natto) and found that only the ΔcwlO strain exhibited increased γ-PGA production. They also studied the effects of single deletions of pgdS and ggt (gene encoding γ-glutamyltranspeptidase) and found that these deletions had no effect on γ-PGA production. Scoffone *et al*. (2013) were the first to study multiple deletions of genes for γ-PGA-degrading enzymes. A mutant strain with both of its pgdS and ggt genes deleted had doubled γ-PGA yield compared with the wild-type strain. They also studied the effects of multiple deletions of the pgdS, ggt and cwlO genes on γ-PGA production in *B. amyloliquefaciens* NK-1 strain (Feng *et al*., 2014a). In contrast to the results of Scoffone *et*

al. (2013) which found that double deletion of the pgdS and ggt genes had no effect on γ-PGA production; but the strain with double deletion of the pgdS and cwlO genes exhibited a 93% increase in γ-PGA production. Although many studies have been conducted on this topic, most of them have focused on only one synthetic bottle neck in every round of cellular engineering and no systematic studies of the effects of modifying multiple synthetic bottle necks on γ-PGA production in a single strain have been performed using modular pathway engineering.

Feng *et al*. (2014a) simultaneously optimize the entire biosynthesis pathways, and fine-tune the synthetic pathways and balance the metabolism in the glutamate-independent *B. amyloliquefaciens* NK-1 strain. Urushibata *et al*. (2002) working with clones of γ-PGA genes from *Bacillus subtilis* IFO16449, isolated from fermented soybeans determined the role of each of the four genes in the open reading frames of the cloned fragment. They disrupted *ywsC*, *ywtA*, and *ywtB*, genes to determine which plays a central role in γ-PGA biosynthesis. In the case where *ywsC* and *ywtA* were disrupted, no PGA was produced indicating that both these genes are essential for γ-PGA production. They went further to determine the function of YwsC protein by tagging the protein with histidine (Urushibata *et al*., 2002). They produced YwsC (YwsC-His) in the *ywsC* strain and purified from the lysozyme-treated lysate of the transformant by Ni-nitrilotriacetic acid affinity chromatography. They were able to establish that the protein consists of two subunits; 44 kDa and 33 kDa proteins, which are encoded by in-phase overlap in the *ywsC* gene (There were four open reading frames in the clone with 4.2 kb) (Urushibata *et al*., 2002). Labeling the protein with 14C-label, they synthesized γ-PGA by the purified proteins from L-(14C)-glutamate in the presence of ATP and $MnCl_2$, through an acylphosphate intermediate,

indicating that the *ywsC* gene encodes for γ-PGA synthetase showing that *ywsC* of *Bacillus subtilis* IFO16449 is identical to *ywsC* and *ywtABC* genes of *B. subtlis* 168 (Urushibata *et al*., 2002).

Scoffone *et al*. (2013) produced γ-PGA from *B. subtilis* strain 168, proving that strain 168 offers the advantage of a producer characterized by a well-defined genetic framework and simple manipulation techniques. The knockout of genes for the major γ-PGA degrading enzymes, pgdS and ggt, leads to a considerable improvement in the polymer yield, which attains levels analogous to the top wild γ-PGA producer strains. They were able to highlights the convenience of using the laboratory strain of *B. subtilis* over wild isolates in designing strain improvement strategies aimed at increasing γ-PGA productivity (Scoffone *et al*., 2013).

A work by Feng *et al*. (2014b) on an engineered glutamate-independent *Bacillus amyloliquefaciens* LL3 strain was able to establish the effect of single, double and triple deletion of γ-PGA degrading enzymes. The genes ggt, pgdS and cwlO were experimented on. They showed that a markerless double deletion of pgdS and cwlO genes results in 93% higher yield compared to the wild-type LL3 strain (the yield in the NK-pc mutant and the LL3 strains were 7.12 g/L and 3.69 g/L respectively). The triple-gene-deletion strain NK-pgc on the other hand had a decrease in yield of 28 % (2.69 g/L). Their work also showed that, the cell of the mutants are shorter compared to the wild type strains. This is suggestive that γ-GTP (encoded by ggt) could be involved in the inhibition of cell elongation (Feng *et al*., 2014b). Xu *et al.* (2014) reported a novel aerobic plant fibrous-bed bioreactor (APFB) for enhanced γ-PGA production and compared its productivity with traditional free-cell fermentation medium. The APFB proved to be more efficient and produces higher turnover

per unit time with γ-PGA concentration and productivity of 71.21 g/L and 1.246 g/L/h respectively by cells immobilized in bagasse during APFB process. They were able to reuse bagasse eight times over a period of twenty days. Suggesting APFB is a promising technique for γ-PGA production in future industrial applications (Xu *et al*., 2014).

2.4 Recombinant DNA Technology in γ-PGA Production

A number of work have been done with recombinant *B. subtilis*, one was reported by Lin *et al*. (2016). They cloned and expressed γ-PGA synthetase gene *pgs*BCA of *Bacillus subtilis*, using pWB980 plasmid and transfected the recombinant expression vector pWB980-*pgs*BCA into *Bacillus subtilis* WB600 under the action of P43. Their recombinant *B. subtilis* WB600 was able to synthesize γ-PGA and secretes the product into the fermentation broth, with a product yield of about 1.74 g/L. When they purified and characterized the γ-PGA produced, the hydrolysate was in single form, constituting simply glutamic acid units only. The FT-IR spectra of the produced γ-PGA agree with that of γ-PGA standard, with molecular weights within the range of 500–600 kDa.

Another recombinant expression of γ-PGA is the work of Cao *et al.* (2010). Working with recombinant strains of *Escherichia coli* JM109 and *Corynebacterium glutamicum* ATCC13032 were able to express pgsBCA in *C. glutamicum ATCC13032* with a yield of 0.69 g/L and 97% proportion of L-glutamate monomer showing that the strain is glutamic independent (Cao *et al.,* 2010). This is a proof that γ-PGA biosynthesis directly from glucose by genetic engineering is feasible and significant. The genes were cloned from *B. licheniformis* NK-03 isolated from fermented *natto* with pgsB, pgsC and pgsA open reading frames in the cloned fragment which are similar to those found in typical *Bacillus subtilis* strains (Cao *et al.,* 2010). They went further to study the amino acid sequence

alignment and showed that PgsC was the most conservative of the three subunits of the pgsBCA complex. The recombinant plasmid they used; pXMJ19-PGS was constructed by a shuttle vector pXMJ19, and successfully transformed and expressed in the recombinant strains respectively (Cao *et al*., 2010).

Another report noteworthy also is that of Cao *et al*. (2013) where they tried to establish the relationship between functional synthetic enzyme and the strains' yield or substrate dependency. They cloned *pgsBCA* and glutamate racemase gene *racE* from both L-glutamate-dependent γ-PGA producing *Bacillus licheniformis* NK-03 and L-glutamate-independent *B. amyloliquefaciens* LL3 strains. From their findings, the *RacE* and *PgsA* from the two strains have identity of 84.5% and 78.53% respectively, with *PgsB* and *PgsC* subunits having greater similarity of 93.13% and 93.96%. They were able to induce the co-expression of *pgsBCA* and *racE* in *Escherichia coli* strains and the strains were able to synthesize γ-PGA. A recombinant strain, LL3 derived PgsBCA had higher catalytic activity and enhanced productivity than NK-03 when they grow them in Luria–Bertani medium containing glucose and L-glutamate. However, the differential effect was weakened when providing sufficient immediateness L-glutamate substrate, that is, the supply of substrate could be served as the ascendance upon γ-PGA production. Furthermore, *RacE* integration could enhance γ-PGA yield through improving the preferred D-glutamate content. This is the first report about co-expression of *pgsBCA* and *racE* from the two *Bacillus* strains, which will be of great value for the determination of the biosynthetic mechanism of γ-PGA (Cao *et al*., 2013).

2.5 Nutrients Requirement for *γ*-PGA Production in *Bacillus subtilis*

Bacteria that can produce γ-PGA are classified into two groups based on their nutrient requirement thus, L-glutamic acid-dependent and L-glutamic acid-independent strains (Ogunleye *et al*., 2015). The L-glutamic acid-dependent bacteria include *B. licheniformis* 9945a (Birrer *et al*., 1994), *B. subtilis* chungkookjang (Ashiuchi *et al*., 2001a), *B. subtilis* CGMCC 0833 (Wu *et al*., 2010), *B. licheniformis NK-03* (Cao *et al*., 2010) and *B. subtilis* (natto) ATCC 15245 (Bhat *et al*., 2013). L-glutamic acid-independent strains on the other hand include; *B. subtilis* C1 (Shih *et al*., 2005), *B. amyloliquefaciens* LL3 (Cao *et al*., 2011) and *B. subtilis* C10 (Zhang *et al*., 2012).

Cromwick and Gross, (1995) demonstrated with the use of radioactive labelled carbon in the utilization of citrate and glutamate supplied exogenously. They proved that the supplied glutamate was used to a large extent for polymer formation with both retention of the carbon skeleton as well as decarboxylation at C-1. The supplied citrate on the other hand was also used as a source of carbon to form repeat units of the produced γ-PGA. Ko and Gross (1998) showed in their work that glucose does not significantly impact on the yield of γ-PGA. They also showed an interesting relationship between glycerol utilization to glutamic acid utilization. Xu *et al. (*2005) reported a contradictory work on carbon sources utilization for γ-PGA production. In their report; glucose, sucrose, maltose and starch when added to the medium, produced γ-PGA without formation of unwanted polysaccharide.

When citric acid as a carbon source was added to a glutamic acid medium containing L-glutamic acid and ammonium sulphate, a large amount of pure γ-PGA was produced. On the other hand, when glucose was added to the glutamic acid medium, a by-product was produced, which seemed to be a polysaccharide. Moreover, the mode of hydrolysis was

investigated with PGA in aqueous solutions at 80, 100, and 120°C by monitoring the time-dependent changes in the molecular weights. Hydrolytic degradation of PGA was found to proceed through a random chain scission (Goto and Kunioka, 1992).

Bajaj and Singhal (2009a) carried out a study "Enhanced production of poly (γ-glutamic acid) from *Bacillus licheniformis* NCIM 2324 by using metabolic precursors" from the study they also emphasized the roles of glycerol, ammonium sulphate and L-glutamic acid in the improvement of the yield. The report showed that these three factors significantly enhanced the production of γ-PGA with significant effects of interactions of the factors (Bajaj and Singhal 2009a).

Soliman *et al.* (2005) evaluating nutrient requirement that affects γ-PGA production by *B. licheniformis* SAB26, using Plackett–Burman statistical design examined 15 independent variables casein hydrolysate, ammonium sulphate, ammonium chloride, glucose, citrate, glycerol, K_2HPO_4, KH_2PO_4, $MgSO_4$, $MnSO_4$, $FeSO_4$, $CaCl_2$ and $ZnCl_2$ were investigated. From the analysis of regression coefficients carried out, it showed that; KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$ and casein hydrolysate were the most significant factors increasing γ-PGA production, whereas NH_4Cl and pH significantly decrease the γ-PGA production (Soliman *et al.*, 2005).

2.6 Activity of *γ*-PGA Degrading Enzyme (*γ*-GTP)

Poly-γ-glutamic acid degrading enzymes are mainly common in bacteria (Kimura and Fujimoto, 2010). The degrading enzyme acts differently in different species and are essential in facilitating or antagonizing physiological functions of γ-PGA. The degrading enzymes recognize not only the γ-peptide bond but also the stereochemistry of the polymer. Among the γ-PGA degrading enzymes are; *B. anthracis* CapD and *Bacillus subtilis* γ-GTP (Kimura and Fujimoto, 2010).

2.7 Inhibition Studies on γ-GTP

The inhibition studies of γ-GTP were mostly done with human γ-GTP. So far, there has not been any report on the inhibition of γ-GTP for γ-PGA production. But from general assumptions, there is similarity between human GGT and *B. subtilis* γ-GTP. King *et al*. (2009) reported a novel GTT inhibitor OU749 which acts uncompetitively in contrast to acivicin which is a glutamine analogue. OU749 lead compound has an intrinsic Ki of 17.6 μM. Inhibition of GGT by OU749 is species-specific; inhibiting GGT isolated from human kidney with 7- to 10-fold greater potency than GGT isolated from rat or mouse kidney and does not inhibit GGT from pig cells at all. It inhibits glycyl-glycine acceptor in a competitive manner, which indicates that OU749 occupies the acceptor site while binding to the γ-glutamyl substrate complex (King *et al*., 2009).

Shuai *et al*. (2011) were able to purify and characterize an extracellular γ-glutamyl transpeptidase with a specific activity of 683.4 U/mg from homogenized culture filtrate of *B. subtilis* SK11.004. They discovered that the purified γ-GTP is composed of two subunits with a molecular weight of 62 kDa with optimal pH and temperature of 10.0 and 37°C, respectively. Also in their report was the Km values of hydrolysis and of transpeptidation for L-Gln of 3.16 mM and 0.83 mM, respectively, suggesting that the γ-GTP likely synthesizes valuable γ-glutamyl peptides using L-Gln as γ-glutamyl donor (Shuai *et al*., 2011). The effects of inhibitors on the enzyme suggested that the tryptophan residues and hydroxy groups of serine or threonine are essential to its activity. Based on the biochemical characteristics of the enzyme and lack of homology to previously identified proteins, it can be concluded that the GGT from *B. subtilis* SK11.004 is a novel enzyme (Shuai *et al*., 2011).

2.8 Production and Optimization of γ-PGA

It is believed that the largest source of γ-PGA is primarily through microbial fermentation by gram-positive bacteria (genus: *Bacillus*, class: *Bacilli*). Although the production is inherent to the microorganisms, it has been reported that the wild type do lose their production capability due to unstable heritability, easily leading to a reduction or loss in the ability to synthesize γ-PGA during fermentation (Lin *et al.*, 2016).

Tork *et al.* (2015) worked with *Bacillus licheniformis* NRC20, produced a γ-PGA with molecular weight of 1266kDa comprising of both L- and D- residues. The γ-PGA production in NRC 20 was reported to be independent of L-glutamic acid but the polymer production enhanced when cultivated in medium containing L-glutamic acid as the sole nitrogen source (Tork *et al.*, 2015).

Zeng *et al.*, (2017) on the other hand, isolated a thermotolerant glutamate-independent *Bacillus subtilis* GXG-5 and optimized the fermentation process by single-factor test and obtained a yield of 19.50 g/L with substrate conversion efficiency of 78% at 50°C in 10L fermentor. They further compared the GXG-5 and GXA-28 (glutamate-dependent strain) under respective optimal fermentation conditions. Their result showed that a 19.0% higher in GXG-5 than that of GXA-28, and GXG-5 was also superior to GXA-28 in the availability of carbon sources and substrates (Zeng *et al*., 2017). Screening bacterial strains to find a more efficient producer, Ju *et al*. (2014) isolated *Bacillus subtilis* strain MJ80 as a γ-PGA producer from soil samples which was able to produce γ-PGA from both glutamic acid and soybean powder (Ju *et al*., 2014). Kongklom *et al*. (2015) also worked with *Bacillus licheniformis* TISTR 1010 in a fed-batch production with simultaneously and continuously feeding glucose and NH_4Cl manually in a 7L stirred fermenter produced γ-PGA concentration and productivity of 27.5 g/L/ and 0.286 g/L/h, respectively (Kongklom

et al., 2015). In the same vein, Zhan *et al*. (2017) improved glycerol catabolism in *B. licheniformis* for enhanced γ-PGA by substituting the native glpFK promoter with the constitutive promoter (P43), ytzE promoter (PytzE), and bacABC operon promoter (PbacA), respectively. The glycerol consumptions of the corresponding mutant strains WX02-P43glpFK, WX02-PytzEglpFK, and WX02-PbacAglpFK were 30.9, 26.42, and 18.8% higher than that of the WX-02 strain, respectively (Zhan *et al*., 2017).

Kumar and Pal (2015) designed a system of γ-PGA production from renewable carbon source in which yield and productivity of γ-PGA was enhanced through substrate-product inhibitions of traditional batch production system with the rate of 0.91 g/L/h (Kumar and Pal, 2015). The design incorporated a screen-ultrafiltration membrane module which helped to concentrate the produced γ-PGA while ensuring recovery and recycling of unconverted carbon source resulting in yield of 0.6 g/g along with high product purity (Kumar and Pal, 2015).

Bhat (2012) carried out a study to compare the production of γ-PGA by eight *B. subtilis* strains; *B. subtilis* 23856, *B. subtilis* 23857, *B. subtilis* 23858, *B. subtilis* 23859, *B. subtilis* natto, *B. licheniformis* 1525, *B. licheniformis* 6816 and *B. licheniformis* 9945a. From the study, he obtained a yield of up to 22.3 g/L in shake flasks and molecular weights ranging from 3 kDa to 871 kDa (Bhat, 2012). Using *Bacillus licheniformis* NCIM 2324, Bajaj *et al*. (2008) performed an enhanced production of γ-PGA in solid state fermentation (SSF). Using one factor-at-a-time method to investigate the effect of solid substrates, initial moisture content, pH, and additional carbon and nitrogen source on PGA production to screen for significant factors which he finally optimized by response surface methodology (RSM). He obtained an optimized yield of 98.64±1.61mg/g/day of γ-PGA (Bajaj *et al*., 2008).

2.9 Uses of γ-PGA

Poly-γ-glutamic acid has been successfully exploited for a wide array of useful applications due to its multifarious properties (Ogunleye *et al.,* 2015).

2.9.1 Metal chelator

Poly-γ-glutamic acid has been proven to be an excellent metal chelator both for clinical and industrial applications. Inbaraj and Chen (2012) carried out a metal chelating study with γ-PGA coated super paramagnetic iron oxide NPs (γ-PGA-SPIONs) in an *in vitro*-simulated gastrointestinal fluid and a metal solution. The results suggest that γ-PGA-SPIONs could be used as a metal chelator for clinical treatment of metal poisoning (Inbaraj and Chen 2012). Mark *et al.,* (2006) carried out a study using γ-PGA obtained from *Bacillus licheniformis* ATCC 9945 evaluated its heavy metals biosorbent potential using Langmuir adsorption isotherm model were able to show that γ-PGA had a copper capacity approaching 77.9 mg/g and a binding constant of 0.5 mM at pH 4.0 and 25°C which is reasonable compared to other methods of heavy metal removal from waste water (Mark *et al.,* 2006).

Ritchie *et al.* (1999) functionalized γ-PGA on silica-composite membranes and showed that it is very important for the production of metal sorption material on microfiltration (MF) membrane supports. The result showed high capacity sorbents that represent a significant advancement over conventional silica-based sorbents with excellent acid/solvent stability and higher throughput convective flow (Ritchie *et al.,* 1999).

Lead ions removal from aqueous solution by a combined nano-membrane separation technique was investigated by Hajdu *et al.* (2012) using γ-PGA and its cross-linked nanoparticles to capture lead ions by forming nano sized particles. The filtrate they

obtained satisfied the standard for drinking water recommended by the World Health Organization (WHO) (Hajdu *et al*., 2012).

2.9.2 Drug carrier/deliverer

The use of safe biologically nontoxic materials as building blocks of nanomaterials is crucial and a necessary transition for clinical application. Kim *et al.*, (2019) constructed a polymeric nanomaterial with *γ*-PGA and dopamine as building blocks for carrying hydrophobic anticancer drugs. The *in vivo* safety profile of polymeric nanoparticles allowed Paclitaxel (PTX) to be delivered at 3.6 fold higher dose than was possible with PTX solubilized in surfactant. The nanoparticle delivered PTX was able to circumvent the side effects of the surfactant. Their results supported the multifunctional potential of polymeric nanoparticles for the delivery of various hydrophobic drugs (Kim *et al.*, 2019).

γ-PGA delivery system loaded PGA–Asp–maleimide–cisplatin–peptide complex (PAMCP) for cancer therapies possesses potential to overcome side effects. Using PAMCP loaded with cis-diamminedichloridoplatinum (II) (CDDP) and conjugated with the transferrin receptor (TFR)-targeting peptide through a maleimide functional linker. The PAMCP reduced the toxicity of CDDP, suppressed tumour cell growth, and achieved efficient anti-tumour effects *in vivo* making PAMCP potentially safe and effective in anticancer pharmaceutical formulation (Zhang *et al.*, 2018b).

γ-PGA with covalently attached cisplatin was shown to reduce the toxicity of cisplatin, whilst efficiently decreasing the tumour size of xenografted human breast tumours in nude mice as well as lengthen the survival of nude mice grafted with breast cancer (Bcap-37) tumour cells (Ye *et al*., 2006)

A macromolecular conjugate of PTX and γ-L-PGA called paclitaxel poliglumex exhibited outstanding benefits over conventional PTX. Paclitaxel poliglumex was accumulated in tumour tissue, where it gradually discharged the active agent paclitaxel (Singer, 2005).

Ahn *et al.* (2018) showed in a study that γ-PGA attenuated NLRP3, NLRC4 and AIM2 inflammasome activation and also upregulates pro-inflammatory cytokine expression in human and murine macrophages. Although γ-PGA had conflicting effects on cytokine production and maturation, they were able to show that it clearly alleviated the severity of lipopolysaccharide-induced endotoxin shock in animal model. Thus, it is suggestive that γ-PGA is a potent candidate to control inflammasome-mediated disorders (Ahn *et al.*, 2018). Inflammasomes are intracellular multi-protein complexes which promote acute and chronic inflammation via interleukin-1β or interleukin-18 maturation, and they are known targets for metabolic syndromes and cancer (Ahn *et al.,* 2018).

It has been established that most commercially available amphotericin B (AmB) formulations are limited either by cytotoxicities, lower efficacies, shelf-life related issues or high production costs. Dinh *et al*. (2017) prepared and tested an AmB complexes based on γ-PGA for their efficacies against AmB-deoxycholate (Fungizone®) and liposomal AmB (AmBisome®). *In-vitro,* AmB/γ-PGA complexes exhibited an improved and comparable cytotoxicity profile as compared with Fungizone® and AmBisome® respectively, with respect to haemolytic activity and up-regulation of cytokine productions (TNF-α and IL-1ß). AmB/γ-PGA complexes were significantly more efficacious *in-vivo* than both Fungizone® and AmBisome® in experimental murine *candidiasis*. These results provide strong evidence that AmB/γ-PGA complexes have a better efficacy and safety profile than the currently approved AmB products (Dinh *et al*., 2017).

2.9.3 Tissue engineering

Poly-γ-glutamic acid's free carboxyl side groups allow for simple chemical functionalization, which makes it a versatile candidate for molecular scaffolds. Gentilini *et al.* (2012) suggested that esterified γ-PGA polymer scaffolds are new and versatile candidates for tissue engineering applications when carrying out investigation with a series of water-resistant fibrous scaffolds by ethyl (Et), propyl (Pr) and benzyl (Bn) esterifications of γ-PGA. The scaffolds were non-cytotoxic and γ-PGA-Bn showed increased cell adhesion of human mesenchymal stem cells (hMSCs) while cells on γ-PGA-Bn showed three fold higher viability and significantly higher adhesion when compared with poly-L-lactic acid (PLLA) scaffolds, despite having a similar hydrophobicity (Gentilini *et al.,* 2012).

A technique for synthesizing biocompatible hydrogels by cross-linking calcium-form γ-PGA, alginate sodium, and pluronic F-127 was created by Chan *et al.* (2015), where alginate was cross-linked by Ca^{2+} from Ca–γ-PGA directly and γ-PGA molecules introduced into the alginate matrix to provide pH sensitivity and hemostasis. The experiment unravels the potential of γ-PGA as biocompatible scaffold material for application in bone treatment (Chan *et al.,* 2015). Another interesting work that supports the use of γ-PGA is the methacrylation of γ-PGA introduced to the spongy poly (ethylene glycol)-*co*-2-hydroxyethyl methacrylate (PEG-HEMA) cryogels. The γ-PGA cryogels was found to promote homogeneous mineralization, while higher concentration of γ-PGA inhibited the mineralization. The deposited minerals mainly consisted of hydroxyapatite, carbonated apatite, and calcium phosphate, and were independent on the concentration of γ-PGA. Showing that the mineralized cryogels can support adhesion, viability, and migration of rat bone marrow stromal cells (rMSCs) (Liu, *et al.,* 2015).

A γ-PGA/chitosan composite biomaterial showed potential application in tissue engineering as it is more hydrophilic and cytocompatible than conventional chitosan matrices (Hsieh *et al.,* 2005). Polyelectrolyte complexes (PEC) of chitosan and γ-PGA were demonstrated to be useful in wound dressing. The complex presented sufficient moisture content and showed good mechanical properties, which would allow the dressing to be easily removed from the wound surface without destroying renewed tissues (Tsao *et al*., 2011).

2.9.4 Cryoprotectant

Bhat *et al.* (2013) carried out a study to investigate the cryoprotective properties of γ-PGA in the preservation of probiotics during freeze drying. They discovered that 10% γ-PGA protects *Lactobacillus paracasei* significantly better than 10% sucrose, whereas it showed comparable cryoprotectant activity to sucrose when it was used to protect *Bifidobacterium breve* and *Bifidobacterium longum* (Bhat *et al.,* 2013). Cryoprotectants basically are chemical compounds which can successful preserve the normal function of cells or tissues at reduced temperature below which biochemical reactions and physiological activities take place (Mullen and Critser, 2007; Bhattacharya, 2016).

2.10 6-Diazo-5-oxo-L-Norleucine (DON)

The compound 6-diazo-5-oxo-L-norleucine (DON) (Figure 2.4) is a diazo non-natural occurring amino acid analogue of L-glutamine isolated from Streptomyces in 1953 (Kisner *et al.,* 1980). Due to its reactive diazo group, DON can alkylate several glutamine-utilizing enzymes such as glutaminase, nicotinamide adenine dinucleotide (NAD) synthase, cytidine triphosphate (CTP) synthetase and formylglycinamide ribonucleotide (FGAR) aminotransferase in the purine and pyrimidine biosynthetic pathways, respectively (Rais *et al.,* 2016). The mechanism is a competitively and irreversibly binding to the active sites of

the glutamine-utilizing enzymes, permanently inhibiting their catalytic activities (Manivannan *et al.,* 2016). It has been used in clinical trials in the 1980s which proved effective against tumor cells, but with dilapidation due to substantial toxicity from prolonged treatment (Lynch *et al.,* 1982; Earhart *et al*., 1990). Other studies have showed that DON can; inhibit lymphocyte proliferation, graft rejection and also decrease *Sindbis virus* (SINV)-induced memory impairment and improve outcomes for mice with cerebral malaria (Manivannan *et al.,* 2016).

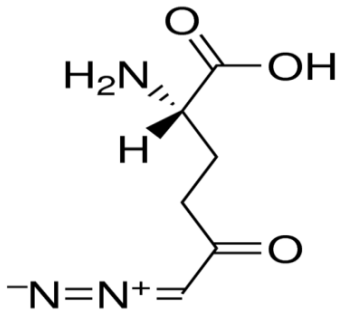

Figure 2.4: Structure of 6-diazo-5-oxo-L-norluecine (Sigma Aldrich, 2019)

2.11 Gamma-Glutamyl Transpeptidase (γ-GTP) Inhibition by DON

Gamma-glutamyl transpeptidase or gamma-glutamyl transferase is located on the surface of the cell and cleaves extracellular gamma-glutamyl compounds (Terzyan *et al.,* 2017). It has two nonidentical subunits (Figure 2.5) and is rapidly inactivated with respect to its transpeptidase and hydrolase activities by the γ-glutamyl analogs 6-diazo-5-oxo-L-norleucine in mammals (Tate and Meister, 1977). The reaction is specific, covalent, and stoichiometrical at the γ-glutamyl site of the enzyme localized to the light subunits (Tate and Meister, 1977). DON inhibits human GGT in an irreversible manner with a second order rate constant of inactivation values of $0.052 mM^{-1}min^{-1}$ and the *Ki* of 2.7 ± 0.7 mM

(Terzyan *et al*., 2017) and has an IC_{50} value of 0.371 mM for human GGT (hGGT) (Lin *et al*., 2018) with LD_{50} of 0.81μM on dividing 786-O cells (Sigma Aldrich, 2019).

The crystal structure of DON-inactivated hGGT1 contained a molecule of DON without the diazo-nitrogen atoms in the active site. The three dimensional structure of the hGGT1-DON complex resembled the structure of the apoenzyme with some shifts in the loop forming the oxyanion hole and elements of the main chain that form the entrance to the active site. The structure of hGGT1-DON complex revealed two covalent bonds between the enzyme and inhibitor which were part of a six membered ring includes Thr381 (Terzyan *et al*., 2017). The structure of DON bound hGGT1 has led to the discovery of a new mechanism of inactivation by DON that differs from its inactivation of other glutamine metabolizing enzymes (Terzyan *et al*., 2017).

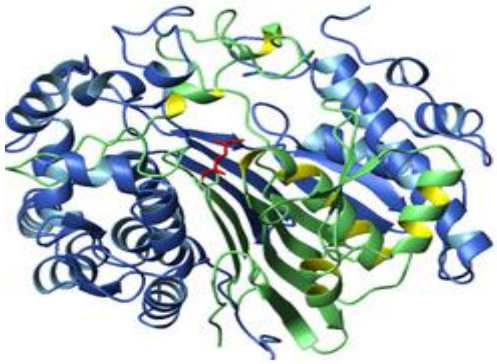

Figure 2.5: Structure of DON-hGGT1complec (Terzyan *et al*., 2017).

2.12 Rice Husks

The use cellulosic waste is increasingly gaining attention in recent years because of the readily availability of these raw materials and the ease of them being degraded by microorganism (Johar *et al.*, 2012). Rice being the largest staple cereal crop produced globally generates a lot of waste during the milling process. The husk from rice is found in

heaps at various locations in Nigeria which is common sights when you travel along the major high ways. Globally, rice husk constitute more than 20% of the over 500 million tons of paddy produced worldwide (Runge *et al.,* 2019). The annual rice production for Nigeria was put at 7.2 million tonnes (4.435 million tonnes, milled basis) in 2018 which is an upward increase of 3% on the previous year. While at the continental level, improved weather and continued state assistance to agricultural development in Africa yielded a 4% output recovery in 2018 with an aggregate harvest of 32.1 million tonnes (20.9 million tonnes, milled basis) (FAO, 2018).

Rice husk was estimated to contain about 33% cellulose, 26% hemicellulose, and 7% lignin (Johar *et al.,* 2012) which make it a good source of carbon from readily available agricultural waste. The use of this agricultural waste for the production of useful industrial end products by microbial fermentation is a major step in the utilization of such waste material to produce important polymers and enzymes. Amongst the use of rice husk are; production of biofuels (Ezeonu, *et al*., 2018), palp, fertilizers and animal feed (Ibitoyea *et al*., 2020); fuel for making fire in in brick kilns, furnaces, parboiling process of rice, raw material for sodium silicate production, cleaning/polishing agent in metal and machine industry and briquettes molecular sieve (Patil *et al*., 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/reagents

All chemicals and reagents used were of analytical and molecular grades purchased from Sigma Aldrich Missouri, USA; Bio-Rad California, USA; Inqaba Biotec, West Africa; JHD, Germany and Titan Biotech Rajasthan, India. Medium components include: Nutrient agar, ammonium sulphate, glucose, glutamic acid, citric acid, glycerol, 6-diazo-5-oxo-L-norluecine, biotin (vitamin H), $CaCl_2 \cdot 6H_2O$, concentrated HCl, concentrated NaOH, copper sulfate solution, dH_2O, $FeC1_3 \cdot 6H_2O$, KCl, KH_2PO_4, $MgCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 2H_2O$, $MnSO_4$, Na_2HPO_4, NaCl, KCl, normal saline, peptone water, tryptone, yeast extracts, lysogeny broth (LB)-Lennox. Buffers, PCR and SDS-PAGE reagents: phosphate buffered saline (PBS), cation-adjusted- Mueller-Hinton broth (CAMHB), Mueller Hinton broth, tricine buffer, Na-K phosphate buffer, and Tris-HCl (pH 8.0), Taq 10x buffer, SDS-PAGE buffer.

3.1.2 Apparatus and equipment

High performance liquid chromatography (HPLC) machine (Agilent 1200 series), FT-IR Spectrometer (Agilent Cary 630), UV-VIS Spectrophotometer (722N, CGOLDENWALL); PCR thermocycler (Labnet MultiGene OptiMax), SDS-PAGE Apparatus (Biorad), Microscope (LX300, Labomed, Inc.), Autoclave (YX-280, Healicon Medical Equipment), Centrifuge (Heraeus Labofuge 300, Fisher Scientific), pH meter (pH 009, Henan), Weighing balance (FA2004, China), Incubator (IH-150, Gallenkamp), Water bath (SHA-C, China), Shaker (HY-A4, China), Eppendorf tube, Volumetric flask, Beaker, Erlenmeyer flask, Filter paper, Glass vial, Incubator, McCartney bottle, Micropipette, Milling machine,

Test tubes, Petri-dish, Pico-Tag apparatus, Sakaguchi flasks, Glass slides, and Sterile syringes.

3.1.3 Samples and sample collection

Rice husks were obtained from a rice mill at Hayin Dogo, adjacent to Model Learning Secondary School, Samaru, and taken to the central laboratory services unit of National Animal Production Research Institute (NAPRI), Ahmadu Bello University, Zaria, for processing before being returned to the Industrial laboratory, Department of Microbiology, Ahmadu Bello University main Campus, for the study.

Soil samples were collected from roots of *Sorghum bicolor* (Guinea corn) in a farm land behind Pensioners Quarters, Hayin Dogo Samaru, Zaria. The water samples were obtained from the ABU dam at the University main campus, Samaru, Zaria, while *daddawa* was purchased from market women in Samaru market. The soil, water and *daddawa* samples were collected in clean and sterile sample containers which were properly labeled and conveyed to the laboratory for the isolation of *B subtilis*.

3.2 Methodology

3.2.1 Experimental flowchart

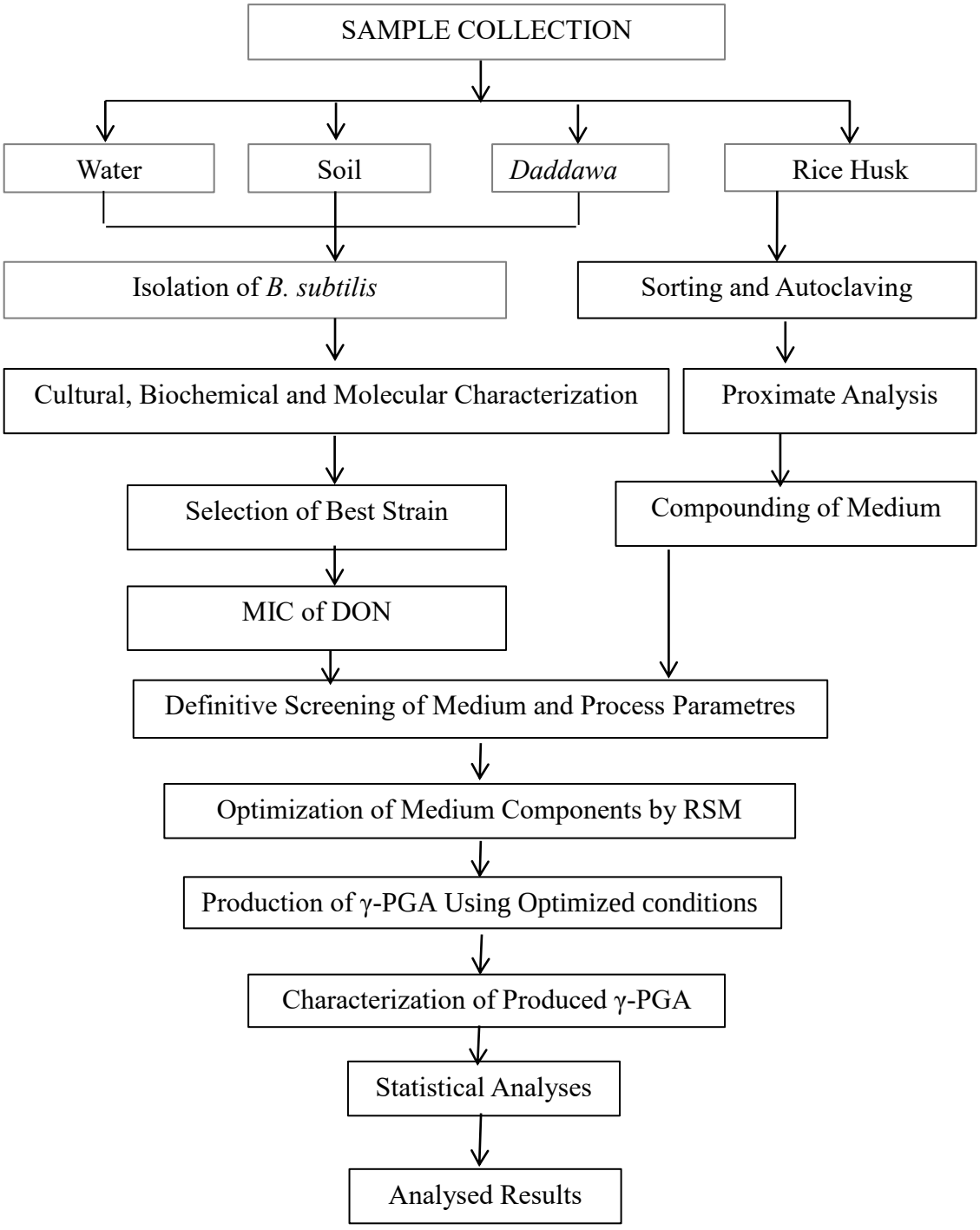

Figure 3.1: Flowchart of Experimental Activities Conducted in the Study

3.2.2 Preparation of biomass and proximate analyses

The sample of rice husk was oven dried to remove moisture and milled using a laboratory size milling machine. The milled husk was sieved into different particle sizes using ISO standard no.: 0.150, 0.210 and 0.420 mm and finally autoclaved. The different sieved rice husks were labelled and used as solid base for the solid state fermentation (SSF) for the production of γ-PGA using *B. subtilis* as the fermenting organism (Merkus, 2009). The proximate analyses were carried out according to the method described by the Association of Official Analytical Chemists (AOAC, 2005). These analyses were carried out to provide insights into the proximate contents of rice husk used as the substrate for solid state fermentation.

3.2.3 Strain and culture condition

3.2.3.1 Isolation of microorganisms

Nutrient agar was prepared by dissolving 28 g in 1000 mL of distilled water and about 20 mL of autoclaved and cooled media were poured into Petri-dishes and allowed to solidify. A loop-full of the samples were streaked on the surface of the solidified media and labelled accordingly and incubated at 37ºC for 24 hour in an incubator (Kim *et al*., 2012).

Bacillus subtilis strains were isolated from the sample of water, soil and from the local soup condiment; *daddawa* (Figure 3.1). The isolations were carried out according to the method described by Kim *et al*. (2012). Exactly 10 g of soil and 1 g of *daddawa* were taken aseptically and dissolved in 50 mL and 10 mL distilled water respectively and thoroughly shaken to dissolve. The suspended samples were incubated at ambient temperature for 10 min and allowed to cool. For the water sample, 10 mL was taken and incubated at the same time with the other samples. The samples were picked with a loop and streaked on separate

plates of nutrient Agar. Colonies formed on the plates were picked and sub-cultured on nutrient agar plates until pure cultures were obtained (Kim *et al*., 2012).

Colonies obtained after incubation were observed and the creamy, viscous rod-shaped bacterial colonies were selected and sub-cultured on nutrient agar and incubated for another 24 hour at 37^0C. Gram staining reactions and cell morphology from heat fixed smears were used to select Gram-positive colonies based on the method of microbial identification described by Cowan and Steel (1966).

The isolated *B. subtilis* were grown aerobically under aseptic conditions. The *B. subtilis* isolates were identified first by morphological and biochemical means and then by 16S rRNA gene sequence analysis. The pure cultures of *B. subtilis* were preserved on nutrient agar slants and stored at 4ºC.

3.2.3.2 Gram staining techniques

A thin smear of each of the pure 24 hour old culture was prepared on clean grease-free slides, fixed by passing over flame. Each heat-fixed smear was stained by addition of 2 drops of 0.3% crystal violet solution for 60 s and rinsed with water. The smears were again flooded with liquol iodine for 30 s and rinsed with water, decolourized with 70% alcohol for 15 s and were rinsed with distilled water. They were then counter stained with 2 drops of Safranin for 60 s and finally rinsed with water, then allowed to air dry. The smears were mounted on a light microscope and observed under oil immersion objective lens (×100). (Gram negative cells appeared pink or red while gram positive organisms appeared purple (Fawole and Oso, 2004)).

3.2.3.3 Spore staining technique

Heat-fixed smears of the organisms were prepared on separate slides and flooded with 5% Malachite green solution and steamed for 1 min which were thereafter washed off with water and counter stained with 2 drops of Safranin solutions for 20 s. The slides were allowed to air dry and examined under oil immersion objective (×100) lens. (Revealing stained endospores) (Cheesbrough, 2006).

3.2.3.4 Biochemical tests

Motility test: Motility tests were carried out according to the method described by Olutiola *et al*. (2000). A sterile needle was used to pick a loop-full of 24 hour old cultures and stabbed onto nutrient agar in test tubes which were incubated at 37°C for 48 hours. (Motility was confirmed by pulsating growth emerging from the stabbed margin, while non-motile isolates had growth confined to the stab line with definite margins without spreading to surrounding areas).

Methyl red test: Exactly 5 mL of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for 48 hours. At the end of incubation, few drops of methyl red solution were added to each test-tube and colour change was observed. (A red colour indicates a positive reaction) (Olutiola *et al*., 2000).

Voges-proskaeur test: Exactly 5 mL of glucose phosphate broth (1 g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 mL distilled water) were dispensed in clean test tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 37°C for

48 h. After incubation, 6% α-naphhtol and 6% sodium hydroxide were added to 1 mL of the broth culture. (A strong red colouration formed within 30 min indicates positive reaction) (Olutiola *et al*., 2000).

Indole test: Tryptone broth (5 mL) was placed into different test tubes after which a loop-full of the bacterial isolates was inoculated into the test tubes, leaving one of the test tubes uninoculated to serve as a control. The test tubes were then incubated at 37°C for 48h. After incubation, 0.5 mL of Kovac's reagent was added and shaken gently; it was allowed to stand for 20 min for the reagent to rise. (A red or red-violet coloured ring at the top surface of the tube indicates a positive result while yellow colouration indicates a negative result) (Cheesbrough, 2006).

Citrate test: This test detects the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of citrate agar was dissolved in 100 mL of distilled water. About ten millilitre (10mL) of citrate medium was dispensed into each tubes and covered, then sterilized and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface. (A change from green to blue indicates utilization of the citrate) (Cheesbrough, 2006).

Oxidase test: A piece of filter paper was soaked with a few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organism and smeared on the filter paper. Oxidase producing organism oxidizes the phenylenediamine in the reagent to a deep purple colour (Cheesbrough, 2006).

3.2.4 Molecular characterization of isolated *B. subtilis*

3.2.4.1 DNA extraction

The 16S rRNA gene of the isolates of *Bacillus subtilis* were extracted from 24 hour pure cultures grown in Luria-Bertani (LB) media at 37^{o}C. The cultures were centrifuged for 2 min to sediment the cells from which cell lysis was performed using 10% SDS and 20 mg/ml proteinase K in TE buffer and incubated for 1 hour at 37^{o}C. Exactly 100 µL of NaCl (5 M) and 100 µL of CTAB/NaCl were added and incubated for 10 min at 65^{o}C. DNA was extracted using chloroform/isoamyl alcohol, then phenol/chloroform/isoamyl alcohol. Aqueous phase was transferred to a fresh tube and DNA was extracted with isopropanol. Finally, DNA was resuspended in 100 µL of TE buffer. The amount of DNA extracted was electrophoresed on 0.8% agarose gel (Liles *et al.*, 2004).

3.2.4.2 PCR amplification of bacterial 16S rDNA gene

Oligonucleotide primers were used to amplify the full length 16S rRNA for the *B. subtilis* isolates by using two universal primers; forward: (AGAGTTTGATCCTGGCTCAG) and reverse: (AAGGAGGTGATCCAGCCGCA). 16Sr RNA was amplified from the obtained DNA in a reaction mixture of PCR conditions as follows: 10x*Taq* buffer, 1.25 U Taq DNA polymerase, 2 mM dNTP mixture, 25 mM $MgCl_2$, 0.7 µg DNA, double-distilled water mixed in a final volume of 50µL. The program for PCR was as follows: 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and extension at 72^{o}C for 7min. Amplification was done using MultiGene OptiMax Thermal Cycler. Amplicons were visualized by electrophoresis on 1% agarose gel after staining with ethidium bromide (Hengstmann, 1999).

3.2.4.3 Sequencing B. subtilis 16S rDNA PCR product

The 16S rDNA PCR product was extracted from gel using gel extraction kit (QIAquick Qiagen) while Sanger DNA sequencing technique was used to sequence the amplicons extracted from the gel. Sequence analysis was performed with the sequences in the NCBI database (www.ncbi.nlm.nih.gov/blast) using the Basic Local Alignment Search Tool, BLAST (Camacho *et al*., 2009).

3.2.5 Selection of promising isolate and preservation

3.2.5.1 Selection of promising strain

The isolates were screened for their γ-PGA producing ability in submerged fermentation. A starter culture of each isolate was prepared by inoculating the isolates in LB and grown overnight. The inoculum concentrations were adjusted to 1×10^7 CFU/mL using cation-adjusted Mueller-Hinton broth (CAMHB) (Appendix I) solution ($1OD_{600} \sim 10^9$ CFU/ml) according to the method described by Cowan and Steel (1966). From the adjusted starter broth of the isolates, 2 mL which is equivalent to 2×10^7 CFU each were used to inoculate 25 mL in 100mL Erlenmeyer flask of the production medium (Cheesbrough, 2006).

The biopolymer production medium comprises of 8.0% glucose, 2.0% glutamic acid, 0.4% $NaH_2PO_4{\cdot}2H_2O$, 0.08% $MgSO_4{\cdot}2H_2O$, and 0.3µg/ml biotin per 100mL. The media was autoclaved at 121°C for l5 min before cultivating aerobically with shaking at 120 rpm and 37°C for 5 days as described by Zhao *et al*. (2005).

3.2.5.2 Strain preservation

The selected strains were then cultivated in an aerobic condition using a media prepared in a 100 ml flask containing 1 g of polypeptone, 0.2 g of yeast extract, and 0.1 g of $MgSO_4{\cdot}7H_2O$ at 37°C for 20 hour. The culture broth was then added to the same volume of

20% (v/v) glycerol solution and annealed at 60°C for 10min so that the bacteria sporulates. The spore suspension was stored at temperature of -20°C and used throughout the study to constitute the starter inoculum for the production or optimization of γ-PGA (Goto and Kunioka, 1992).

3.2.6 Isolation and purification of γ-PGA

Poly-γ-glutamic acid was purified using ethanol precipitation method as modified by Bajestani *et al*. (2018). The solid state fermentation (SSF) media was dissolved in 10 ml of distilled water and agitated at 120 rpm using shaker for 40 min. The supernatant containing γ-PGA was removed by centrifuging at 1000 rpm for 15 min and decanted into fresh tubes. The supernatant was then centrifuged at 6000×*g* for 40min. and the supernatant containing low molecular weight dissolved-substance were discarded. To the pellets in the tubes, 1:4 volume of water to ethanol was then added. The resultant solution was allowed to stand for one day. Thereafter, it was decanted to remove debris that left from the media. The decanted suspension was then centrifuged again at 6,000 rpm for 40 min and the resulting pellets were dried and suspended in 5ml of distilled water. The suspension was further centrifuged at 6,000×*g* for 1 hour to obtain pallets of produced γ-PGA which was dried and stored at 4ºC for further analyses (Shih *et al*., 2002; Anju *et al*., 2018; Bajestani *et al*., 2018).

3.2.7 *In silico* docking of DON on γ-GTP

The three dimensional (3D) crystal structure of γ-GTP (PDB Accession No: AAC60409.1) was downloaded from protein data bank (https://www.uniprot.org/uniprot/Q53260). The structure was optimized using discovery Studio version 4.5 (DS), Accelrys Software Inc., USA. Also water molecules and cofactors were removed before the docking procedure was

carried out. The complex was optimized for interactions using Platinum software web server, followed by docking with PyRx (Vina wizard, Scripps Institute, USA) software. The 3D and 2D molecular interaction models of docked DON on γ-GTP protein were visualized using Discovery Studios (DS) version 4.5 (Ezebuo *et al*., 2016).

3.2.8 Minimal inhibitory concentration (MIC) DON on *B. subtilis*

The MIC of the inhibitor compounds, DON was determined by the method described by Rodriguez-Tudela *et al*. (2003). A single colony of *B. subtilis* was picked from a lysogeny broth (LB)-Lennox (Appendix I) and cultured in 3 ml LB- Lennox broth and incubated overnight at 37°C, 120rpm and dissolved. The optical density was checked at 600nm using UV-VIS spectrophotometer (722N, CGOLDENWALL) (OD_{600} was considered to be equal to 10^9CFU/ml). Serial dilution of the culture solution was carried out using LB broth to about 0.1 OD_{600} suspensions and incubated at 37ºC, 220rpm till mid-log phase in about 3 hour, which was monitored by measuring the turbidity at some interval of time. About 1 ml of the culture solution was transferred into a 1.5 ml Eppendorf tube, centrifuged at 6,000 rpm for 5min and rinsed with 1ml phosphate buffered saline (PBS) solution. The procedure was repeated twice to obtain pure bacterial pellets which were then dissolved with 10 ml cation-adjusted Mueller-Hinton broth (CAMHB) solution (Appendix I). Exactly 1 ml of the prepared bacterial solution was taken and mixed with 9ml Phosphate-buffered saline (PBS1×), thereafter the OD_{600} was checked. The bacterial concentration was estimated and then adjusted to $1x10^7$ CFU/ml with CAMHB solution to produce a solution with 1 OD_{600} approximately equal to 10^9 CFU/ml. From the preparation of the dilute *B. subtilis* bacterial solution from the adjusted inoculum culture, 50 µl of $1x10^7$ CFU/ml concentration were picked with a micropipette and mixed with 850 µl CAMHB and 100 µl solution of 10th

serial dilution of DON. Using *E. coli* bacterial solution as a control, about 900 µl of CAMHB and 100 µl solution of 10th serial concentration of the DON was taken and the OD_{600} measured for comparison as a negative control. The prepared tubes were incubated at 37°C and 120rpm for 24h. The OD_{600} was then measured and recorded accordingly. The MIC endpoint was taken as the lowest concentration of the DON at which there is no visible growth of bacteria, and the difference of measured and background OD_{600} is less than 0.01 (Rodriguez-Tudela *et al*., 2003).

3.2.9 Process and medium parameters for solid state fermentation (SSF)

3.2.9.1 Moisture retaining capacity of rice husk

Solid state fermentation (SSF) was employed using 2 g of rice husk as the solid base which was inoculated with $1x10^7$spores/mL from a 7 day old *B. subtilis* culture. The initial moisture content was set based on the moisture holding capacity of the rice husk. Moisture holding capacity of the rice husk was determined using the method described by Shaktimay *et al*. (2010). Exactly 10 g of rice husk was measured into a beaker and 50mL of water was added and allowed to stay overnight. The amount of water retained was recorded and considered as 100% moisture holding capacity of the substrate. The moisture holding capacity was calculated using Equation 3.1.

$$\text{Water retained} = \text{Water added (mL)} - \text{Water drained (mL)} \quad \ldots\ldots \text{(Equation 3.1)}$$

3.2.9.2 Effects of pH on γ-PGA production

The ranges of pH values for the experiment were set at 6.8 (low) and 8.2 (high). The pH value was adjusted in the cocktail medium by adding 2N NaOH and/or 2N HCl within the level of experimental error of 0±0.1 (Dubey *et al.,* 2011; Adnan *et al.,* 2017).

3.2.9.3 Effect temperatures on γ-PGA production

The temperature ranges were set at 30°C (low) and 40°C (high) in order to investigate the effects of temperature on γ-PGA production (Adnan *et al.,* 2017).

3.2.10 Polymer characterization

3.2.10.1 Amino acid Analysis of the produced γ-PGA

The various peak of γ-PGA digest was determined according to the method described by Ikeda *et al.* (2018) using reverse-phase high-performance liquid chromatography (HPLC) (Agilent 1200 series, Agilent Technologies, USA). Purified γ-PGA was filtered to remove low molecular weight impurities by centrifuging at 4000 rpm for 30 min. About 8 mg of γ-PGA was added into a reaction vial which was mixed with 3ml of 6 N HCl solutions. The γ-PGA was hydrolysed in a vial at 110°C for 3h. It was allowed to stand so as to remove volatile contaminants at room temperature (25°C). The hydrolysed residue was then derivatized using 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) (obtained from Sigma Aldrich, USA following the supplier's instructions). Exactly 100 µL of hydrolysed γ-PGA was placed in a 1.0 mL micro reaction vial with the addition of 200 µL of 1% FDAA in acetone and 40 µL of 1.0M sodium bicarbonate. The vial was heated at 40°C for 1h in a heating module after which it was removed and cooled. Then 20 µL of 2 M HCl was added and stored at 4°C for analysis. The resulting diastereomers were analysed by HPLC.

The reverse phase HPLC was run in isocratic conditions with the mobile phase containing 0.05M triethylamine and 0.05% w/v sodium azide at pH 5.0. The column was run at 37°C under isocratic conditions, with a flow rate of 2.0 ml per minute, an injection volume of 5 µ1, and a detection wavelength of 340 nm. The L- and D-glutamate standard derivatives

were baseline-resolved with elution times of 1.31 and 1.66min, respectively. The γ-PGA stereoisomeric content was calculated based on the relative diastereomer peak areas.

3.2.10.2 FT-IR analysis γ-PGA

The spectra of the produced Purified γ-PGA were analysed using automated Cary 630 FTIR Agilent technologies, USA according to the operational protocol for paste sample analysis. The single reflection diamond attenuated total reflectance (ATR) sample interface was cleaned with acetone and background scan was carried out. The sample was smeared on the Attenuated Total Reflectance (ATR) sample interface and the transmittance measured (Dang *et al*., 2019).

3.2.10.3 The molecular weight of the Produced γ-PGA was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using ExpressPlusTM PAGE precast gel according to the method of Laemmli (1970). After deproteinizing with chloroform n-butanol (1:4 v/v) agent, the purified PGA was mixed with SDS buffer (2% SDS. 30% glycerol. 0.25 M Tris hydroxy aminomethane (pH 6.8) and boiled for 2 min. Exactly 41.86 g of 3-morpholinopropane-1-sulfonic acid (MOPS) running buffer powder (Cat No. M00138) was dissolved in a 1L volumetric flask of deionized water to make 1 L 1x running buffer. The ExpressPlusTM PAGE Gel was removed from the pouch, the seal peeled from the bottom of the gel cassette and properly inserted into the electrophoresis cell and the clip clamped to the gel cassette firmly. The comb was removed from the gel cassette gently and the running buffer was poured into the inner tank of the gel running apparatus to cover the sample wells by 5-7 mm after which the outer tank was also filled with 1x MOPS running buffer to ensure proper cooling. The buffer in the outer tank was made to be slightly above the top level of the sample wells.

The sample wells were rinsed thoroughly with 1x running buffer to remove air bubbles and storage buffer. The purified product was loaded directly into the wells as well as the marker (AccuLadder^{TM} protein size marker). The tank was closed and the power turned on and allowed to run. The electrophoresis was carried out at constant voltage of 100V (Biorad. power pack) for a period of 4 hours. After the front has moved to the bottom, the power was turned off and the gel cassette removed from the apparatus. The gel cassette was carefully opened by carefully inserting the cassette opener into the gap between the two plates and wiggled up and down gently to separate the two plates.

After that, the gel was fixed with 60% ethanol and thoroughly washed with distilled water so as to remove SDS. The gel was then equilibrated with 3% acetic acid and there after stained with a basic dye Coomassie blue-250 to visualize the polymer. The excess dye was washed off with 3% acetic acid several times.

3.3 Experimental Design for the Optimization Process

3.3.1 γ-PGA production by solid state fermentation (SSF)

Solid State Fermentation (SSF) was carried out according to the method described by Iluyemi *et al*. (2006). The procedure was conducted using 10 mL tubes containing 2 g of rice husk. Constituent medium components and process parameters were adjusted based on the experimental design (Table 3.1) and the tubes were incubated at the set temperature. The crude γ-PGA were acquired by mixing the content of the fermentation tubes with 5 ml of sterile distilled water and the contents agitated in a rotary shaker at 120 rpm for 50 min at room temperature to initiate γ-PGA extraction. The dissolved medium was spinned at low speed of 100 rpm for 5 min to remove the debris of solid medium and cell mass. The supernatant was further spinned at 4ºC and 6000 rpm for 30 min to sediment the products.

The pellets were re-suspended in distilled water and the procedure repeated twice to remove impurities so as to obtain purified γ-PGA (Riyadi *et al.,* 2017).

3.3.2 Definitive screening design

Definitive Screening Design (DSD) was used to screen the potential medium compositions and process conditions for γ-PGA production. Eleven factors comprising the medium components (ammonium sulfate, DON, citric acid, glucose, glutamic acid, glycerol, and percentage moisture content) and process conditions (pH, particle size, incubation time and temperature) were screened, with three levels of each factor (low (-1), medium (0) and high (+1) (Table 3.2 and Appendix III and IV). α was set and coded as 1.682 so as to cover 2 standard deviations of the mean of the distribution, assuming the data to be drawn to be approximately normal distributions and runs along any axes drawn from the middle of the cube through the centre of each face cube keeping micronutrient and vitamin requirements constant (0.1 g of KH_2PO_4, 0.1 g of $Na_2HPO_4.12H_2O$, 0.05 g of $MgSO_4 \cdot 7H_20$, 0.002 g of $MnSO_4$, 0.005 g of $FeC1_3 \cdot 6H_2O$, 0.02 g of $CaCl_2$ and 50 µg of biotin per 100 g of production medium) (Appendix V) (Goto and Kunioka, 2014). Quantity of γ-PGA was expressed in mg/gds of response (*y*). A set of 27 experimental designs was generated (Table 3.2) using Design Expert version 11.0 (State Ease, Minneapolis, USA). All the experimental runs were conducted in triplicate and presented as average values, plus or minus standard deviation (Mean ± S.D). Factors with least statistical significance in the DSD were kept constant in the central composite design (CCD) response surface design (Tai *et al.,* 2015).

Table 3.1: Set Ranges and Levels of the Independent Factors for DSD

SN	Factors	Units	Low(-1)	High (+1)
1	$(NH_4)_2SO_4$	g/100g	0.5	1.5
2	DON	µg/100g	1.25	8.5
3	Citric acid	g/100g	2.5	5.5
4	Glucose	g/100g	2.5	5.5
5	Glutamic acid	g/100g	2.5	5.5
6	Glycerol	ml/100g	2.5	5.5
7	Initial pH		6.8	8.0
8	Incubation time	Days	3.0	7.0
9	Temperature	°C	30	40
10	Moisture content	%v/w	40	70
11	Particle size	mm	0.15	0.42

Table 3.2: Design Matrix Code for Definitive Screening Design

Std	Run	A	B	C	D	E	F	G	H	I	J	K
10	1	+1	-1	+1	-1	+1	+1	+1	-1	-1	+1	-1
5	2	+1	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1
3	3	+1	+1	-1	+1	+1	-1	+1	-1	+1	-1	-1
23	4	+1	+1	-1	-1	+1	+1	-1	+1	+1	+1	-1
9	5	+1	+1	-1	-1	-1	-1	+1	+1	-1	-1	+1
16	6	+1	-1	-1	+1	-1	-1	-1	-1	+1	+1	+1
14	7	+1	-1	-1	+1	-1	+1	+1	+1	-1	+1	-1
17	8	-1	-1	-1	+1	+1	-1	-1	+1	-1	-1	-1
4	9	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1	-1
20	10	+1	+1	+1	+1	+1	-1	-1	+1	-1	+1	+1
25	11	0	0	0	0	0	0	0	0	0	0	0
24	12	-1	+1	-1	+1	+1	+1	+1	-1	-1	+1	+1
21	13	-1	-1	+1	+1	+1	+1	-1	-1	+1	+1	-1
12	14	+1	-1	+1	-1	-1	-1	-1	+1	+1	-1	-1
27	15	0	0	0	0	0	0	0	0	0	0	0
22	16	-1	+1	-1	+1	-1	+1	-1	+1	+1	-1	+1
7	17	-1	-1	-1	-1	+1	-1	+1	+1	+1	+1	+1
1	18	+1	-1	-1	-1	+1	+1	-1	-1	-1	-1	+1
13	19	-1	+1	+1	+1	-1	-1	+1	+1	+1	+1	-1
11	20	-1	-1	+1	+1	-1	-1	+1	-1	-1	-1	+1
26	21	0	0	0	0	0	0	0	0	0	0	0
18	22	+1	-1	+1	+1	+1	+1	+1	+1	+1	-1	+1
8	23	-1	-1	-1	-1	-1	+1	+1	-1	+1	-1	-1
15	24	-1	-1	+1	-1	-1	+1	-1	+1	-1	+1	+1
2	25	-1	+1	+1	-1	+1	-1	-1	-1	+1	-1	+1
19	26	+1	+1	+1	+1	-1	+1	-1	-1	-1	-1	-1
6	27	-1	+1	-1	-1	-1	-1	-1	-1	-1	+1	-1

Factor A: $(NH_4)_2SO_4$ (g/100g of medium), Factor B: DON (mg/100g of medium), Factor C: Citric acid (g/100g of medium), Factor D: Glucose (g/100g of medium), Factor E: Glutamic acid (g/100g of medium), Factor F: Glycerol (ml/100g of medium), Factor G: Initial pH, Factor H: Incubation time (Days), Factor I: Temperature (°C), J: Percentage moisture content (%v/w), Factor K: Particle size (mm), Factor L: Response: γ-PGA Yield (mg/gds of medium) and Std: Standard run order from design

3.3.3 Central composite design (CCD) method

Appropriate ranges (Appendix VI) were selected based on the data generated from the DSD analysis. Significant factors; DON, citric acid and glycerol from the DSD were optimized using central composite design of response surface methodology (RSM) to obtain the optimum fermentation conditions for maximal γ-PGA production. A set of 20 experimental runs with 6 centre and 14 axial points was generated as shown in Table 3.3 using Design Expert. Each factor was studied at three levels [low (-1), medium (0) and high (+1)] (Appendix VII and VIII). The results obtained were used to generate a response surface regression model by evaluating the regression coefficient values and analysis of variance (ANOVA). The general representation of the model is shown in Equation 3.2.

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j + e \quad \ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 3.2)$$

Where y is the predicted response (quantity of γ-PGA in mg/L), β_0 represent the intercept, β_i; β_{ij} and β_{ii} are coefficients for linear, interaction and quadratic regressions respectively while, x_i and x_j on the other hand, denote the independent variables (Tsapatsaris and Kotzekidou, 2004; Rajendran and Thangavelu, 2012).

Table 3.3: Design Matrix Code for Central Composite Design

Runs	A	B	C
1	-1	-1	-1
2	+α	0	0
3	-1	-1	+1
4	1	+1	+1
5	0	- α	0
6	-1	+1	+1
7	0	0	0
8	+1	-1	+1
9	-1	+1	-1
10	+1	-1	-1
11	0	0	- α
12	0	0	0
13	0	0	+α
14	+1	+1	-1
15	0	0	0
16	0	0	0
17	- α	0	0
18	0	0	0
19	0	0	0
20	0	- α	0

Where A: DON, B: Citric acid and C: Glycerol

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3.4 Data Analysis

All analyses were carried out in triplicates and expressed as mean±SD. One-way ANOVA ($p \leq 0.05$) were internally generated by design expert software to estimate the levels of significance. 3D response surface plots were used to represent the outcome and effects of independent factors. Also to evaluate the optimization model, ANOVA and the response surface regression procedure of Design Expert version 11.0 (Stat-Ease Inc., Minneapolis, USA) was used.

CHAPTER FOUR

4.0 RESULTS

This chapter presents the results of various laboratory procedures and experiments designed. The results are presented in sequence of the objectives of this research. The proximate analyse of rice husk used in this study is presented in Appendix II.

4.1 Isolates Obtained from Collected Samples of Water, Soil and *Daddawa*

From the colony morphological characteristics of the microbial growth on streaked nutrient agar plates, the colonies that showed white viscous spreading were picked and further sub-cultured. Only isolates from soil samples gave positive results of wide and spreading, white viscous colonies. The isolates from water and *daddawa* samples were likely to be bacteria of other genera, hence were discarded. From the positive colony identified, the best twelve were sub-cultured on nutrient agar four times to further purify the isolate. The purified isolates were labelled S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11 and S12 (Table 4.1) respectively.

4.1.1 Microscopic and biochemical characteristics of the isolates

4.1.1.1 Microscopic Characteristics of the isolates

The microscopic morphology of isolates S1, S2, S4, S5 and S12 was confirmed to be gram-positive as shown in Table 4.1.

4.1.1.2 Biochemical characteristics of the isolates

Isolates S1, S2, S4 and S5 were motile showing diffused growth (Table 4.2). Isolate S12 on the other hand was found to be non-motile because the growth was confined to the stabbed line with definite margins without spreading to surroundings area. Methyl red and Voges-

proskaeur (MR-VP) tests for the production of stable acids end products from fermentation of supplied glucose were all negative for isolates S1, S4 and S5. This showed that the isolates were not mixed acid producers. On the other hand, S2 and S12 gave a positive result of red coloration on addition of methyl red indicator. The α-naphhtol (Voges-proskaeur) test gave positive results on isolates S1, S4 and S5. *B. subtilis* do not decompose tryptophan into indole. When inoculated media of the isolates were treated with Kovac's Reagent (hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol), the yellow coloration of the solution remained indicative of negative results for all the samples. On the citrate utilization test all the sample isolates gave positive results in the presence of bromothymol blue indicator, changing the colour of the medium from light green to blue due to alkaline reaction is an indication of citrate utilization by the test organism. Also, oxidase test was used to detect the presence of cytochrome oxidase system that can catalyse the transport of electrons between electron donors in the bacteria and a redox dye; tetramethyl-*p*-phenylene-diamine. Some of the isolates gave positive results while others were nonreactive. This cannot be used to conclude on the identity of the isolates because it depends on the condition of growth of the microorganism. Hence the biochemical tests and spore staining technique confirmed three out of the five isolates (S1, S4, and S5) as *B. subtilis*. While S2 and S12 were not of the *B. subtilis* isolates. The results of the biochemical and spore staining are presented in Table 4.2.

The results of the spore staining showed centrally located endospores in all the isolates which were typical to *B. subtilis* species. The results of the biochemical analysis for three of the sample isolates; S1, S4 and S5 agree with the standard biochemical tests for *B. subtilis* species, while isolate S2 and S12 failed some of the biochemical tests for *B. subtilis* as such were discarded.

Table 4.1: Gram Staining Characteristics of the Isolates

SN	Isolates	Shape	Colour	Gram Stain Reactions
1	S1	Rod	Purple	+
2	S2	Rod	Purple	+
3	S3	Spherical	Pink	-
4	S4	Spherical	Pink	+
5	S5	Rod	Purple	+
6	S6	Spherical	Purple	-
7	S7	Spherical	Pink	-
8	S8	Spherical	Pink	-
9	S9	Spherical	Pink	-
10	S10	Spherical	Pink	-
11	S11	Spherical	Pink	-
12	S12	Rod	Purple	+

S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11 and S12 are the Isolates;

+: Positive test observation

-: Negative test observation

Table 4.2: Biochemical and Spore Staining Characteristics of the Isolates

SN	Tests	S1	S2	S4	S5	S12
1	Motility	+	+	+	+	-
2	Oxidase	+	-	+	+	-
3	Methyl red	-	+	-	-	+
4	Voges-Proskauer	+	+	+	+	-
5	Indole	-	-	-	-	-
6	Citrates	+	+	+	+	+
7	Spore Staining	Endo	Endo	Endo	Endo	Endo

S1, S2, S4, S5 and S12 are Isolates;

+: Positive test observation

-: Negative test observation

4.2 Molecular characteristics of 16S rRNA gene of the isolates

Segment of the isolates' 16S rRNA gene was successfully amplified from DNA extracted from the isolates. The PCR products for isolate S1 gave band of about 3000bps, isolate S4 gave four bands which could be as a result of breakage of the DNA strand (Figure 4.1). One of the bands corresponds to that of isolate S5 with about 2500bps. The sequence BLAST analysis on National centre for biotechnology information (NCBI) BLAST site (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed close similarity to *B. subtilis* with percentage identity scores of more than 90%. The specific results for each sample isolates were presented in Table 4.3. Isolate S1 had 95.73% percentage similarity and E-value score of 0.00. Isolates S4 and S5 on the other hand have percentage similarity and E-value scores of 91.26%; 91.27% and 0; 4.00E-176 respectively.

4.3 Screening and Selection of Best γ-PGA Producing Isolate

The results of the screening experiment for the selection of the best γ-PGA producer are presented in (Figure 4.2). Isolate S5 gave a significant higher yield of 2.50±0.30 mg/mL compared to isolate S1 and S4 with yields of 1.40±0.20 mg/mL and 1.3±0.00 mg/mL at P-value of 0.05, therefore isolate S5 was selected for further studies and optimization.

4.4 Minimum Inhibitory Concentration of DON against *B. subtilis*

The minimum inhibitory concentration of DON on the isolate that significantly decreased the growth of *B. subtilis* was estimated to be 40 μg/mL by MIC endpoint (Appendix XI).

4.5 *In silico* characteristic binding of γ-PGA and DON

The docking results in Table 4.4 showed nine possible binding sights with binding energies greater than -3 kcal/mol and are all statistically significant.

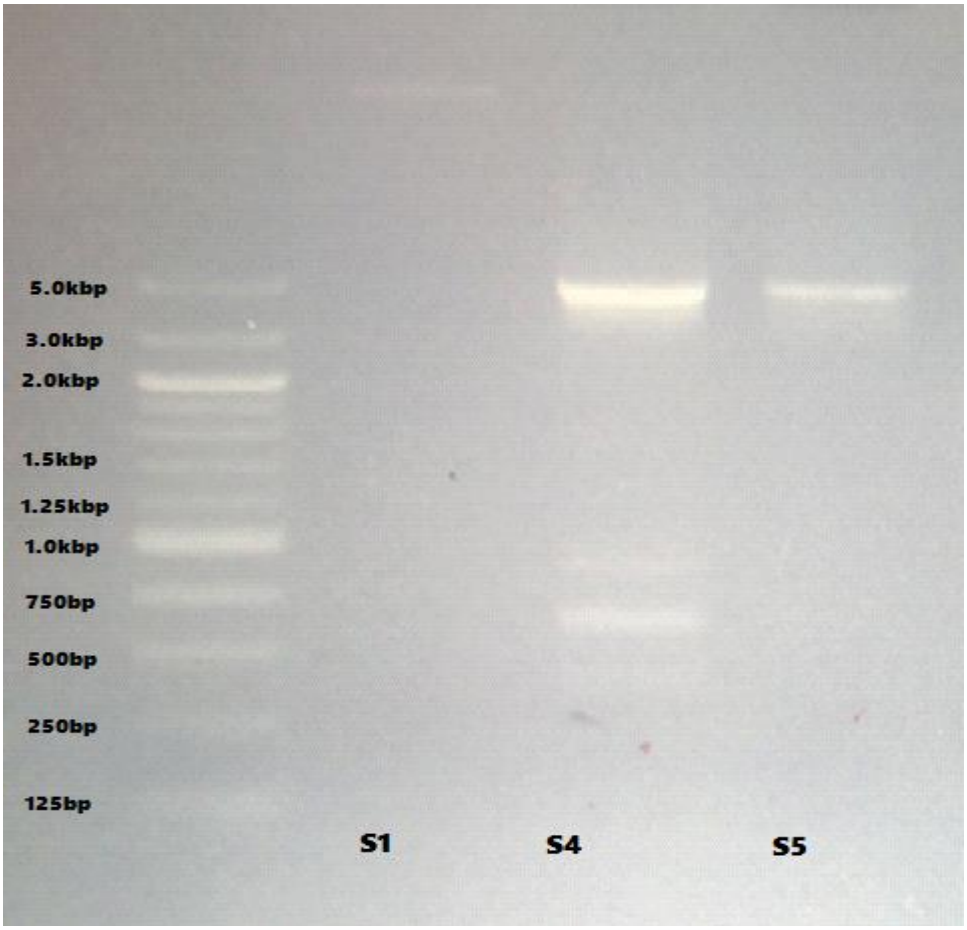


Figure 4.1: Electropherogram of isolates 16S rRNA gene PCR products

Lane 1: Ladder, lane 2: Isolate S1 16S rRNA amplicons, lane 3: Isolate S4 16S rRNA amplicons and lane 4: Isolate S5 16S rRNA amplicons

Table 4.3: Sequence BLAST Analysis of Isolates' 16S rRNA Gene

S/N	Isolates	Matches	NCBI Accession	Max Score	Total Score	E value	Identity
1	S1	*Bacillus subtilis* 16S rRNA gene	MW 785886-785887	1168	1168	0	95.73%
2	S4	*Bacillus subtilis* 16S rRNA gene	MW805751	990	990	0	90.54%
3	S5	*Bacillus subtilis* 16S rRNA gene	MW 805756-805757	616	616	2.00E-174	91.04%

E-value: Expectation of finding a sequence by chance.

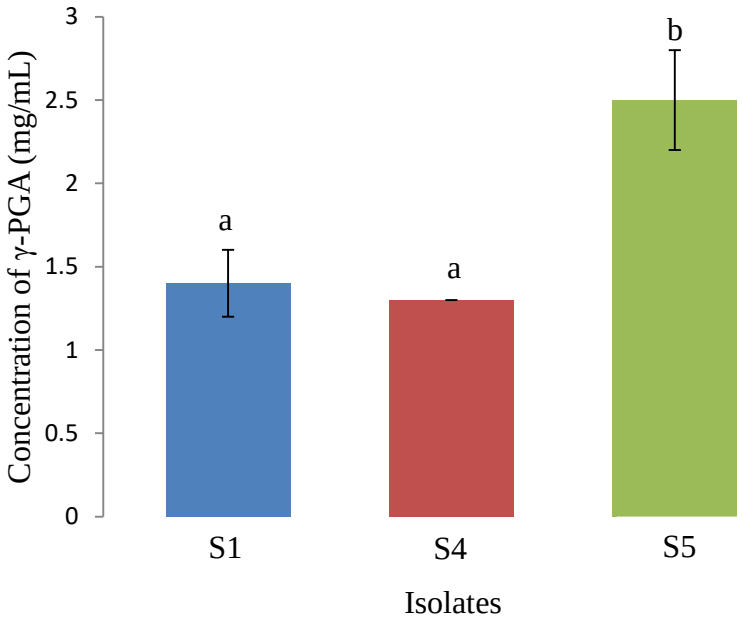


Figure 4.2: γ-PGA yields of isolates in submerged broth

Table 4.4: *In silico* docking of *γ-GTP* Inhibition Kinetics by DON

Mode	Binding Affinity (kcal/mol)	RMSD lower bound	RMSD upper bound
1	-4.2	0.000	0.000
2	-4.2	1.839	4.301
3	-4.1	16.815	18.422
4	-4.1	2.921	5.249
5	-4.0	2.887	4.615
6	-4.0	13.071	13.447
7	-4.0	2.925	4.244
8	-4.0	3.409	4.299
9	-4.0	12.329	12.599

RMSD: Root-mean-square deviation of atomic positions

4.6 Definitive Screening and Optimization of Production of γ-PGA

4.6.1 Definitive screening of medium components and conditions

The results of γ-PGA yield in milligram per gram dry substrate (mg/gds) from the definitive screening experiment are shown in Table 4.5.

4.6.2 Normal analysis of DSD experiments responses

The analysis of the definitive screening design by half and normal plots (Figure 4.3) showed that four of the independent factors have the highest effects on the yield. The effect of temperature was largely negative, while citric acid, glycerol and DON have positive impact on the yield in a descending degree respectively. The rest of the factors; Glucose, moisture contents, particle size showed positive effects but are not statistically significant. On the other hand, glutamic acid, incubation time, ammonium sulphate and initial pH gave statistically insignificant effects. There were interactions also among the factors. The interaction between particle size and moisture content, DON and glutamic acid, glycerol and temperature as well as DON and moisture contents gave statistically insignificant positive effects. In the same vein, DON and incubation time; glycerol and initial pH; ammonium sulphate, DON and citric acid; initial pH and particle size; initial pH and moisture content; DON and particle size; citric acid and incubation time as well as ammonium sulphate and DON gave a negative interaction effects on the γ-PGA yields at (P-value ≤ 0.05).

Table 4.5: γ-PGA Response from DSD of Medium Components and Conditions

STD	A	B	C	D	E	F	G	H	I	J	K	Response
1	1.5	0.05	2.5	2.5	5.5	5.5	6.8	3	30	40	0.42	8.25±4.50
2	0.5	0.35	5.5	2.5	5.5	2.5	6.8	3	40	40	0.42	9.05±3.60
3	1.5	0.35	2.5	5.5	5.5	2.5	8.0	3	40	40	0.15	15.62±1.70
4	0.5	0.35	5.5	2.5	5.5	5.5	8.0	7	30	40	0.15	3.82±2.90
5	1.5	0.35	5.5	2.5	2.5	5.5	8.0	3	40	70	0.42	6.61±1.90
6	0.5	0.35	2.5	2.5	2.5	2.5	6.8	3	30	70	0.15	3.55±0.10
7	0.5	0.05	2.5	2.5	5.5	2.5	8.0	7	40	70	0.42	7.45±1.90
8	0.5	0.05	2.5	2.5	2.5	5.5	8.0	3	40	40	0.15	12.53±7.70
9	1.5	0.35	2.5	2.5	2.5	2.5	8.0	7	30	40	0.42	15.38±7.80
10	1.5	0.05	5.5	2.5	5.5	2.5	8.0	3	30	70	0.15	13.32±1.47
11	0.5	0.05	5.5	5.5	2.5	2.5	8.0	3	30	40	0.42	11.20±1.33
12	1.5	0.05	5.5	2.5	2.5	2.5	6.8	7	40	40	0.15	15.50±1.93
13	0.5	0.35	5.5	5.5	2.5	2.5	8.0	7	40	70	0.15	12.23±1.22
14	1.5	0.05	2.5	5.5	2.5	5.5	8.0	7	30	70	0.15	7.050±2.20
15	0.5	0.05	5.5	2.5	2.5	5.5	6.8	7	30	70	0.42	9.20±5.50
16	1.5	0.05	2.5	5.5	2.5	2.5	6.8	3	40	70	0.42	4.08±0.60
17	0.5	0.05	2.5	5.5	5.5	2.5	6.8	7	30	40	0.15	6.22±1.50
18	1.5	0.05	5.5	5.5	5.5	5.5	8.0	7	40	40	0.42	8.97±6.30
19	1.5	0.35	5.5	5.5	2.5	5.5	6.8	3	30	40	0.15	4.07±0.50
20	1.5	0.35	5.5	5.5	5.5	2.5	6.8	7	30	70	0.42	12.06±1.03
21	0.5	0.05	5.5	5.5	5.5	5.5	6.8	3	40	70	0.15	6.51±3.40
22	0.5	0.35	2.5	5.5	2.5	5.5	6.8	7	40	40	0.42	4.30±2.20
23	1.5	0.35	2.5	2.5	5.5	5.5	6.8	7	40	70	0.15	4.53±0.00
24	0.5	0.35	2.5	5.5	5.5	5.5	8.0	3	30	70	0.42	38.83±4.02
25	1.0	0.2	4.0	4.0	4.0	4.0	7.4	5	35	55	0.285	4.70±0.60
26	1.0	0.2	4.0	4.0	4.0	4.0	7.4	5	35	55	0.285	14.08±1.42
27	1.0	0.2	4.0	4.0	4.0	4.0	7.4	5	35	55	0.285	8.03±0.30

Factor A: $(NH_4)_2SO_4$ (g/100g of medium), Factor B: DON (μg/100g of medium), Factor C: Citric acid (g/100g of medium), Factor D: Glucose (g/100g of medium), Factor E: Glutamic acid (g/100g of medium), Factor F: Glycerol (ml/100g of medium), Factor G: Initial pH, Factor H: Incubation time (Days), Factor I: Temperature (°C), J: Percentage moisture content (%v/w), Factor K: Particle size (mm), Factor L: Response: γ-PGA Yield (mg/gds of medium) and Std: Standard run order from design.

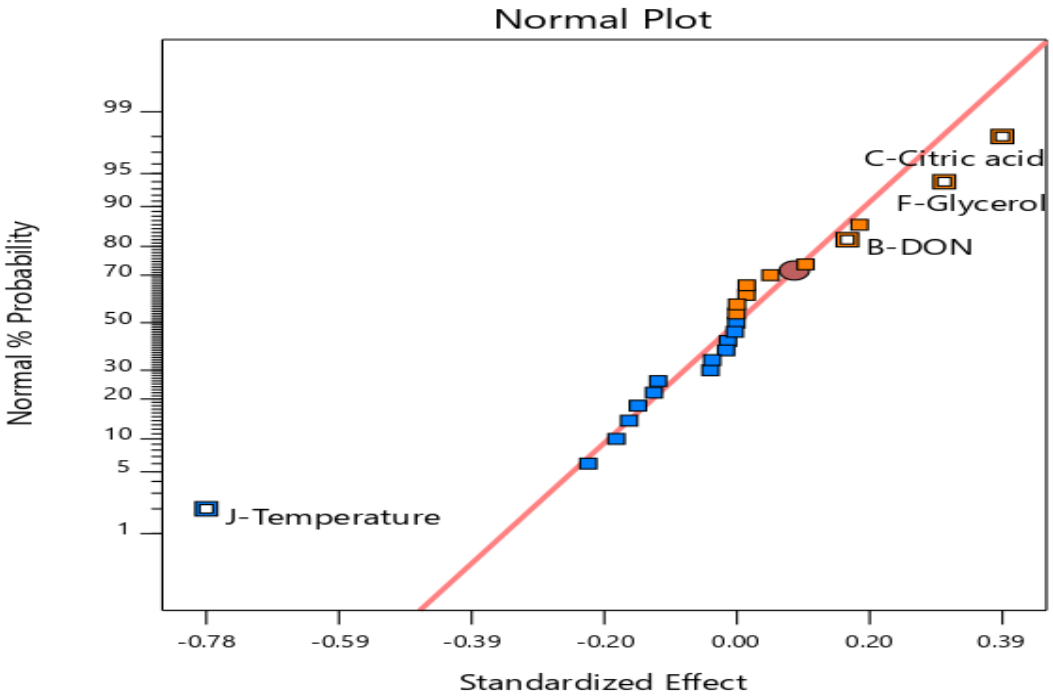


Figure 4.3: Normal plot of effects of independent factors and interactions on γ-PGA yield from the DSD screening

4.6.3 One-way ANOVA of selected significant factorial model's independent factors

The ANOVA and regression analysis of the results showed that the design is partial (III) design. The lack of fit showed that it is not significant with F-value of 2.05 and percentage error probability of 37.93% and core total degree of freedom of 26 as shown in Table 4.6. Of all the factors under study, temperature gave the largest negative effect indicative of the fact that the optimum temperature suitable for the experiment lies far to the lower set value of temperature in the definitive design. Due to the large negative effect of temperature, it was excluded in the optimization experiment and kept constant at the optimum growth temperature of *B. subtilis*. The total sum of squares of the model was 5.35 with four of the significant factors selected. The residual sum of squares from other least effect factors and interaction terms were 6.92 and 22 degrees of freedom while the pure error sum of squares was 0.32 and degrees of freedom of 2 (Table 4.6).

4.6.4 Response surface optimization by central composite design

Three of the significant factors from the screening; Citric acid, glycerol and DON were optimized using the central composite design, keeping the rest of the medium components and conditions constant at the medium level (0) as applied in the DSD. The experiment was design with 6 centre points, 14 axial points based on levels; -α, -1, 0, +1 and +α of each of the factors in the design. The result of the FCCD optimization is presented in Table 4.7. The central points showed reasonable agreement within the level of experimental design with an average of about 15.60 mg/gds. The concentration of γ-PGA tends to increase with decreasing concentrations of DON and citric acid. Glycerol also on the other hand showed a positive trend by increasing yield with increasing concentration glycerol.

4.6.5 Summary of fit statistics of developed model

The fit analysis (Table 4.8) showed that the coefficient of regression (R^2) of 0.9558 and a standard deviation of 5.63 of the mean 159.02mg/gds. The Model F-value of 24.63 (Table 4.9) implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. On the other hand, P-values less than 0.05 indicate model terms are significant. In this case DON, Citric acid and Glycerol are all significant. Values greater than 0.1000 indicate the model terms are not significant. The Lack of Fit F-value of 1.74 implies the Lack of Fit is not significant relative to the pure error. This is a good indicator since we want the model to fit. The difference between adjusted and predicted R^2 was 0.1499 (0.9180-0.7691) which is reasonably within the generally accepted range of 0.2. The percentage coefficient of variability was 3.49 with adequacy precision of 18.736 (Table 4.9).

The ANOVA of selected quadratic terms (Table 4.9) showed that the design is robust and can be used for optimization. All the selected terms for the design were significant with p-values <0.0001 while the interactions were not statistically significant. The sum of squares of model terms 68.21 with mean square of 7.58 and 9 degrees of freedom from the selected factors and interactions that gave significant contributions to the yield with a reasonable F statistic of 24.63 which cannot be explained by noise of just less than 0.1%. The residual sum of squares was 3.08; mean square of 0.3077 and degrees of freedom of 10. With respect to the pure error, it returned the sum of squares of 1.12; 0.2247 mean square and degrees of freedom of 5. Coefficients of variability of the factors, interactions and quadratic terms are presented in Appendix IX.

Table 4.6: One-way ANOVA of Selected significant Factorial Model's Independent Factors

SN	Source	Sum of Squares	Df	Mean Square	F-value	p-value
1	Model	5.35	4	1.34	4.25	0.0107
2	B-DON	0.1619	1	0.1619		
3	C-Citric acid	0.9294	1	0.9294		
4	F-Glycerol	0.5676	1	0.5676		
5	J-Temperature	3.69	1	3.69		
6	Residual	6.92	22	0.3148		
7	Lack of Fit	6.60	20	0.3301	2.05	0.3793
8	Pure Error	0.3225	2	0.1612		
9	Cor Total	12.27	26			

Table: 4.7: Response surface optimization by full central composite design

Std	A:DON (µ/100g)	B:citric acid (%:w/w)	C:glycerol %:v/w)	γ-PGA Yield (mg/gds)
1	(-1)20	(-1)5	(-1)5	18.76±2.20
2	(+1)40	(-1)5	(-1)5	14.76±0.00
3	(-1)20	(+1)10	(-1)5	17.11±0.08
4	(+1)40	(+1)10	(-1)5	12.13±6.50
5	(-1)20	(-1)5	(+1)10	19.55±5.75
6	(+1)40	(-1)5	(+1)10	16.14±7.50
7	(-1)20	(+1)10	(+1)10	17.18±4.90
8	(+1)40	(+1)10	(+1)10	13.33±0.50
9	(-α)13.1821	(0)7.5	(0)7.5	18.95±0.85
10	(+α)46.8179	(0)7.5	(0)7.5	13.16±2.50
11	(0)30	(-α)3.29552	(0)7.5	16.37±2.68
12	(0)30	(+α)11.7045	(0)7.5	13.64±2.20
13	(0)30	(0)7.5	(-α)3.29552	14.87±4.45
14	(0)30	(0)7.5	(+α)11.7045	16.29±2.59
15	(0)30	(0)7.5	(0)7.5	16.33±4.75
16	(0)30	(0)7.5	(0)7.5	15.06±3.75
17	(0)30	(0)7.5	(0)7.5	15.85±6.50
18	(0)30	(0)7.5	(0)7.5	16.14±8.71
19	(0)30	(0)7.5	(0)7.5	16.18±1.25
20	(0)30	(0)7.5	(0)7.5	16.25±1.84

DON: 6-diazo-5-oxo-L-norluecine; γ-PGA: Poly gamma glutamic acid; Std. Standard run by design; +1: High, -1: Low, 0: medium, -α and +α: are axial points of error estimates; Std: Standard run order from design

Table 4.8: Summary of Fit Statistics of Developed Model

Std. Dev.	0.5547	R^2	0.9558
Mean	15.90	Adjusted R^2	0.9180
C.V. (%)	3.49	Predicted R^2	0.7691
		Adeq Precision	18.736

C.V. %: Percentage coefficient of variation; R^2: Coefficient of determination; Std. Dev.: Standard deviation.

Table: 4.9: One-way ANOVA of Selected FCCD Quadratic Model Terms

S/N	Source	Sum of Squares	df	Mean Square	F-value	p-value
1	Model	68.21	9	7.58	24.63	< 0.0001
2	A-DON	49.41	1	49.41	160.58	< 0.0001
3	B-citric acid	14.46	1	14.46	46.98	< 0.0001
4	C-glycerol	2.49	1	2.49	8.08	0.0175
5	AB	0.2521	1	0.2521	0.8191	0.3867
6	AC	0.3698	1	0.3698	1.20	0.2987
7	BC	0.1013	1	0.1013	0.3290	0.5789
8	A^2	0.3553	1	0.3553	1.15	0.3078
9	B^2	0.6612	1	0.6612	2.15	0.1734
10	C^2	0.0017	1	0.0017	0.0056	0.9419
11	Residual	3.08	10	0.3077		
12	Lack of Fit	1.95	5	0.3907	1.74	0.2792
13	Pure Error	1.12	5	0.2247		
	Core Total	71.28	19			

Df: Degree of freedom, AB: interaction of DON and citric acid, AC: interaction of DON and glycerol, BC: interaction of citric acid and glycerol, A^2: product of interaction of DON, B^2: Product of interaction of B and C^2: product of interaction of C

4.6.6 Three Dimensional Response Surface Plots of the Optimized Parameters

A response surface plot provides topography of the effects of interaction of two independent variables. The 3D Surface plot is a projection of contours accompanied with colour shadings in a three dimensional plot. The optimization factors; DON, Citric acid and Glycerol were plotted against each other as showed in Figures 4.4a, b & c. hence the regions of desirable yield from the 3D plot were selected at; DON =20.00 μg/100g , Citric acid =5.00% (w/w) and Glycerol= 10.00% (v/w) which gave a predicted mean of 17.25 mg/gds as shown in Table 4.10.

4.7 Validation Production of γ-PGA

The validation result gave a mean optimized yield of 15.35±5.63 mg/gds as against the predicted optimized yield of 17.25 mg/gds (Table 4.10). The constraints of the validation experiment are presented in Appendix X.

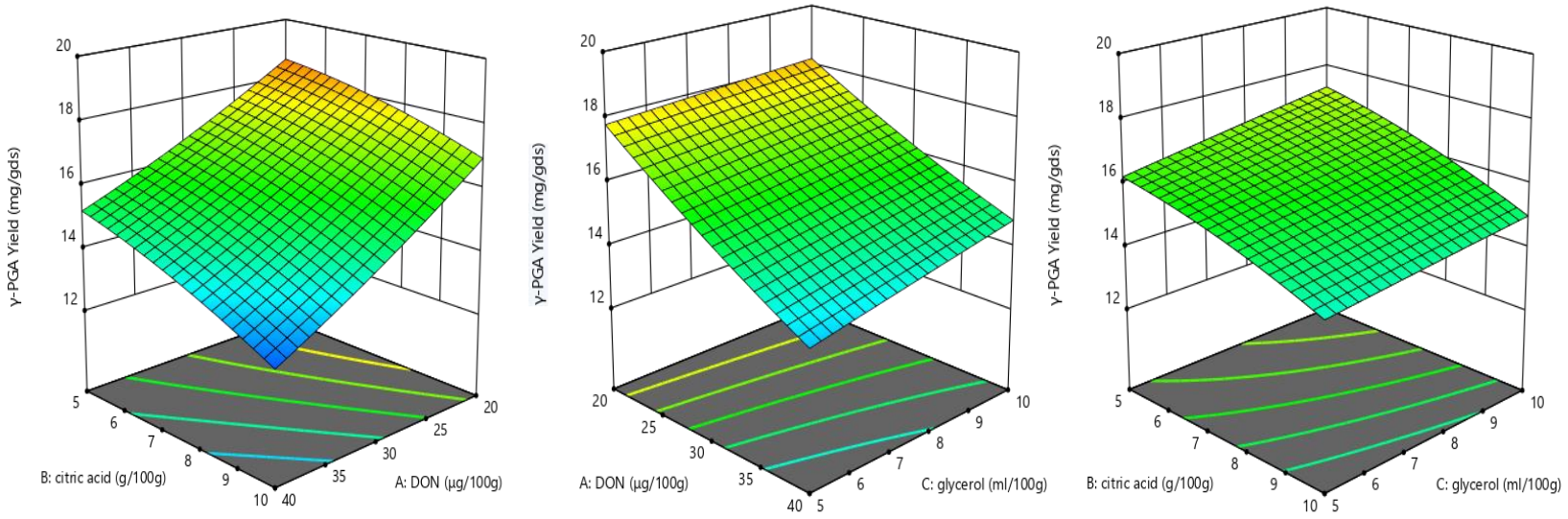
4.8 Model Equation

The second order equation of the developed quadratic model is shown in equation 4.1 with intercept of 15.9 mg/gds and error due to noise of 1.73 mg/gds.

$$y = 15.9 + (-1.95)A + (-1.03)B + (0.42)C + (-0.18)AB + (0.22)AC + (-0.11)BC + (0.16)A^2 + (-0.21)B^2 + (-0.01)C^2 + 1.73$$

…….(Equation 4.1)

Where y is the yield mg/gds and A, B and C are DON, citric acid glycerol respectively.



a b b

Figure 4.4: Response Surface Plots (a = citric acid/DON, b = glycerol/DON and c = citric acid/glycerol) for γ-PGA Optimization

Table 4.10: Confirmation of designed experiment for the Production of γ-PGA

Factors			**Responses**	
DON	Glycerol	Citric acid	Observed	Predicted
25.00 μg/100g	10.00%	7.50%	15.35 mg/gds	17.25 mg/gds

4.9 Polymer Characterization

The amino acid composition and molecular weight of the produced γ-PGA were determined using HPLC amino acid analyser, FT-IR and SDS-PAGE.

4.9.1 Amino acid analysis of produced γ-PGA

The HPLC absorbance peaks of the derivatized γ-PGA digest were seen at 1.75 min and 1.93 min elution time using the FLDA1 fluorescence dictator system and a total concentration of 20 pmol/μL in the sample digest. But it was not able to quantify the amount of the isomer present (Figure 4.5)

4.9.2 FT-IR spectra of produced γ-PGA

The FTIR spectrum of the produced γ-PGA is shown in Figure 4.6. Specific regions of the IR spectrum were considered for the identification of specific functional groups. Those regions include the O-H, C=O stretching vibration characteristic of saturated aliphatic carboxylic acid dimers (1630–1680 cm^{-1}), C-O stretching peak assigned to carboxylic acid dimers (1320–1210 cm^{-1}), N-H/C-N conjugate stretching (1420-1300 cm^{-1}), and the broad O-H stretching (3000-2500 cm^{-1}).

There was a strong absorbance at 2974.4 cm^{-1} (80.56%) which corresponds to the absorbance O-H of carboxylic acid or conjugates of carboxylic acid. Another broad region of absorbance was at 3304.4 cm^{-1} (65.05%), this corresponds to region of absorbance of N-H of primary amines. This can be explained by the presence of free amino ends of the glutamic acid that are not involved in bonding. There was also an interesting absorbance at the N-H/C-N conjugate at 1408.9 cm^{-1} (79.9%) due to the presence of multiple conjugates of these functional groups in the molecule of γ-PGA. Also, C-O stretching absorbance was

observed at 1080.9 cm^{-1} (47.7%), this corresponds to that of primary alcohols and COO^- functional group. The result of the analyses of the spectrum is presented in Table 4.11.

4.9.3 Molecular weight of produced γ-PGA

The SDS-PAGE of the purified samples of the produced γ-PGA is shown in Figure 4.7. The product gave three distinct bands of 35, 44 and 96 kDa molecular weights with a profuse band at region of less than 10 kDa.

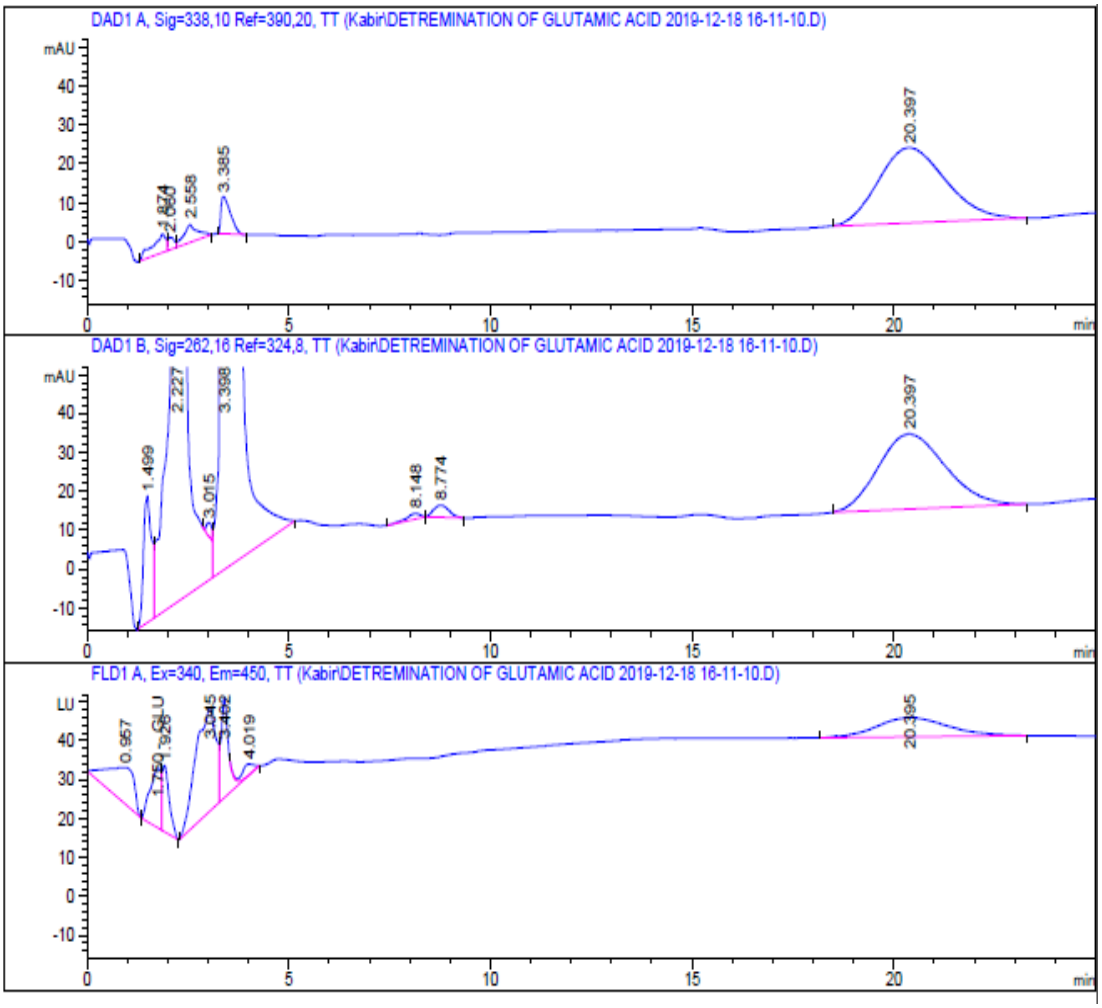


Figure 4.5: Absorbance peaks of γ-PGA digest analysis by HPLC

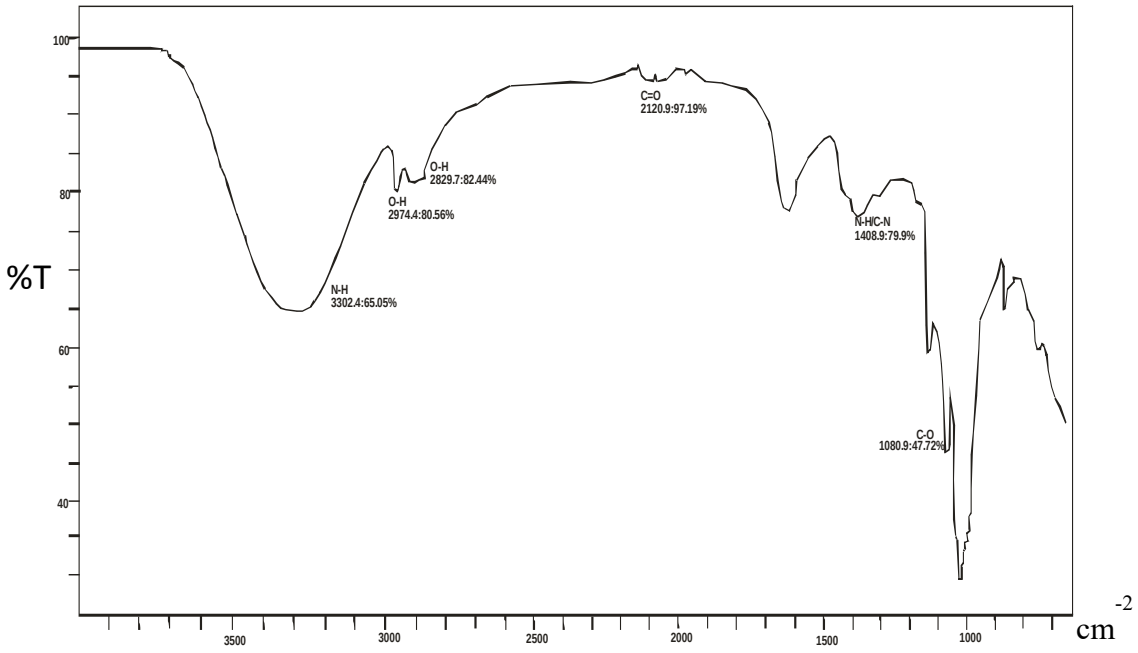


Figure 4.6: FT-IR spectrum of produced γ-PGA

Table 4.11: FT-IR Spectra Analyses of Produced γ-PGA

SN	IR Region (cm^{-1})	Absorbance peak	Functional Group	Compound Class
1	4,000-3,000	3302.4: (65.05%)	N-H stretching	primary amine
2	3,000-2,500	2974.4: (80.56%)	O-H stretching	Carboxylic acid
3	3,000-2,500	2829.7: (82.44%)	O-H stretching	Intermolecular bonds
4	2400-2000	2120.9: (97.90%)	C=O stretching	Conjugate anhydrides
5	1600-1300	1408.9: (79.90%)	N-H/C-N bending	γ-Conjugate bonds
6	1600-1300	1408.9: (47.72%)	C-H bending	Alkyl group
7	1400-1000	1080.9: (47.72%)	C-O stretching	Primary alcohol
8	1400-1000	1021.3: (29.24%)	C-O-O stretching	Anhydride
9	1000-650	879.7: (66.63%)	C-H bending	Substituted alkyl group

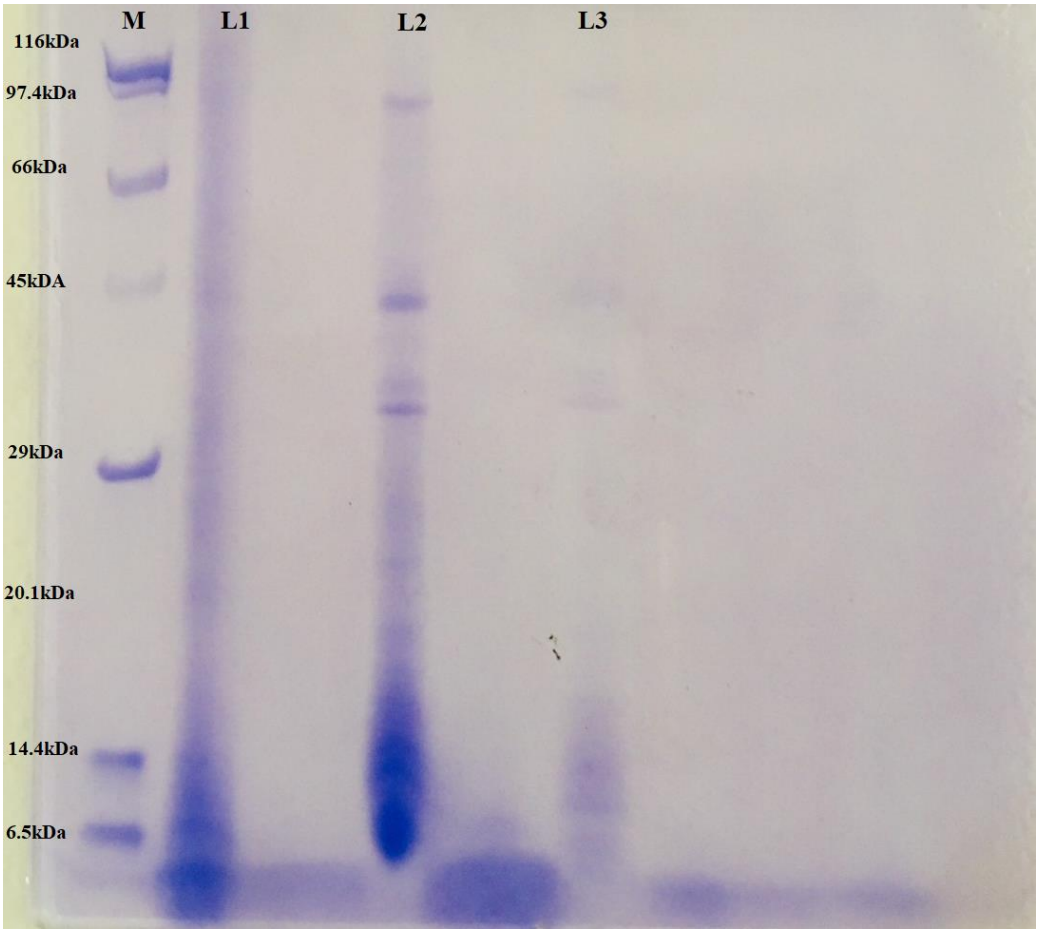


Figure 4.7: SDS-PAGE Electropherogram of Produced γ-PGA

M: Molecular weight marker, L2 and L3 γ-PGA

CHAPTER FIVE

5.0 DISCUSSIONS

From the samples collected for the isolation of *B. subtilis*, 3 were confirmed to be *B. subtilis* based on the total number of biochemical tests carried out. The molecular characterization of the isolates 16S rRNA gene also confirmed the isolate to have close similarities with other sequences submitted to the NCBI gene bank. i.e percentage similarities of 95.73%, 91.26% and 91.27% for isolate S1, S4 and S5 respectively. The E-values on the other hand are; 0 for isolate S1 and S4 and 2E-174 for isolate S5. The sequences can be accessed using the assession numbers of; MW785886-785887, MW805751 and MW 805756-805757 for isolate S1, S4 and S5 respectively.

The screening production gave the highest yield of 2.5 mg/mL with isolate S5. This agrees with the work of Ogunleye *et al*. (2015) where higher yield of 11.69 g/L were obtained by submerged production; Wang *et al*. (2020) with yield of 1.85 g/l/h productivity; Lin *et al*. (2016); Mohanraj *et al*. (2019) and John *et al*. (2020) where yield of 6.23 mg/g was obtained.

The minimum inhibitory concentration value of 40 μg/mL could be as the result of the established toxicity of DON due to the presence of the reactive diazo group that can alkylate other glutaminase enzymes such as Nicotinamide adenine dinucleotide synthase, Cytidine triphosphate synthase and Formylglycinamide ribonucleotide aminotransferase as reported by Rais *et al*. (2016). The *in silico* binding on the other hand is due to strong interaction in between the Histidine and other amino acid moiety and the reactive diazo group as previously reported by Teryan *et al*. (2017).

Furthermore, the results of the preliminary screening showed that citric acid, glycerol, DON, glucose, rice husk's particle size and percentage moisture have positive effects on γ-PGA yield in the medium. But only Citric acid, Glycerol and DON have significant positive effects on γ-PGA yield. The effect of citric acid on γ-PGA yield can be explained by the fact that citric acid is a TCA cycle intermediate and can directly contribute to the biosynthesis of γ-PGA in the cell.

The finding from this research showed that citric acid is an excellent carbon source for microbial fermentation of γ-PGA agrees with work of Goto and Kunioka (1992) who demonstrated the role of citric acid as excellent additional carbon source in γ-PGA fermentation using a glutamic acid dependant *B. subtilis* IF03335. Other TCA intermediates (L-malic acid, succinic acid and fumaric acid) showed no reasonable γ-PGA synthesis as reported by Goto and Kunioka (1992). Another research of note that supports this finding is that of Zhang *et al.* (2012). On the contrary, Xu *et al.* (2005) reported that citric acid has no significant effects on the yield of γ-PGA when working with *B. subtilis* NX-2. This could be as a result of strain metabolic peculiarities.

The effect of glycerol could be as a result of increase in the synthesis of phospholipids (Phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl serine, phosphatic acid and cardiolipin) in the cells of *B. subtilis* which enhances the effective secretion of produced γ-PGA being a basic polymer (Uttlova *et al*., 2016). In a study, Uttlova *et al*. (2016) using glycerol as supplementary carbon source demonstrated an increase in secretion of γ-PGA into the medium when the ratio of glycerol in the medium was increased without corresponding increase in cell growth. This increase could be as result of increase in secretion of γ-PGA into the extracellular environment with increase in the amount of glycerol in the medium as postulated that glycerol increases the biosynthesis of

phosphatidyl glycerol amongst other things. Xu *et al.* (2005) also reported a significant yield of γ-PGA when glycerol was used as additional carbon source. When they added 30 g/l of glycerol, they were able to obtain a yield of 24.3 g/L of γ-PGA.

It has been generally reported that glycerol influences γ-PGA production by stimulating poly glutamyl synthetase and minimizes γ-PGA chain length. Due to this fact, the viscosity of medium with higher amount of glycerol is usually lower compared to medium with little or no glycerol. This phenomenon allows for the uptake of more substrate hence improving cell growth and γ-PGA production (Ashiuchi, 2001a). Wu *et al.* (2010) demonstrated that glycerol significantly increases the production of γ-PGA in *B. subtilis* when they added 20 g/L glycerol into the medium; the yield was increased from 26.70 to 31.70g/l. In the same vein, they recorded lower molecular weight γ-PGA with higher amount of added glycerol. The molecular weight dropped from $2.43\pm0.07\times10^6$ to $1.86\pm0.06\times10^6$ Da.

The inhibitor 6-diazo-5-oxo-L-norleucine is a well-established inhibitor of human glutamyl transpeptidase. So far no work has been reported on the use of DON as inhibitor of γ-GTP enzyme. DON was able to decrease the degradation of the synthesized γ-PGA over time. Most of the reported works done without the inhibition of the degrading enzyme, three days was the length of time that gave highest yield without breakdown of the produced γ-PGA (Mohanraj *et al*., 2019; Zhang *et al*., 2019; Wang *et al*., 2020).

For glutamate independent *B. subtilis* strain, the utilization of glucose is central to the cellular biomass accumulation of the bacteria. A number of reports indicate that, glucose can increase the yield of γ-PGA when combined with other carbon sources such as glycerol, citric acid and other sugars. But when the glucose content exceeds a certain

optimum, it leads to the production of other polysaccharides alongside γ-PGA. When Wu *et al.,* (2008) maintained glucose concentration 10 g/L or lower, 24 g/L Poly-Glutamic acid by *Bacillus sp.* was produced without formation of polysaccharide side products. These reports are in concord with the results from this research. Addition of glucose increases the yield of γ-PGA in the medium with a contribution factor of about 1%.

The role of ammonium sulphate in the production of γ-PGA has been understood to be by donating ammonium ion to α-ketoglutarate and glutamine transaminase. Kunioka (1995) has demonstrated that the optimum concentration for best yield of γ-PGA in 100ml of broth is 1% (w/v). In concert to the results of Goto and Kunioka (1992), there was no reasonable effect by ammonium sulphate on the yield of γ-PGA. The lower concentration gave higher yield than when the concentration is increased above 1.5%.

A number of researchers have postulated that exogenous glutamic acid independent strain had the capability to synthesize glutamic acid as an essential substrate for the biosynthesis of γ-PGA (Zhang *et al.,* 2012). In this study, the addition of exogenous glutamic acid did not have any positive effect on the yield of γ-PGA; which makes it reasonable to conclude that the isolated strains is a glutamic independent strain. Therefore, it means that the glutamic acid molecules that were used by the isolate to synthesize γ-PGA came from the conversion of other supplied nutrients such as the citric acid, and nutrients from the rice husk. Endogenous glutamic acid can be derived from 2-oxoglutaric acid from the TCA cycle intermediate; α-ketoglutaric acid (Kunioka, 1997; Zhang *et al.,* 2012). Hence, the outflow of α-ketoglutarate from TCA cycle could be channelled by the microorganism to synthesize γ-PGA.

The effect of initial moisture content of γ-PGA yield was marginally significant. This result agrees with the report by Jian *et al.,* (2005) which demonstrated that moisture content is an important factor in γ-PGA production. The challenge mostly encountered during SSF is maintaining the moisture content at constant value due to evaporation since the culture environment is not mostly air tight. They also reported that the optimum percentage of moisture content for γ-PGA production is 60%–65%.

When rice husk was used as the only nutrient source without supplementation, the yield of γ-PGA obtained was low compared to when the medium components were optimized; 15.350±5.63 mg/gds. This result fairly agrees with the work of Song *et al*., (2019). In their work, they showed that the optimal yield of 22.64g/L of γ-PGA was obtained by submerged fermentation using 38.6 g/L of rice bran. A similar work was reported by Nie, *et al., (*2015) where an optimal yield of 144.5mg/g was reported using a mixture of rice husk and soy beans as the base substrate for the co-production of *Natto* kinase and γ-PGA. Odeniyi and Avoseh (2018), screened for cheap biomass and reported that cassava peels produced a γ-PGA yield of 22.26 g/L.

The result of the particle size showed a marginal positive effect on the yield of γ-PGA, the larger particle by implication showed the best result. This could be attributed to the fact that the larger particle allows for the circulation of air molecules better than the finer particles. *B. subtilis* is an aerobic microorganism that requires oxygen supply during fermentation. The large particle can be seen as good index for the utilization of rice husk in solid state fermentation to improve aeration. Largely the challenge with submerged fermentation for the production of γ-PGA is increased viscosity of the medium which limits the growth of the organism and consequently the production of the biopolymer.

The time course was discovered to be statistically not significant in influencing the yield of γ-PGA in the designed experiment. The yield variation between day 3 and day 7 were not significantly different. Although most reports have it that the maximum yield of γ-PGA were mostly in the day 3 of production, but with subsequent depletion of the product with time (Chen *et al.*, 2005; Jiang *et al.*, 2019). Jiang *et al.*, (2019) reported that the yield of γ-PGA showed a growing trend in the first 60 hours, especially in 18-60 hours by SSF and then decreased gradually. This result could be an excellent indication of the inhibition of γ-GPT enzyme activity by DON since there was no significant depletion of the produced γ-PGA with time after the first 3 days of culture growth. Also most workers have reported a decrease in cell growth after day 3 of culture which can be explained by the depletion of nutrient in the medium and increasing accumulation of toxic waste metabolites in the medium.

The effect of pH on the yield of γ-PGA was not significant. This could be as a result of a number of factors of which the metabolic diversity of *B. subtilis* is a major contributor. Secondly the spreading of the selected pH within a very narrow range around the optimal growth pH of the organism (pH 7.5) can be annulled with time. Jiang *et al.* (2019) reported that initial pH played a significant role on γ-PGA production when investigated under solid-state fermentation process. They showed that a slightly alkaline environment was favourable for the accumulation of γ-PGA with pH 8 giving the highest yield. Cromwick *et al.* (1995) studied the effect of pH in submerged fermentation at pH values of 5.5, 6.5, 7.4, and 8.25. The pH value of 8.25 gave the best yield at extended time. In a similar vein, they compared the controlled pH experiment with an uncontrolled set up and were able to show that there was no significant difference between pH 5.5 and the uncontrolled pH which disagree with the report of Jiang *et al*. (2019).

The molecular weight of the produced γ-PGA was determined by SDS-PAGE. From the result of the analysis, three distinct bands of 34, 45 and 96 kDa were obtained. The molecular weight of γ-PGA is dependent on fermentation organism, process conditions and medium's components (Najar and Das 2015). A number of works have reported similar results with solid state fermentation while higher molecular weights were obtained from submerged fermentation with shorter fermentation time.

Yong *et al.,* (2011) optimized the production of γ-PGA by *B. amyloliquefaciens* C1 in solid state fermentation using dairy manure compost and monosodium glutamate production residues as basic substrates and obtained γ-PGA with molecular weight of about 130 kDa. An interesting thing about our findings is the distinct molecular weight obtained.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This research focuses on the production of γ-PGA from rice husk considering the amount of underutilized husk produced from rice annually as well as enhancing production by inhibiting the activity of γ-PGA degrading enzyme in a newly isolated *B. subtilis* strain from Samaru, Zaria. Three strains (S1, S4 and S5) of *B. subtilis* were isolated of which S5 was had a significant yield of 2.5 mg/mL which can be considered a promising strain for the production of low molecular weight γ-PGA from rice husk. The concentration of DON that minimally inhibits the growth of *B. subtilis* was found to be 40 μg/mL.

The definitive screening revealed temperature, glycerol citric acid and DON to have the most significant impact on the yield of γ-PGA in solid state production using rice husk. The optimised product yield by central composite design was 15.35±5.63 g/gds of γ-PGA in solid state fermentation. In the same vein, addition of DON in production medium of γ-PGA by *B. subtilis* impact on the yield. Contributions to knowledge are presented in Appendix XII.

6.2 Recommendations

i. It is recommended that isolate S5 should be used for the production of low molecular weight γ-PGA using rice husk
ii. Other cheap biomass can also be studied using the obtained isolates to see if they can generate higher yield of γ-PGA.
iii. Further studies on the mechanisms and kinetics of inhibition of γ-GTP by DON should be carried out in order to better understand the kinetics of the inhibition.

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APPENDICES

Appendix I: Buffer preparations

1. Cation-Adjusted Mueller-Hinton Broth

Cation-Adjusted Mueller-Hinton Broth was prepared by weighing 23g Mueller Hinton broth into a beaker and dissolved with about 20ml of distilled water. The content of the beaker was then transferred into a 1000ml volumetric flask and make up with about 900ml of distilled water and the pH adjusted to 7.2 using concentrated hydrochloric acid. It was then made up to 1000ml mark with distilled water which was sterilized by autoclaving at 121ºC for 15min. After cooling to room temperature, 2ml 10g/L Ca^{2+} (3.68g $CaCl_2 \cdot 6H_2O$ in 100ml distilled water) and 1ml 10g/L Mg^{2+} (8.36g $MgCl_2 \cdot 2H_2O$ in 100ml distilled water) will be added.

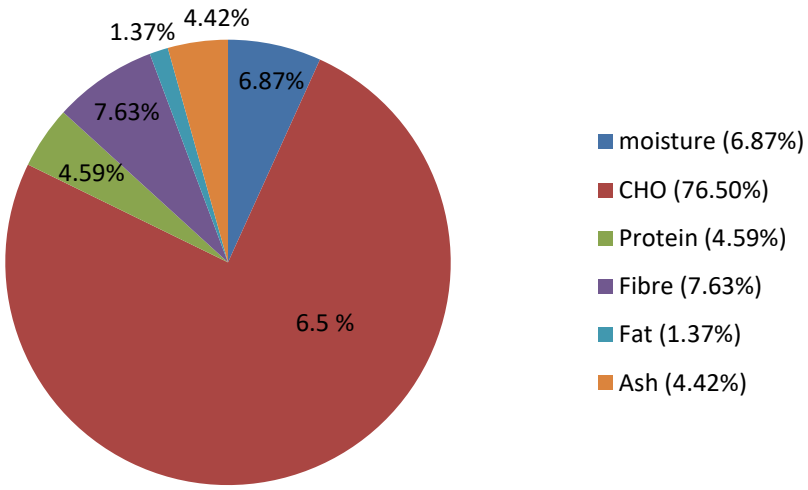
2. The phosphate buffered saline

The phosphate buffered saline was prepared by weighing; 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 into a beaker and adding about 20ml of distilled water to dissolve. It was then poured into a 1000ml volumetric flask and the beaker rinsed thoroughly into the flask. About 900ml of distilled water was added to the volumetric flask and thoroughly shaken to dissolve completely; the final pH was adjusted to pH to 7.2 using concentrated HCl acid and sterilized by autoclaving at 121°C for 15min.

3. Lysogeny broth (LB)-Lennox

Lysogeny broth (LB)-Lennox on the other hand, was prepared by weighing; 10g tryptone, 5g yeast extracts and 5g NaCl into a beaker. It was then dissolved with about 50ml of distilled water then transferred into a 1000ml flask autoclaved at 121ºC for 15min.

Appendix II: Proximate content of rice husk used in the study



Appendix III: Table of factors for Definitive Screening Design

Factors	**Units**	**Type**	**Changes**	**Std. Dev.**	**Low**	**High**
(NH4)2SO4	g/100g	Factor	Easy	0	0.5	1.5
DON	µg/100g	Factor	Easy	0	1.25	8.75
Citric acid	g/100g	Factor	Easy	0	2.5	5.5
Glucose	g/100g	Factor	Easy	0	2.5	5.5
Glutamic acid	g/100g	Factor	Easy	0	2.5	5.5
Glycerol	ml/100g	Factor	Easy	0	2.5	5.5
Initial pH		Factor	Easy	0	6.8	8
Incubation time	Days	Factor	Easy	0	3	7
Temperature	°C	Factor	Easy	0	30	40
Moisture content	%v/w	Factor	Easy	0	40	70
Particle size	Mm	Factor	Easy	0	0.15	0.42
γ-PGA Yield	mg/gds	Response		0		

Appendix IV: Design Matrix for Definitive Screening Design in actual set values

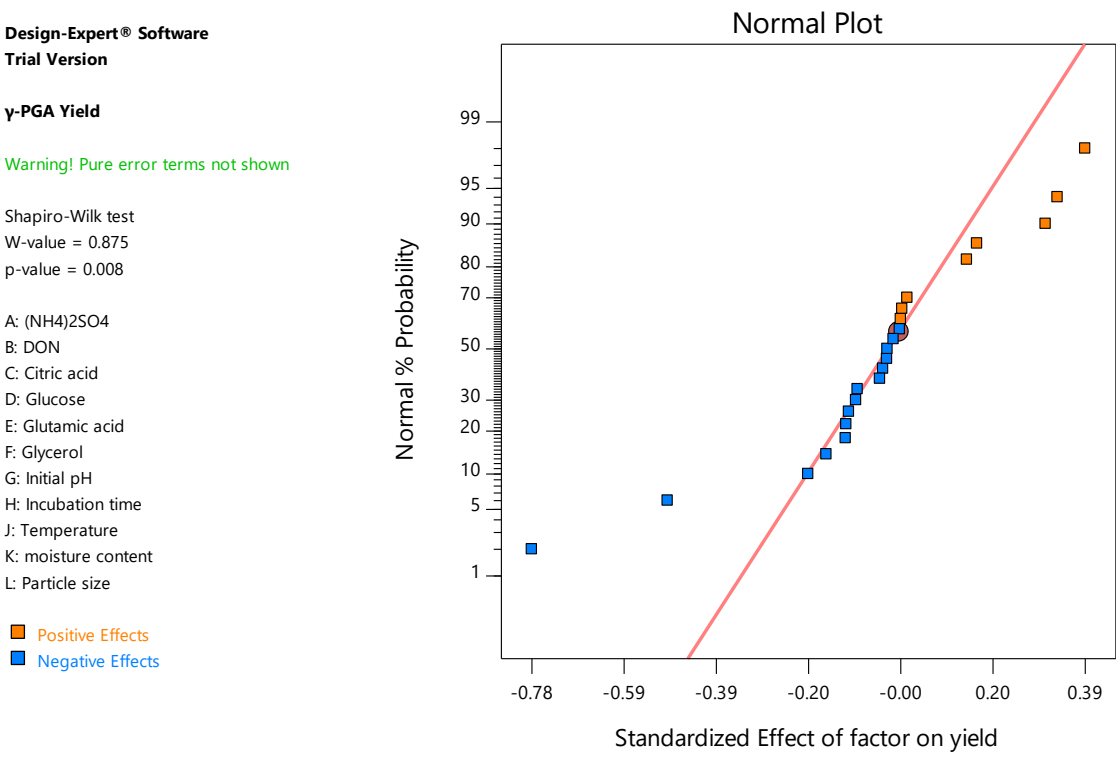
S/No	A	B	C	D	E	F	G	H	I	J	K
1	1.5	0.05	5.5	2.5	5.5	2.5	8.0	3	30	70	0.15
2	1.5	0.35	5.5	2.5	2.5	5.5	8.0	3	40	70	0.42
3	1.5	0.35	2.5	5.5	5.5	2.5	8.0	3	40	40	0.15
4	1.5	0.35	2.5	2.5	5.5	5.5	6.8	7	40	70	0.15
5	1.5	0.35	2.5	2.5	2.5	2.5	8.0	7	30	40	0.42
6	1.5	0.05	2.5	5.5	2.5	2.5	6.8	3	40	70	0.42
7	1.5	0.05	2.5	5.5	2.5	5.5	8.0	7	30	70	0.15
8	0.5	0.05	2.5	5.5	5.5	2.5	6.8	7	30	40	0.15
9	0.5	0.35	5.5	2.5	5.5	5.5	8.0	7	30	40	0.15
10	1.5	0.35	5.5	5.5	5.5	2.5	6.8	7	30	70	0.42
11	1.0	0.2	4.0	4.0	4.0	4.0	7.4	5	35	55	0.285
12	0.5	0.35	2.5	5.5	5.5	5.5	8.0	3	30	70	0.42
13	0.5	0.05	5.5	5.5	5.5	5.5	6.8	3	40	70	0.15
14	1.5	0.05	5.5	2.5	2.5	2.5	6.8	7	40	40	0.15
15	1.0	0.2	4.0	4.0	4.0	4.0	7.4	5	35	55	0.285
16	0.5	0.35	2.5	5.5	2.5	5.5	6.8	7	40	40	0.42
17	0.5	0.05	2.5	2.5	5.5	2.5	8.0	7	40	70	0.42
18	1.5	0.05	2.5	2.5	5.5	5.5	6.8	3	30	40	0.42
19	0.5	0.35	5.5	5.5	2.5	2.5	8.0	7	40	70	0.15
20	0.5	0.05	5.5	5.5	2.5	2.5	8.0	3	30	40	0.42
21	1.0	0.2	4.0	4.0	4.0	4.0	7.4	5	35	55	0.285
22	1.5	0.05	5.5	5.5	5.5	5.5	8.0	7	40	40	0.42
23	0.5	0.05	2.5	2.5	2.5	5.5	8.0	3	40	40	0.15
24	0.5	0.05	5.5	2.5	2.5	5.5	6.8	7	30	70	0.42
25	0.5	0.35	5.5	2.5	5.5	2.5	6.8	3	40	40	0.42
26	1.5	0.35	5.5	5.5	2.5	5.5	6.8	3	30	40	0.15
27	0.5	0.35	2.5	2.5	2.5	2.5	6.8	3	30	70	0.15

Factor A: $(NH_4)_2SO_4$ (g/100g of medium), Factor B: DON (mg/100g of medium), Factor C: Citric acid (g/100g of medium), Factor D: Glucose (g/100g of medium), Factor E: Glutamic acid (g/100g of medium), Factor F: Glycerol (ml/100g of medium), Factor G: Initial pH, Factor H: Incubation time (Day), Factor I: Temperature (°C), J: Percentage moisture content (%v/w) and Factor K: Particle size (mm).

Appendix V: Concentrations of medium components and moisture content adjustment for definitive screening design

Run	$(NH_4)_2SO_4$	DON	Citric acid	D:Glucose	Glutamic acid	F:Glycerol	Minerals	K:Moisture	Residual
	g/100g (ml)	mg/100g (ml)	g/100g (ml)	g/100g (ml)	g/100g (ml)	ml/100g (ml)	(ml)	%v/w(ml of H_20)	(ml of H_2O)
1	1.5(0.24)	1.25(0.03)	5.5(0.22)	2.5(0.10)	5.5(0.22)	2.5(0.20)	0.2	70(2.856)	1.646
2	1.5(0.24)	8.75(0.21)	5.5(0.22)	2.5(0.10)	2.5(0.10)	5.5(0.22)	0.2	70(2.856)	1.566
3	1.5(0.24)	8.75(0.21)	2.5(0.10)	5.5(0.22)	5.5(0.22)	2.5(0.20)	0.2	40(1.632)	0.262
4	1.5(0.24)	8.75(0.21)	2.5(0.10)	2.5(0.10)	5.5(0.22)	5.5(0.22)	0.2	70(2.856)	1.566
5	1.5(0.24)	8.75(0.21)	2.5(0.10)	2.5(0.10)	2.5(0.10)	2.5(0.10)	0.2	40(1.632)	0.582
6	1.5(0.24)	1.25(0.03)	2.5(0.10)	5.5(0.22)	2.5(0.10)	2.5(0.10)	0.2	70(2.856)	1.866
7	1.5(0.24)	1.25(0.03)	2.5(0.10)	5.5(0.22)	2.5(0.10)	5.5(0.22)	0.2	70(2.856)	1.746
8	0.5(0.08)	1.25(0.03)	2.5(0.10)	5.5(0.22)	5.5(0.22)	2.5(0.10)	0.2	40(1.632)	0.682
9	0.5(0.08)	8.75(0.21)	5.5(0.22)	2.5(0.10)	5.5(0.22)	5.5(0.22)	0.2	40(1.632)	0.382
10	1.5(0.24)	8.75(0.21)	5.5(0.22)	5.5(0.22)	5.5(0.22)	2.5(0.10)	0.2	70(2.856)	1.456
11	1(0.16)	5.00(0.125)	4(0.16)	4(0.16)	4(0.16)	4(0.16)	0.2	55(2.44)	1.315
12	0.5(0.08)	8.75(0.21)	2.5(0.10)	5.5(0.22)	5.5(0.22)	5.5(0.22)	0.2	70(2.856)	1.606
13	0.5(0.08)	1.25(0.03)	5.5(0.22)	5.5(0.22)	5.5(0.22)	5.5(0.22)	0.2	70(2.856)	1.666
14	1.5(0.24)	1.25(0.03)	5.5(0.22)	2.5(0.10)	2.5(0.10)	2.5(0.10)	0.2	40(1.632)	0.642
15	1(0.16)	5.00(0.125)	4(0.16)	4(0.16)	4(0.16)	4(0.16)	0.2	55(2.44)	1.315
16	0.5(0.08)	8.75(0.21)	2.5(0.10)	5.5(0.22)	2.5(0.10)	5.5(0.22)	0.2	40(1.632)	0.502
17	0.5(0.08)	1.25(0.03)	2.5(0.10)	2.5(0.10)	5.5(0.22)	2.5(0.10)	0.2	70(2.856)	2.026
18	1.5(0.24)	1.25(0.03)	2.5(0.10)	2.5(0.10)	5.5(0.22)	5.5(0.22)	0.2	40(1.632)	0.522
19	0.5(0.08)	8.75(0.21)	5.5(0.22)	5.5(0.22)	2.5(0.10)	2.5(0.10)	0.2	70(2.856)	1.726
20	0.5(0.08)	1.25(0.03)	5.5(0.22)	5.5(0.22)	2.5(0.10)	2.5(0.10)	0.2	40(1.632)	0.682
21	1(0.16)	5.00(0.125)	4(0.16)	4(0.16)	4(0.16)	4(0.16)	0.2	55(2.44)	1.315
22	1.5(0.24)	1.25(0.03)	5.5(0.22)	5.5(0.22)	5.5(0.22)	5.5(0.22)	0.2	40(1.632)	0.282
23	0.5(0.08)	1.25(0.03)	2.5(0.10)	2.5(0.10)	2.5(0.10)	5.5(0.22)	0.2	40(1.632)	0.802
24	0.5(0.08)	1.25(0.03)	5.5(0.22)	2.5(0.10)	2.5(0.10)	5.5(0.22)	0.2	70(2.856)	1.906
25	0.5(0.08)	8.75(0.21)	5.5(0.22)	2.5(0.10)	5.5(0.22)	2.5(0.10)	0.2	40(1.632)	0.502
26	1.5(0.24)	8.75(0.21)	5.5(0.22)	5.5(0.22)	2.5(0.10)	5.5(0.22)	0.2	40(1.632)	0.222
27	0.5(0.08)	8.75(0.21)	2.5(0.10)	2.5(0.10)	2.5(0.10)	2.5(0.10)	0.2	70(2.856)	1.966

Appendix VI: Normal plot of medium components and condition from definitive screening design



Appendix VII: Coded Levels of central composite design

Name	Units	Type	Changes	Std. Dev.	Low	High
DON	μ/100g of medium	Factor	Easy	0	-1	+1
citric acid	g/100g of medium	Factor	Easy	0	-1	+1
Glycerol	ml/100g of medium	Factor	Easy	0	-1	+1
γ-PGA Yield	mg/gds	Response				

Appendix VIII: Concentrations of medium components and moisture content adjustment for central composite design

Run	A:DON	B:citric acid	C:glycerol	Glucose	Glutamate	Mineral	(NH4)2SO4	Inoculum	Residual moisture
	μ/100g(ml)	g/100g(ml)	ml/100g(ml)	(ml/2g)	(ml/2g)	(ml/2g)	(ml/2g)	(ml/2g)	ml/2g
1	30(0.3)	7.5(0.2)	11.7045(0.234)	0.2	0.2	0.2	0.2	0.2	0.516
2	30(0.3)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.6
3	40(0.4)	10(0.27)	5(0.1)	0.2	0.2	0.2	0.2	0.2	0.48
4	46.8179(0.47)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.43
5	30(0.3)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.6
6	30(0.3)	11.7045(0.31)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.49
7	20(0.2)	10(0.27)	5(0.1)	0.2	0.2	0.2	0.2	0.2	0.32
8	40(0.4)	10(0.27)	10(0.2)	0.2	0.2	0.2	0.2	0.2	0.38
9	30(0.3)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.6
10	30(0.3)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.6
11	30(0.3)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.6
12	30(0.3)	7.5(0.2)	3.29552(0.07)	0.2	0.2	0.2	0.2	0.2	0.68
13	20(0.2)	10(0.27)	10(0.2)	0.2	0.2	0.2	0.2	0.2	0.58
14	13.1821(0.13)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.9185
15	20(0.2)	5(0.13)	10(0.2)	0.2	0.2	0.2	0.2	0.2	0.72
16	40(0.4)	5(0.13)	10(0.2)	0.2	0.2	0.2	0.2	0.2	0.52
17	40(0.4)	5(0.13)	5(0.1)	0.2	0.2	0.2	0.2	0.2	0.62
18	20(0.2)	5(0.13)	5(0.1)	0.2	0.2	0.2	0.2	0.2	0.82
19	30(0.3)	7.5(0.2)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.6
20	30(0.3)	3.29552(0.09)	7.5(0.15)	0.2	0.2	0.2	0.2	0.2	0.71

Appendix IX: Coefficients of variability in terms of coded factors of independent and interaction terms

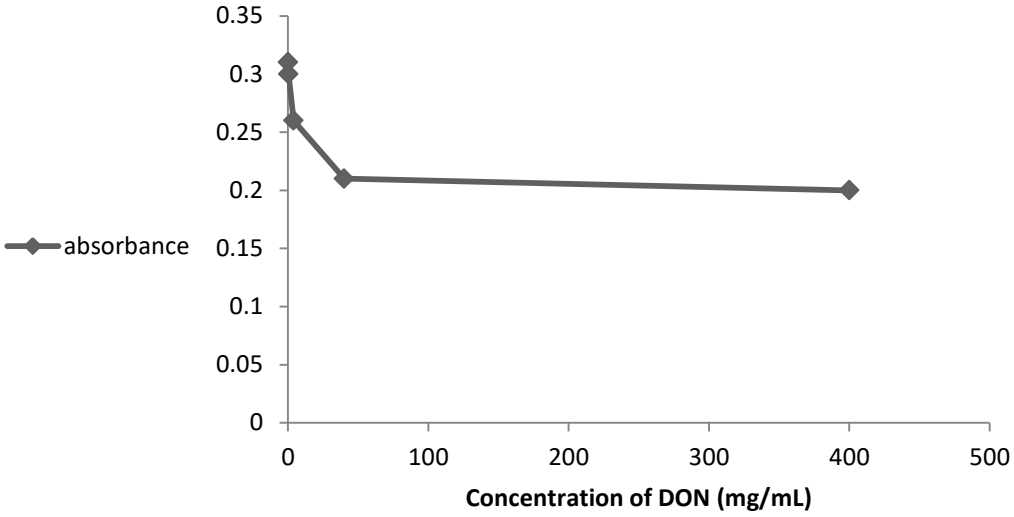
Factor	Coefficient Estimate	df	Std Error	95% CI Low	95% CI High	VIF
Intercept	15.95	1	0.2262	15.44	16.45	
A-DON	-1.900	1	0.1501	-2.24	-1.57	1.0000
B-citric acid	-1.03	1	0.1501	-1.36	-0.6944	1.0000
C-glycerol	0.4268	1	0.1501	0.923	0.7612	1.0000
AB	-0.1775	1	0.1961	-0.6145	0.2595	1.0000
AC	0.2150	1	0.1961	-0.2220	0.6520	1.0000
BC	-0.1125	1	0.1961	-0.5495	0.3245	1.0000
A^2	0.1570	1	0.1461	-0.1686	0.4826	1.02
B^2	-0.2142	1	0.1461	-0.5398	0.114	1.02
C^2	-0.0109	1	0.1461	-0.3365	0.3147	1.02

A: DON, B: Citric acid, C: Glycerol, AB: DON and Citric acid, AC: DON and Glycerol, BC: Citric acid and Glycerol, A2: DON and DON, B2: Squared Citric acid, C2: squared Glycerol. Also; CI: Confidence Interval, VIF: Variance inflation factor and df: degree of freedom.

Appendix X: Constraints of Independent Factors for validation/confirmation experiment

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:DON	maximize	20	25	1	1	4
B:citric acid	maximize	5	7.5	1	1	3
C:glycerol	maximize	7	10	1	1	3

Appendix XI: Minimum inhibitory concentration end point of DON on *B. subtilis*



Appendix XII: Contributions to Knowledge

The findings in this study show that:

i. The optimal fermentation conditions for maximum production of γ-PGA of 19.55±5.75mg/g by *B. subtilis* using rice husk as substrate were 25 μg/100g DON, 7.5% citric acid and 9.96% glycerol.

ii. The minimal inhibitory concentration of DON that allows minimal growth of *B. subtilis* was found to be 40μg/L of broth medium.

iii. The produced γ-PGA had molecular weight within the range of 35 kDa to 96 kDa band in SDS-PAGE.