MICROBIAL SYNTHESIS OF ANTIBIOTIC PRECURSOR KANOSAMINE AND VALUE-ADDED CHEMICAL SHIKIMIC ACID

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ABSTRACT

MICROBIAL SYNTHESIS OF ANTIBIOTIC PRECURSOR KANOSAMINE AND VALUE-ADDED CHEMICAL SHIKIMIC ACID

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Kanosamine is a naturally occurring antibiotic in several bacterial species including *Bacillus* and *Amycolatopsis* and the biosynthetic precursor of 3-amino-5-hydroxybenzoic acid, the building block for ansamycin family of antibiotics. It is also the nitrogen source in the aminoshikimate pathway. The kanosamine-synthesizing operon from *Bacillus subtilis* includes glucose-6-phosphate 3-dehydrogenase-encoding *ntdC*, 3-oxo-glucose-6-phosphate:glutamate aminotransferase- encoding *ntdA*, and kanosamine-6-phosphate phosphatase-encoding *ntdB*. Heterologous expression in *E. coli* SP1.1 strain produces approximately 8 g/L kanosamine in both glucose-rich and glucose-limited fed-batch fermenter conditions. This study confirms the proposed biosynthesis of kanosamine, in addition to providing a reproducible microbial synthesis for the antibiotic precursor.

Starch-based feedstocks, the primary choice of carbon source for commercial production of biobased fuels and chemicals, must be replaced by non-food-based feedstocks derived from agricultural biomass and residues to avoid competition between chemical uses and food uses of carbohydrates. To illustrate this concept, 1 g/L shikimic acid was produced in shake flask experiments of the strain *E. coli* SP1.1/pKD12.138 using anaerobic digestion hydrolysate containing glucose, xylose, and various compounds like furfural, 5-hydroxymethyl furfural, ferulic acid and coumaric acid as the carbon source. Model fed-batch fermentations with the same microbe using a 4% (w/v) glucose-xylose feed equivalent to the sugar concentrations in the AD hydrolysate resulted in the synthesis of 4 g/L shikimic acid and 6 g/L quinic acid.

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Lastly, a huge thank you to my extended family and friends for their prayers and good wishes.

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KEY TO ABBREVIATIONS

Ac	acetyl
AD	anaerobic digestion
ADP	Adenosine diphosphate
AHBA	3-amino-5-hydroxybenzoate
aminoDAHP	4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate
aminoDHQ	5-amino-5-deoxy-3-dehydroquinic acid
aminoDHS	5-amino-5-deoxy-3-dehydroshikimate
aminoF6P	3-amino-3-deoxy-D-fructose 6-phosphate
K6P	kanosamine 6-phosphate
AMP	adenosine monophosphate
Amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
Cm	chloramphenicol
DAHP	3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate
DCIP	dichloroindophenol
DHQ	3-dehydroquinate
DHS	3-dehydroshikimate
DNA	3-deoxyribonucleic acid
D.O	dissolved oxygen
E4P	D-erythrose 4-phosphate
F6P	D-fructose 6-phosphate
FAB	fast atom bombardment
FBR	Feedback resistant
G6P	D-glucose 6-phosphate
Glu	L-glutamate
g	gram

h	hour
His	L-histidine
iminoE4P	1-imino-1-deoxy-D-erythrose 4-phosphate
IPTG	isopropyl β– D-thiogalactopyranoside
KG	2-ketoglutaric acid
LB	luria broth
M	molar
mg	milligram
mL	milliliter
μL	microliter
mM	millimolar
MS	mass spectrometry
min	minute
NAD	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NMR	nuclear magnetic resonance
OD	optical density
PEP	phosphoenolpyruvate
PCR	polymerase chain reaction
PMS	phenazine methosulfate
ppm	parts per million
psi	pounds per square inch
rpm	revolutions per minute
rt	room temperature
SA	shikimic acid
Tc	tetracycline
TLC	thin-layer chromatography
TMS	trimethylsilyl
TSP	sodium 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid
UDP	uridine 5'-diphosphate
UMP	uridine 5'-monophosphate

UV	ultraviolet

CHAPTER ONE

MICROBIAL SYNTHESIS OF ANTIBIOTIC PRECURSOR KANOSAMINE

1. INTRODUCTION

Amino sugars are sugar derivatives in which at least one of the hydroxyl groups is substituted with an amino group, free or substituted. Most amino sugars found in nature are components of more complex molecules - in naturally occurring antibiotics such as kanamycin, streptomycin, erythromycin, nystatin, neomycins, muramic acid and in bio-macromolecules that occur in microbial cell walls and other tissues like chitin, glycoproteins, lipopolysaccharides, mucopolysaccharides (Figure 1).[1] More than sixty amino sugars have been reported to date, almost all of which were never found as monosaccharides in nature.

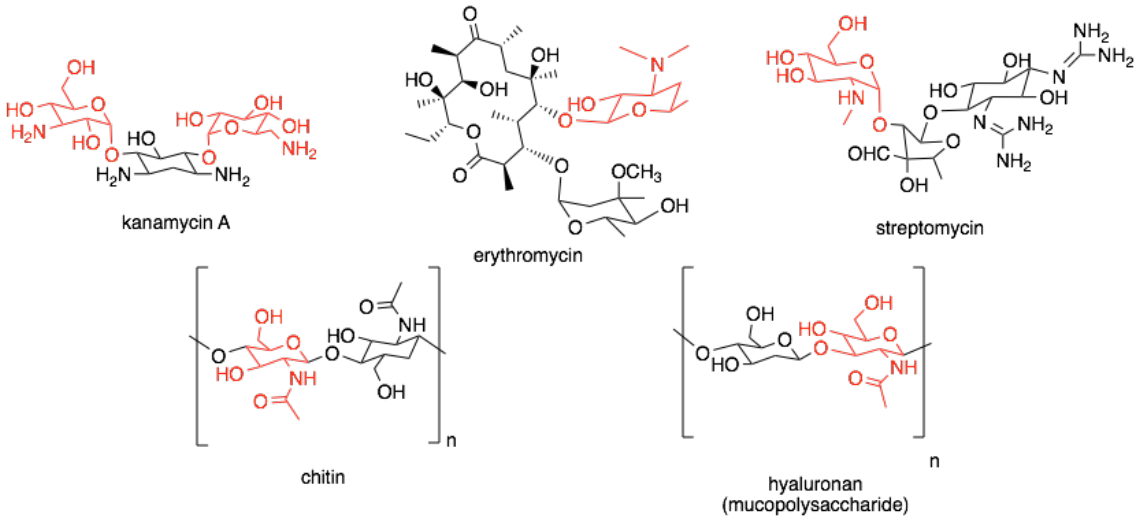
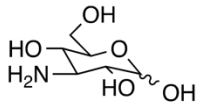


Figure 1. Structures of kanamycin A, erythromycin, streptomycin, chitin, and hyaluronan. Aminosugars are highlighted in red.

Kanosamine (3-amino-3-deoxy-D-glucose, Figure 2) was the first amino sugar to be reported as a monosaccharide antibiotic by itself,[1] produced in *Bacillus aminoglucosidicus* (now known as *Bacillus pumilus*).[2] It is also a key component in another antibiotic, kanamycin, produced by *Streptomyces kanamyceticus,*[3] in addition to being a secondary metabolite in various *Bacillus*,

Actinomycetes and *Amycolatopsis* species. The antibiotic properties of kanosamine were discovered when Iwai et al. observed that kanosamine inhibited the growth of *Staphylococcus aureus* and *Sarcina lutea* within a concentration range of 50-100 μg/mL.[4] They reported that kanosamine was the first monosaccharide antibiotic which showed inhibition of bacterial cell wall synthesis.[4] Janiak et al. supported this conclusion by demonstrating that kanosamine was transported into the cytoplasm of bacterial or fungal cells and was converted into kanosamine-6-phosphate by the action of either bacterial or fungal hexokinase.[5] Kanosamine-6-phosphate would then interact with the enzyme L-glutamine: D-fructose 6-phosphate amidotransferase to inhibit the formation of uridine-5'-diphospho-*N*-acetylglucosamine, which is an important metabolite in bacterial and fungal cell wall synthesis.[5] Kanosamine is just one monosaccharide among the many antifungal agents produced by *Bacillus* species, which are increasingly being considered as effective and environmentally safe biological control agents against soil borne fungal diseases such as take-all.[6] In 2015, Yang et al. reported that the strain *Bacillus subtilis* YB-05 was very effective in suppressing wheat take-all caused by *G. graminis var. tritici*,[7] which aligns with the conclusion of this study that the *ntd* operon from *B. subtilis* is responsible for the biosynthesis of kanosamine.



kanosamine

Figure 2. Structure of kanosamine (3-amino-3-deoxy-D-glucose)

In this chapter, an overview of literature on kanosamine biosynthesis in the microbial species *Amycolatopsis mediterranei*, *Bacillus pumilus*, and *Bacillus subtilis* will be given. Microbial synthesis of kanosamine by transformation of pSN1.292 (pBbA1a-*ntdCntdAntdBserA*), a medium copy number plasmid containing the *B. subtilis* genes *ntdC*, *ntdA*, and *ntdB* into *E. coli* SP1.1 cells will be described. A series of glucose-rich and glucose-minimal fed-batch

fermentations of the kanosamine synthesizing strain SP1.1/pSN1.292 will serve as proof-of-concept for the previously reported but untested *ntd* operon in *B. subtilis*.

One of the first elucidations of a possible metabolic route for the formation of kanosamine (Figure 3) in a series of *B. pumilus* shake flask experiments was conducted utilizing [1-^{14}C]-, [2-^{14}C]-, [6-^{14}C]- and [U-^{14}C]-D-glucose as starting materials.[8] Incubation of [U-^{14}C]-D-glucose along with co-factors ATP, UTP, NAD^+, glutamine and Mg^{2+} in dialyzed cell-free lysate of *B. pumilus* led to the formation of ^{14}C-kanosamine.[8] Similarly, experiments using [6-^{14}C]-D-glucose and [1-^{14}C]-D-glucose resulted in a measured radioactivity of 58% and 73%, respectively.[8] This supported the hypothesis that the whole carbon skeleton of D-glucose is incorporated into kanosamine.[8]

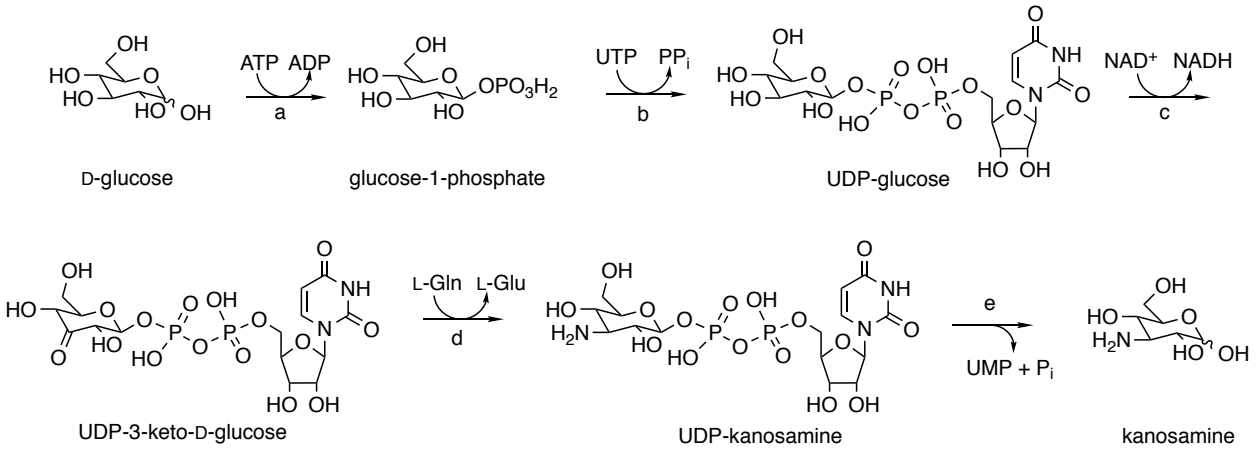


Figure 3. The first proposed kanosamine biosynthetic pathway in *Bacillus pumilus*.[8,a,b] (a) i. glucokinase; ii. phosphoglucomutase; (b) UDP-glucose pyrophosphorylase; (c) UDP-3-keto-D-glucose dehydrogenase; (d) UDP-3-keto-D-glucose transaminase; (e) UDP-kanosamine phosphatase. Abbreviations: ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; L-Gln, L-glutamine; L-Glu, L-glutamate; UDP, uridine 5'-diphosphate; UMP, uridine 5'-monophosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate.

Substitution of [U-^{14}C]-D-glucose with UDP-[U-^{14}C]-D-glucose as the substrate resulted in ^{14}C-kanosamine as the product, indicating that UDP-[U-^{14}C]-D-glucose is a key intermediate in the biosynthesis of kanosamine.[8] These observations led them to the most probable biosynthetic pathway[8] as shown in Figure 3. D-glucose is converted into UDP-glucose, and the requirement for

NAD^+ suggests the oxidation of UDP-D-glucose into UDP-3-keto-D-glucose, which has been reiterated as a key step in literature but UDP-3-keto-D-glucose was not isolated to date. The oxidized intermediate was believed to undergo transamination in the presence of glutamine, resulting in kanosamine.

Renewed interest in kanosamine began when it was postulated to be the source of nitrogen in the aminoshikimate pathway found in *A. mediterranei*. Floss and collaborators discovered that the aminoshikimate pathway leads to the formation of 3-amino-5-hydroxybenzoic acid (AHBA), a precursor to the biosynthesis of ansamycins and mitomycins.[9] They identified and characterized the *rif* biosynthetic gene cluster that encodes the aminoshikimate pathway,[10,11] named after the shikimate pathway found in many plants and microorganisms (Figure 4). The similarity between the two pathways was established with the discovery of 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP) as the key intermediate in the aminoshikimate pathway.[12] Subsequent reports about *A. mediterranei* cell-free lysate reactions confirmed the between the two pathways where aminoDAHP cyclized to 5-deoxy-5-aminodehydroquinic acid

Shikimate pathway[a,b]:

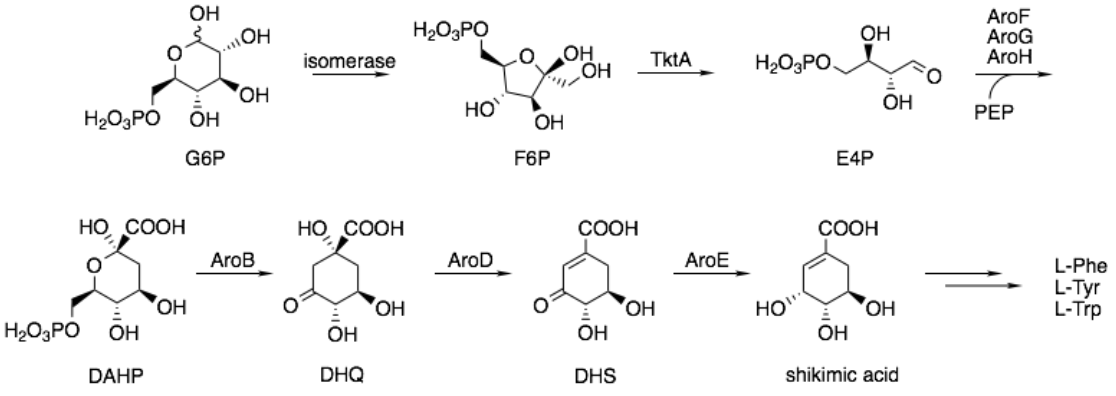
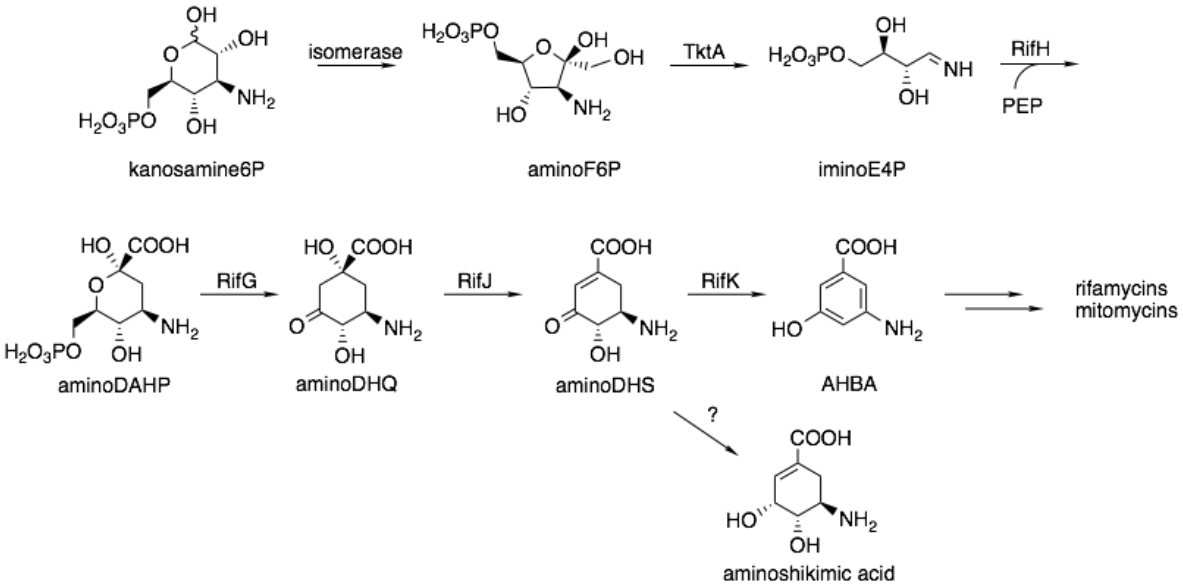


Figure 4. Comparison of metabolic intermediates in the shikimate and aminoshikimate pathways.

Figure 4 (cont'd).
Aminoshikimate pathway[a,b]:



(a) Encoding gene, enzyme: *tktA*, transketolase; *aroF*, *aroG*, *aroH*, DAHP synthase; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *rifH*, aminoDAHP synthase; *rifG*, aminoDHQ synthase; *rifJ*, aminoDHQ dehydratase; *rifK*, AHBA synthase. (b) Abbreviations: G6P, D-glucose-6-phosphate; F6P, D-fructose-6-phosphate; E4P, D-erythrose-4-phosphate; PEP: phosphoenolpyruvate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimic acid; kanosamine6P, kanosamine-6-phosphate; aminoF6P, 3-amino-3-deoxy-D-fructose-6-phosphate; iminoE4P, 1-imino-1-deoxy-D-erythrose-4-phosphate; aminoDAHP, 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate; aminoDHQ, 5-amino-5-deoxy-3-dehydroquinate; aminoDHS, 5-amino-5-deoxy-3-dehydroshikimic acid; AHBA, 3-amino-5-hydroxybenzoic acid.

(aminoDHQ), dehydrated to give 5-deoxy-5-aminodehydroshikimic acid (aminoDHS), and then aromatized to AHBA.[12]

In 2002, Guo and Frost reported the formation of aminoDAHP via the intermediacy of 1-deoxy-1-imino-D-erythrose 4-phosphate (iminoE4P) in cell free lysate of *A. mediterranei* when 3-amino-3-deoxy-D-fructose 6-phosphate (aminofructose6P) was taken as the substrate.[13] It was deduced that aminofructose6P was isomerized from kanosamine 6-phosphate (kanosamine6P) because both substrates led to the formation of aminoDAHP with approximately the same yields in cell free lysate of *A. mediterranei.* UDP-6,6-[$^2H_2$]-D-glucose was incubated in the cell-free lysate with NAD^+ and glutamine to give kanosamine in 5% yield.[14] Moreover, kanosamine was

produced in 6% yield when UDP-3-keto-D-glucose was incubated with NADH and glutamine.[14] These observations confirmed that kanosamine was biosynthesized from UDP-glucose via UDP-3-keto-D-glucose in *A. mediterranei*.

As the *rif* biosynthetic gene cluster has been studied extensively,[10,11,15] the enzymes for kanosamine6P biosynthesis were determined based on their homologies to well-known classes of enzymes. The *rifL* gene was seen to have high sequence homology to Pur10, an oxidoreductase involved in puromycin biosynthesis whereas *rifK* has homology to aminotransferases, and *rifM* has homology to phosphatases belonging to the CBBY family.[16] This suggested that the joint action of *rifL*-encoded oxidoreductase and *rifK*-encoded transaminase converts UDP-glucose to UDP-kanosamine, which is subsequently hydrolyzed by *rifM*-encoded kanosamine phosphatase to

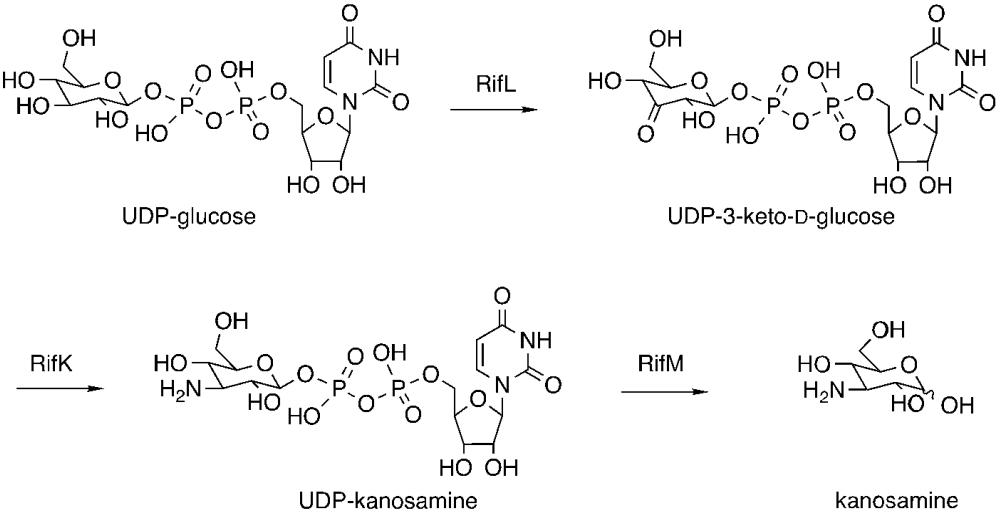


Figure 5. Kanosamine biosynthesis in *A. mediterranei.* [16] (a) Encoding gene, enzyme: *rifL*, UDP-3-keto-D-glucose dehydrogenase; *rifK*, UDP-3-keto-D-glucose transaminase; *rifM,* UDP-kanosamine phosphatase.

yield kanosamine (Figure 5). The following *A. mediterranei* cell-free lysate reactions were performed by Guo which confirmed enzyme activities of RifL and RifK: 1. UDP-glucose was incubated with *rifL*-encoded oxidoreductase in the presence of electron acceptor dichloroindophenol (DCIP) and intermediate electron carrier phenazine methosulfate (PMS),

which led to the formation of UDP-3-keto-D-glucose in 69% yield; 2. UDP-3-keto-D-glucose, glutamine and PLP were incubated with *rifK*-encoded transaminase, which resulted in a 25% yield of UDP-kanosamine.[24]

Attempts to isolate the enzymes in *B. pumilus* that are responsible for kanosamine biosynthesis was initially fruitful when UDP-3-keto-D-glucose dehydrogenase was isolated and characterized.[24] Because of the close relationship between UDP-3-keto-D-glucose dehydrogenase and an NAD^+-dependent enzyme, an NAD^+-dependent enzyme assay was selected to determine the specific activity of the former.[24] Guo was able to identify the enzyme activity during the early stages of purification; however, enzyme activity was lost in the later stages, and this was attributed to the loss of NAD^+-dependent enzyme during purification.[24] Hence, instead of isolating and expressing *B. pumilus* genes in *E. coli*, Guo and Frost devised a two-microbe pathway (Figure 6) where kanosamine was acquired from *B. pumilus* fermentation for their purposes.[17]

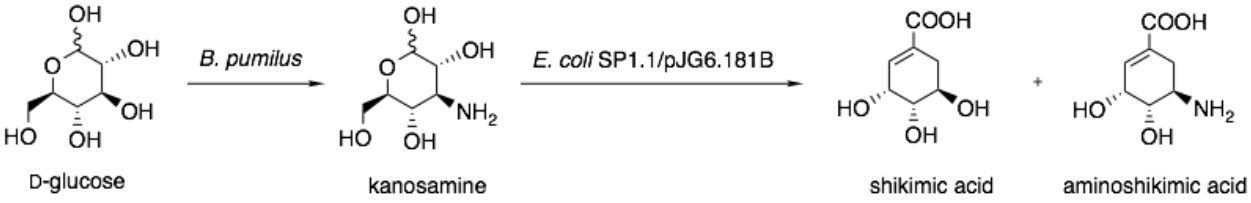


Figure 6. Two-microbe pathway designed by Guo and Frost.

In 2008, Thomas and coworkers discovered the *kab* (*k*anos*a*mine *b*iosynthesis) operon in *Bacillus cereus UW85*[18] during their investigation of the enzymes involved in the biosynthetic route for the antibiotic, zwittermicin A (ZmA). Comparison between the gene clusters in *B. cereus AH1134* and *B. cereus UW85* led them to identify five extra genes in the latter organism.[18] After unsuccessful efforts at PCR amplification of these five genes based on the available *B. cereus UW85* genomic sequence, they concluded that these genes are not involved in ZmA production.

Furthermore, they added that the five genes resemble an operon in *B. subtilis* described by Inoaka et al. that produces the aminosugar antibiotic, 3,3'-neotrehalosadiamine (NTD, Figure 7).

Even though NTD was never detected in the *B. cereus UW85* culture, since it is a dimer of the aminosugar kanosamine, the authors conjectured that the *kab* operon regulates, biosynthesizes and exports kanosamine from *B. cereus UW85* in a simple three step pathway[18] as shown in Figure 8. However, this proposed route was not substantiated by any experimental evidence.

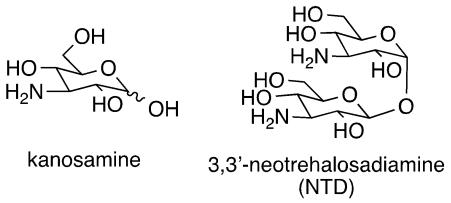


Figure 7. Structures of kanosamine and its dimer, 3,3'-neotrehalosadiamine.

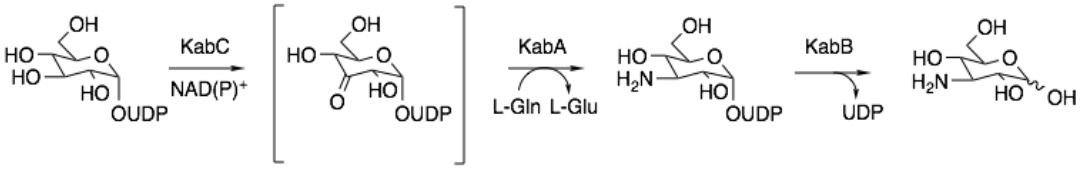


Figure 8. Proposed biosynthetic pathway for kanosamine in *B. cereus UW85.*[18] (a) Encoding gene, enzyme: *kabC*, UDP-glucose C3 dehydrogenase; *kabA*, aminotransferase; *kabB*, UDP-kanosamine hydrolase. (b) Abbreviations: $NAD(P)^+$, nicotinamide adenine dinucleotide (phosphate); L-Gln, L-glutamine; L-Glu, L-glutamate; UDP, uridine diphosphate

The *ntd* operon, defined as the complete set of genes required to synthesize NTD,[19] was first reported by Inoaka and collaborators. While studying the effects of introducing mutations into the genomic sequence of *B. subtilis* to develop rifampicin resistance, they observed an overproduction of NTD which has previously been isolated from *B. pumilus* and *B. circulans*.[19] Screening of 1800 mutant isolates with mini-*tn10* insertions led them to three mutants which were unable to produce NTD, all of which were found to have disrupted the polycistronic operon, *ntdABC*.[19] Another gene, *ntdR,* upstream from the *ntdABC* operon was believed to be a putative *lacI* family transcriptional regulator.[19] A recombinant *E. coli* BL21(DE3) strain containing the high copy number plasmid pUC18-*ntdABC* produced about 5 μg/mL of NTD, as evidenced by the

peak at m/z 363 belonging to NTD $[M+Na]^+$ ion in the ESI-MS spectral analysis.[19] A putative identification for *ntdA* as PLP-dependent aminotransferase, *ntdB* as hydrolase and *ntdC* as NADH-dependent dehydrogenase was given,[19] later followed by Thomas et al. for their studies in *B. cereus UW85*. Moreover, it was established that NTD works as an auto inducer for transcription of the *ntd* operon.[19] However, addition of the dimers trehalose (α,α-linkage) and neotrehalose (α,β-linkage) to the fermentation at a concentration of 1000 μg/mL did not increase the production of NTD regardless of presence or absence of externally added NTD,[19] which led them to infer that amination occurs before dimerization of kanosamine moieties.

In 2013, Vetter et al. reported that the *ntd* operon was in fact responsible for the biosynthesis of kanosamine, and not NTD.[20] They hypothesized that there must be an additional nucleotidyltransferase and/or a glycosyltransferase to dimerize kanosamine[20] since Inoaka et al. predicted that the formation of glycosidic bond occurred after amination. Neither of the three *ntd* genes show sequence similarities to such enzymes.[20] They examined the encoded proteins NtdA,

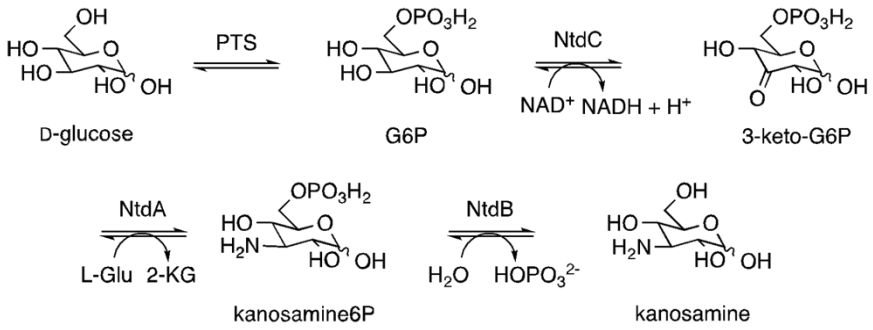


Figure 9. Kanosamine biosynthetic pathway in *B. subtilis.*[20] (a) Genes, encoding enzymes: *ntd*C, glucose-6-phosphate-3-dehydrogenase; *ntd*A, 3-keto-glucose-6-phosphate:glutamate aminotransferase; *ntd*B, kanosamine-6-phosphatase; (b) Abbreviations: PTS, phosphoenolpyruvate-phosphotransferase system; G6P, glucose 6-phosphate; 3-keto-G6P, 3-keto-glucose 6-phosphate; L-Glu, L-glutamate; 2-KG, 2-ketoglutarate.

NtdB and NtdC and gave an initial prediction based on sequence similarities that NtdA is a PLP-dependent aminotransferase, NtdB is a phosphatase from the HAD superfamily, and NtdC is a NAD dependent dehydrogenase.[20] Cloning of *ntdA, ntdB* and *ntdC* into the vector pET-28b and

expression in *E. coli* BL21-Gold cells afforded them purified hexahistidine-tagged proteins to conduct a series of enzyme activity experiments to confirm their proposed pathway as shown in Figure 9.[20]

As UDP-glucose was always reported to have been the substrate in any kanosamine biosynthetic pathway, it was tested as a substrate for NtdC, the first enzyme in the current proposed pathway. There was no activity observed spectrophotometrically at 340 nm in the NtdC-catalyzed enzymatic UV assay.[20] The presence of a phosphatase, NtdB at the end of the pathway was suggestive of a phosphorylated substrate at the beginning of the pathway.[20] This observation was confirmed by the appearance of NADH at 340 nm in the UV assay when glucose-6-phosphate was taken as a substrate.[20] They found a second absorbance at 310 nm when NADH was recycled using lactate dehydrogenase and pyruvate, which corresponded to 3-keto-glucose-6-phosphate, the hemiacetal form of a 1,3-dicarbonyl, the class of compounds that absorb near 300 nm in basic aqueous solutions.[20] To confirm the role of NtdA, an NtdC-NtdA coupled assay was performed in which an excess of L-glutamate and purified NtdA were added to an NtdC-catalyzed reaction.[20] This resulted in the elimination of the absorption at 310 nm indicating that it belonged to 3-keto-G6P, the substrate to NtdA.[20] Because these enzymes were predicted to be reversible, an addition of NtdA and 2-ketoglutarate to kanosamine-6-phosphate should yield an absorbance at 310 nm.[20] Kanosamine6P was synthesized chemically from kanosamine using hexokinase and ATP, a method established by Guo and Frost.[20] Utilizing reverse phase ion-pairing HPLC with evaporative light scattering detection, they confirmed the consumption of kanosamine6P and production of glucose6P when NtdC and NADH were added to the NtdA-catalyzed reaction.[20] Various phosphorylated substrates like NTD-6-phosphate, NTD-6,6'-bisphosphate, glucose-6-phosphate, glucose-1-phosphate were explored for NtdB activity.[20] NtdB showed high selectivity in

hydrolyzing kanosamine6P to kanosamine at the 3-position.[20] However, the authors noted that NtdB did show trace amount of activity in hydrolyzing glucosamine-6-phosphate as well.[20] Thus, the enzymatic experiments helped determine the substrates and products of each enzyme in the *ntd* operon. However, the production of kanosamine using the entire operon was not reported. Hence, the focus of this chapter will be the heterologous cloning and expression of the *ntd* operon in *E. coli* to successfully biosynthesize kanosamine with an 8 g/L titer value.

2. RESULTS

2.1. Cloning *Bacillus subtilis* genes into a Bglbrick vector

2.1.1. Overview

The aim of this project is to clone *ntdC, ntdA,* and *ntdB* genes from the genomic DNA of *B. subtilis* into a suitable vector, transform the resulting plasmid into a compatible *E. coli* strain and biosynthesize kanosamine to provide proof for the proposed biosynthesis published by Vetter et al.[20] It was reported that the *ntd* operon found in *B. subtilis* contained the complete set of genes that are required to biosynthesize kanosamine.[20] The authors performed UV-visible spectrophotometric enzyme assays and demonstrated *in vitro* that NtdC is a glucose-6-phosphate 3-dehydrogenase, NtdA is a pyridoxal phosphate-dependent 3-keto-glucose-6-phosphate:glutamate aminotransferase, and NtdB is a kanosamine-6-phosphate phosphatase.[20] In this study, we report the first heterologous biosynthesis of kanosamine in *E. coli*, thus supplying *in vivo* evidence to support the proposed pathway in *B. subtilis*.

In order to accommodate the exploration of the effects of different inducible expression systems, transcription promoters, antibiotic resistance and plasmid copy numbers on the titer value of kanosamine, a standardized cloning technique developed by Lee et al. was utilized.[21] Conventional restriction enzyme cloning techniques necessitate the creation of multiple plasmids

in the course of heterologously expressing all the enzymes required to make a metabolic pathway. Furthermore, there is a possibility of introducing mutations into our desired DNA sequence every time a particular gene is PCR amplified. This kind of *ad hoc* cloning methodology would limit the efficiency of changing all possible variables to achieve high titer values of the target chemical. The Bglbrick cloning technique involves only one PCR amplification step to acquire the desired gene from genomic DNA and subsequent cloning using the four standard restriction sites - EcoRI, BamHI, BglII, XhoI - into a standardized vector yields a Bglbrick.[21] After sequencing and confirming the absence of any mutations, it can be added to our Bglbrick combinatorial library in long term storage, thus eliminating the need for more pre-assembly PCR amplifications or post-assembly sequencing.

2.1.2. Plasmid Construction

Genomic DNA was isolated from ATCC 23857 *B. subtilis* strain 168 and subsequent cloning into the vector pJF118EH[25] yielded plasmid pKD16.211 (Figure 10).[23] Plasmid pBbA1a-RFP was chosen as the desired Bglbrick vector for the following reasons: 1. It has a medium copy number because of the p15A origin of replication that would help to avoid metabolic burden on the host cell; 2. It has an IPTG-inducible, strong *trc* promoter; 3. It has ampicillin as the antibiotic resistance.[21] Q5 High Fidelity DNA polymerase was employed for the one and only PCR amplification step as it has an error rate about 280 times less than Taq DNA polymerase. The successive cloning of *ntdA* and *ntdB* followed the same pattern (Figure 11 and Figure 12): PCR amplification using Q5 High Fidelity DNA polymerase, enzyme digest of both insert (*ntdA, ntdB*) and vector (pBbA1a) with the enzymes XhoI and BglII, ligation and transformation of the respective plasmids pBbA1a-*ntdA* and pBbA1a-*ntdB* into DH5α electrocompetent cells. In the case of *ntdC* (Figure 13), an extra step of mutagenizing the BglII restriction enzyme site was done as

this site will interfere with making use of BglII in the XhoI/BglII standardized enzyme digestion to ligate the insert with the Bglbrick vector, pBbA1a. Silent mutation which involves the conversion of AGATCT sequence of BglII encoding the amino acids Arginine and Serine to AGATCA, still encoding Arginine and Serine, disrupted the offensive BglII site in the plasmid pKD16.211 containing *ntd*C. The rest of the procedure was same as that of *ntd*A and *ntd*B. All three *ntd* Bglbricks were sequenced, checked for mutations and stored as glycerol freezes in DH5α electrocompetent cells at -80^{o}C.

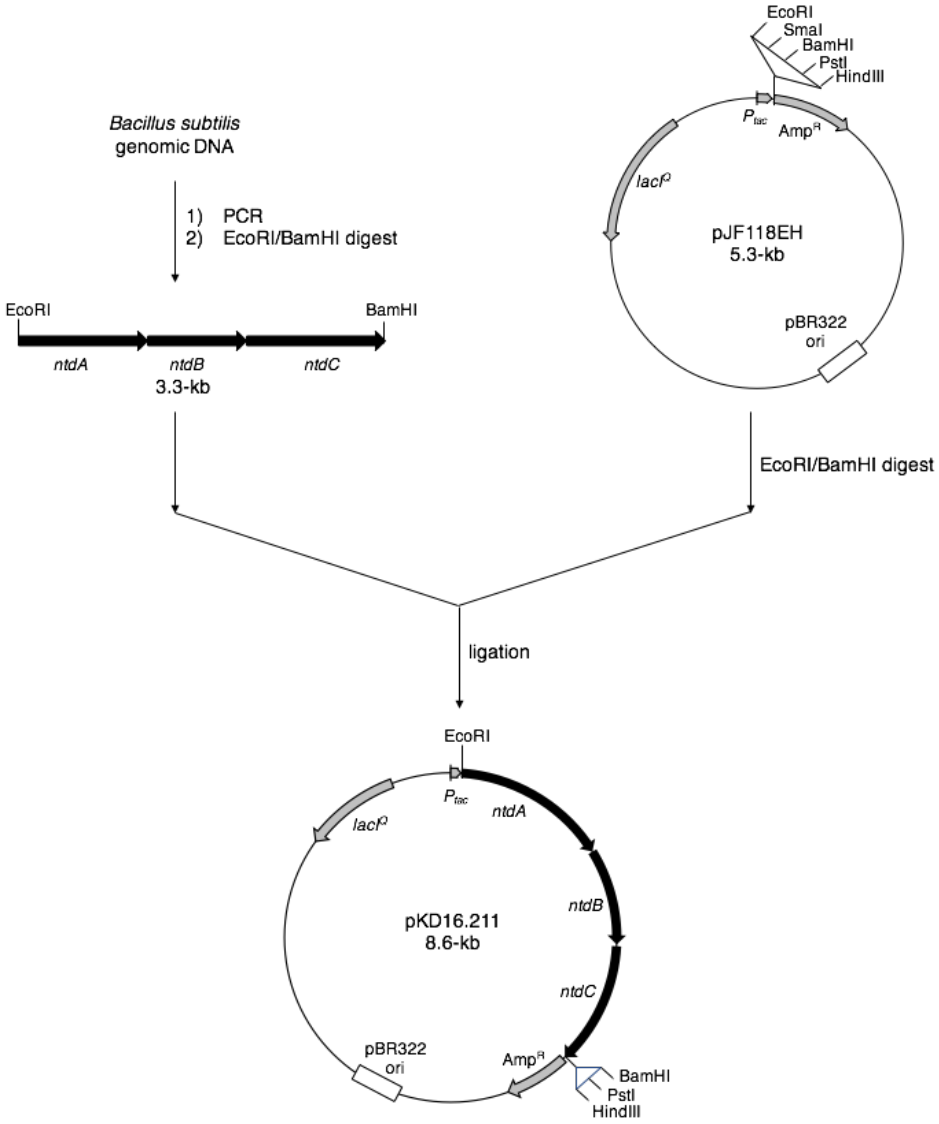


Figure 10. Preparation of Plasmid pKD16.211.

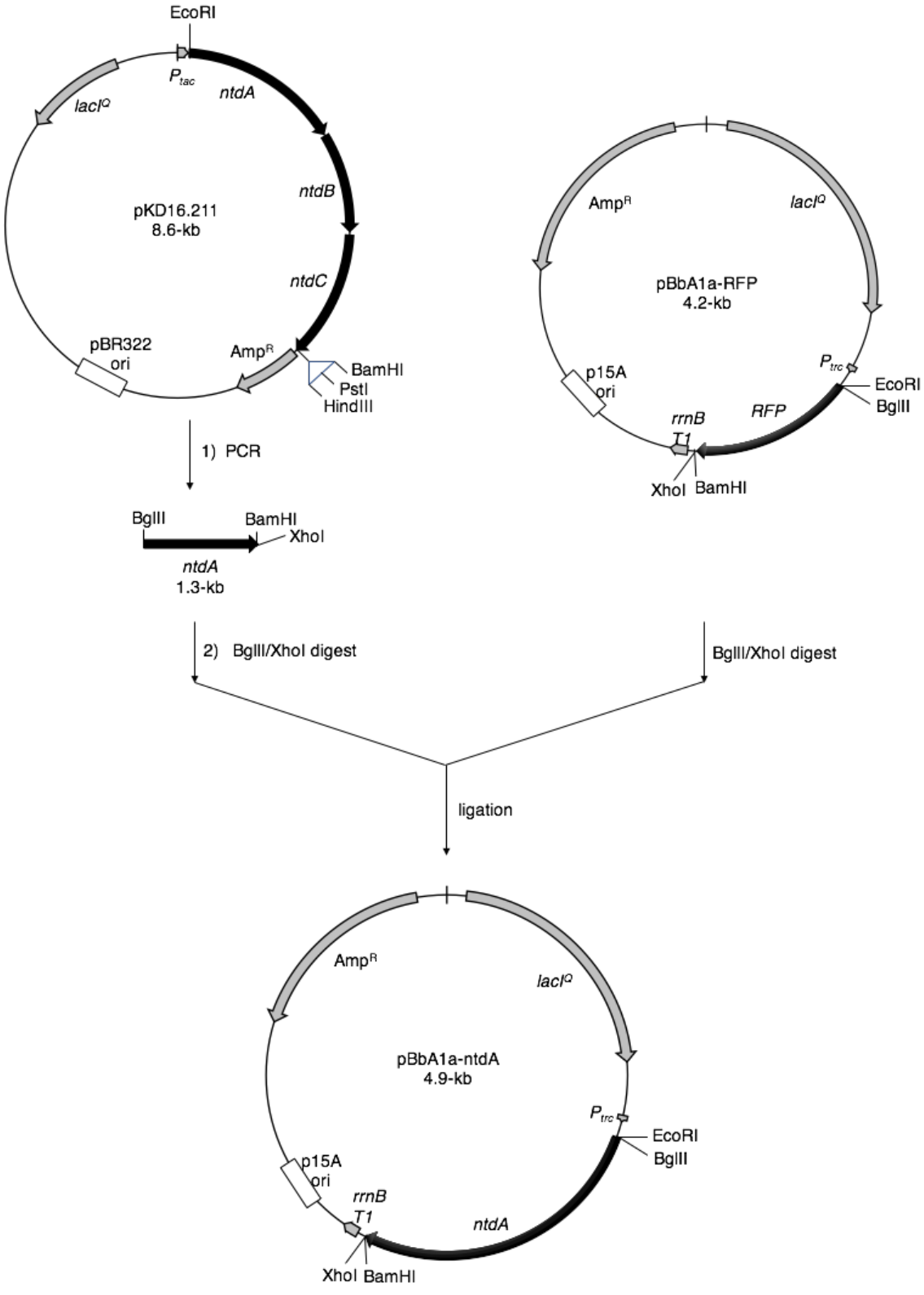


Figure 11. Preparation of Bglbrick Plasmid pBbA1a-ntdA.

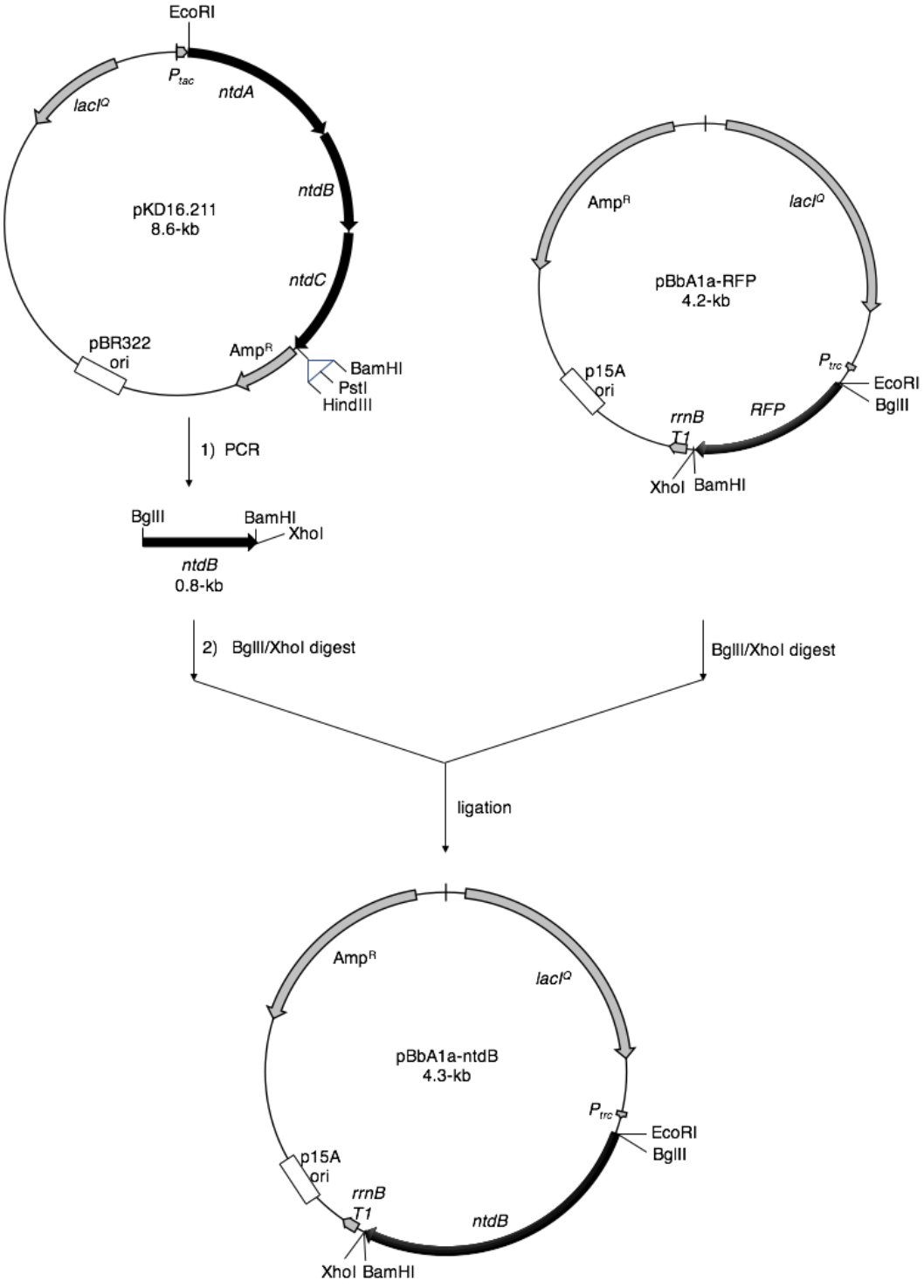


Figure 12. Preparation of Bglbrick Plasmid pBbA1a-ntdB.

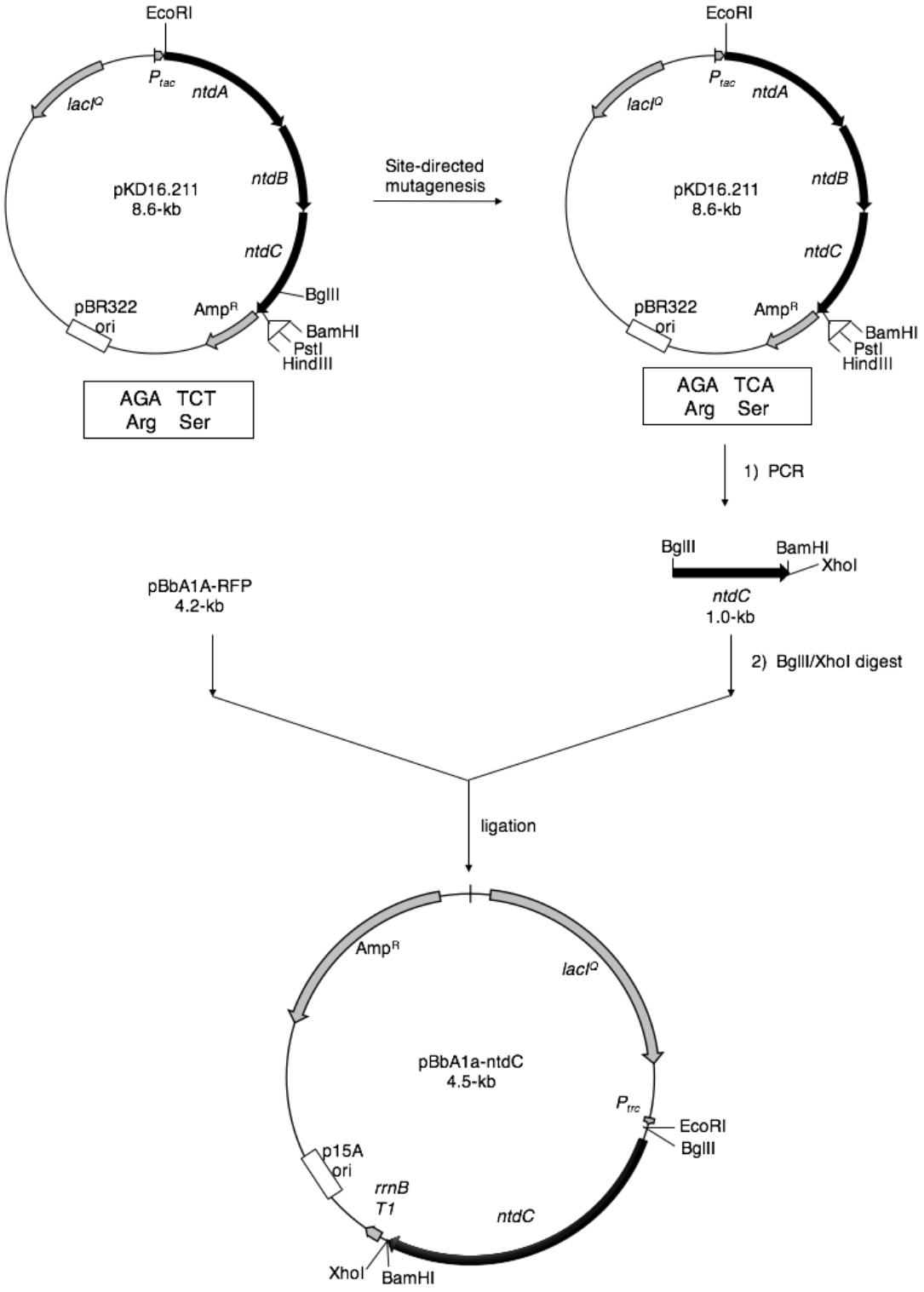


Figure 13. Mutagenesis of offensive BglII site in *ntd*C and Preparation of Bglbrick Plasmid pBbA1a-ntdC.

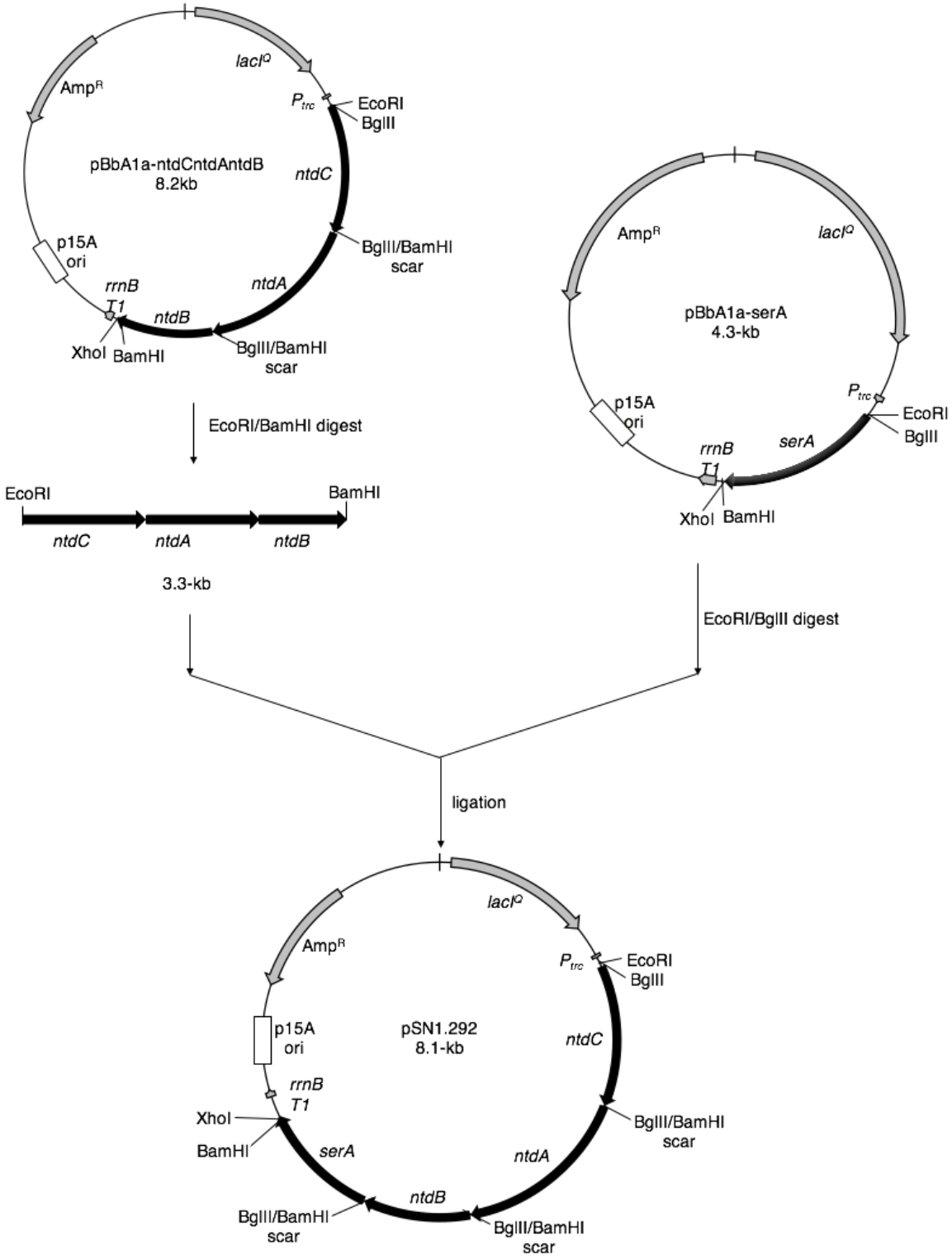


Figure 14. The last step in the preparation of plasmid pSN1.292.

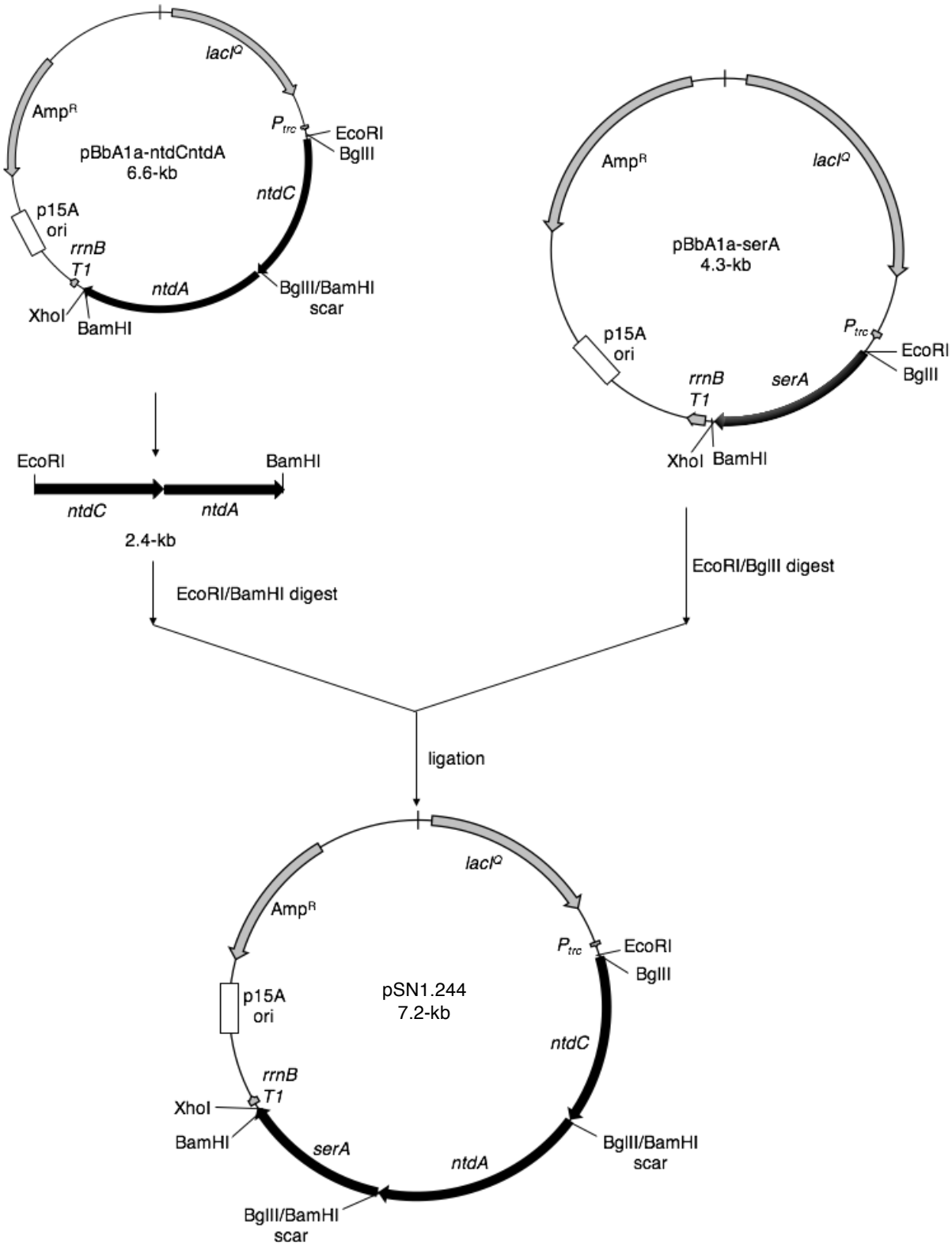


Figure 15. Preparation of Plasmid pSN1.244.

To obtain the kanosamine-producing plasmid pSN1.292, first pBbA1a-*ntdC* was digested with EcoRI and BamHI to yield *ntdC*, which was then inserted into EcoRI-BglII digested pBbA1a-*ntdA* which resulted in the plasmid pBbA1a-*ntdCntdA.* Consequently, the latter was digested with EcoRI and BamHI to acquire the *ntdCntdA* cassette, which was inserted into pBbA1a-*ntdB* through the exact same procedure as above yielding pBbA1a-*ntdCntdAntdB.* Finally, the *ntd* cassette was created by cloning *ntdCntdAntdB* into pBbA1a-*serA* which yielded pSN1.292 (pBbA1a-*ntdCntdAntdBserA,* Figure 14). The order of the genes was a conscious effort to place *ntdC* closer to the transcription promoter P_{trc}, as it is the first gene in the kanosamine biosynthetic pathway and this arrangement would ensure the maximum transcription of *ntd*C.

2.2. Microbial synthesis of kanosamine by SP1.1/pSN1.292

E. coli SP1.1 (RB791 *serA*::*aroB aroL*::*Tn*10 *aro*K::Cm^R) was chosen as the host strain to transform the plasmid pSN1.292 for two reasons: 1. The gene *serA* encoding 3-phosphoglycerate dehydrogenase, an enzyme responsible for the synthesis of L-serine is vital for cell growth when fermented in minimal salts. If *serA* locus is disrupted in a bacterial strain and simultaneously introduced into pBbA1a-*ntdCntdAntdB*, in the absence of L-serine supplementation, the cell would be compelled to maintain the plasmid for its survival. 2. The shikimate kinase isozymes, *aroK* and *aroL*, are inactivated in the *E. coli* SP1.1 strain, thus ensuring the accumulation of shikimic acid. Furthermore, SP1.1 strain would be the ideal host strain to accumulate aminoshikimic acid when the kanosamine pathway is incorporated into the first half of the aminoshikimate pathway. Hence, employing the same strain to analyze kanosamine production will be beneficial in gauging the carbon flow from glucose to kanosamine.

Variables in the fed-batch fermenter runs – temperature (33 ^oC), pH (7.0), dissolved oxygen (D.O.) levels (10% saturation) and glucose feed (60% w/v) - were maintained by Proportional

Integral Derivative (PID) control loops. A 5 mL culture of M9 media supplemented with glucose and aromatic vitamins was grown for 15 h overnight at 37 ^{o}C with agitation at 250-300 rpm until they were turbid. 4 mL of this overnight culture was transferred into a fresh 100 mL culture of M9 media supplemented with aromatic amino acids and aromatic vitamins, which was grown at 37 ^{o}C with agitation until the OD_{600} reached 1.3-1.7 i.e., the exponential phase of the cells. This 100 mL culture was then added to the fermenter vessel. Fermentations were performed under both glucose-rich and glucose-minimal conditions, as described below.

In the case of glucose-rich conditions, the glucose concentration was maintained at 20-25 g/L throughout the run until the glucose feed was fully consumed. The D.O. level was maintained at 10% in three stages by the PID control loops. In the first stage, the airflow was kept constant at 0.06 L/min and the impeller speed increased from 50 rpm to 750 rpm. When the impeller speed reached 750 rpm, the airflow was increased from 0.06 L/min to 1.0 L/min. These two stages were completed around 11-12 h from the time of inoculation and the initial glucose feed of 30 g sustained the exponential growth of bacteria in this time-period. The third and final stage consisted of keeping the airflow constant at 1.0 L/min and the impeller speed set to vary between 750-1800 rpm, the minimum of which was reduced to 400 rpm after 36-48 h of the fermenter run. 1 mL of 100 mM isopropyl β-D-thiogalactopyranoside (IPTG) solution was added to the culture medium every six hours from the time of beginning of stage three to induce transcription of enzymes from the plasmid pSN1.292.

In the case of glucose-minimal fermentation, the initial glucose feed was 20 g, which is 10 g less than glucose-rich conditions. Again, D.O level was controlled by a PID loop. The first two stages of maintaining the D.O level at 10% were identical to the glucose-rich conditions. After the impeller speed and airflow reached their set maxima, 750 rpm and 1.0 L/min, the D.O. level

dropped to zero, at which point the D.O. probe was linked to the glucose intake of the cells. An oxygen nutrient controller maintained the glucose concentration at less than 0.5 g/L, providing the cells just enough glucose based on the fluctuating D.O. levels.

Table 1. Final concentrations of kanosamine and shikimic acid in fermentation broth of pSN1.292 and pSN1.244

Entry	Construct[b]	Glucose conditions	K[a] (g/L)[c]	SA[a] (g/L)[c]
1	SP1.1/pSN1.292	Glucose rich	8	10.8
2	SP1.1/pSN1.292	Glucose minimal	6	1.3
3	SP1.1/pSN1.244	Glucose rich	1.7	7.6

[a] Abbreviations: K, kanosamine; SA, shikimic acid; [b] pSN1.292: pBbA1a-*ntdCntdAntdBserA*; pSN1.244: pBbA1a-*ntdCntdAserA*; [c] concentrations are calculated using the ^{1}H-NMR resonance peaks at δ 4.40, t, 1H for SA and δ 5.27, d, 0.45 H for K by comparing with the integral corresponding to 10 mM TSP (δ = 0.00 ppm)

Evidence from both glucose-rich and glucose-minimal conditions (Table 1, entries 1 and 2) suggests that there was no significant difference between the amount of kanosamine produced. The data for the glucose-rich fermentation was shown in Figure 16. Shikimic acid was a predominant byproduct in this case (Table 1, entry 1) because the SP1.1 does not have the ability

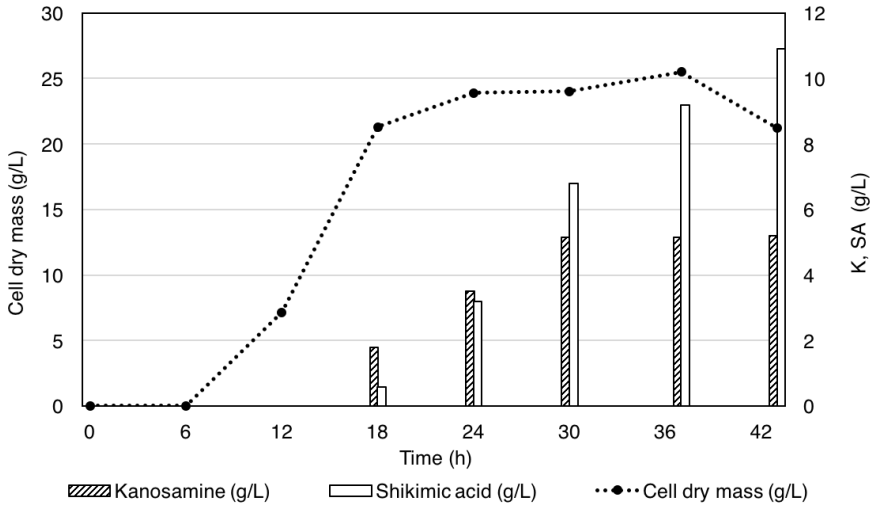


Figure 16. Glucose-rich fermentation of *E. coli* SP1.1/pSN1.292

to metabolize shikimic acid as it is *aroK* and *aroL* deficient. In the glucose-minimal conditions, not much shikimic aid was produced due to the lack of excess glucose in the fermentation vessel (Table 1, entry 2).

A different plasmid pSN1.244 containing the genes *ntdA*, *ntdC* and *serA* was constructed without *ntdB* to verify whether kanosamine-6-phosphate can be dephosphorylated by a native enzyme of *E. coli*. Glucose-rich fermentation of SP1.1/pSN1.244 was run similarly to the previous ones. ^{1}H-NMR analysis revealed only trace amounts of kanosamine. One of the possible reasons might be that kanosamine 6-phosphate has been carried down the pathway to give iminoE4P which then hydrolyzed to E4P and produced shikimic acid. Since the production of shikimic acid is not greater than the production for glucose-rich fermentation of SP1.1/pSN1.292, this might not be the case after all. The incidence of aminoshikimic acid was not found on NMR spectra.

A typical kanosamine fermentation lasts between 48 h to 54 h depending on the glucose conditions. Samples (1-2 mL) of the fermentation culture were collected every six hours from the first addition of IPTG. Cell densities were determined by dilution of fermentation broth with M9 medium (1:100) followed by measurement of OD_{600}. Dry cell weight (g/L) was calculated using a conversion coefficient of 0.43 g/L/OD_{600} and the titer value seen in Table 1 was calculated in g/L from ^{1}H-NMR analysis. Brown-colored kanosamine was obtained from the fermentation broth by applying it to Dowex-50WX8, a strong cation exchange column and eluting fractions with a linear gradient of 0-1N HCl. Fractions testing positive to ninhydrin assay and analyzed as clean by ^{1}H-NMR were collected and concentrated. ^{1}H-NMR and ^{13}C-NMR for the isolated kanosamine yielded from this study can be seen in Figure 21 and Figure 22 respectively.

2.3. Production of kanosamine by fermentation of *Bacillus pumilus*

In 2004, Guo and Frost published a two-microbe pathway to biosynthesize aminoshikimic acid. Instead of heterologously expressing the genes, they decided to synthesize kanosamine from *B. pumilus* itself. According to Guo, 20g/L kanosamine was synthesized in a culture medium of soy flour, soytone and NaCl.[19] The above fermentation was repeated in this study for two reasons: 1. To obtain kanosamine in order to chemically synthesize kanosamine6P; 2. To assess the specific activity of NtdA where kanosamine6P can be utilized as the substrate in the enzyme assay for the reversible biochemical reaction (Figure 17). Kanosamine6P cannot be isolated from fermentation broth because cells do not readily export phosphorylated intermediates. Therefore, it was produced through hexokinase-catalyzed phosphorylation from bio-synthesized kanosamine. Since the construction of the recombinant kanosamine-producing *E. coli* was still in the early stages at that time, kanosamine was biosynthesized using *Bacillus pumilus* ATCC 21143 strain with soy flour as the nitrogen source.

Figure 17. Formation of kanosamine-6-phosphate. (a) Genes: NtdA, 3-keto-glucose-6-phosphate:glutamate aminotransferase; (b) Abbreviations: 3-keto-G6P, 3-keto-glucose 6-phosphate; kanosamine6P, kanosamine-6-phosphate.

Purified *B. pumilus* ATCC 21143 was grown on solid nutrient agar plates at 37 °C for 20 h. Growth of inoculants was initiated by introduction of single colonies from the nutrient agar plate into 100 mL SSNG (soy flour, soytone, NaCl) culture media. Since SSNG[24] is a heterogeneous medium (a suspension of soy flour), the stage of cell growth was monitored through the pH of the culture. The growth of inoculant took 40 h until the pH was between 6.0 to 8.5, at which point it was transferred to the fermenter vessel equipped with baffles. Temperature, pH and D.O. levels

were controlled by PID loops at 30 ^{o}C, 7.0, and 10% respectively. The fermentation lasted for about 90 h. The titer value for kanosamine (10-13 g/L) was calculated through ^{1}H-NMR integration. The broth was applied to a Dowex-50WX8, a strong cation exchange column. Elution with a linear gradient of 0-1N HCl yielded 6 g (60% yield) of brown-colored solid kanosamine.

Hexokinase-catalyzed phosphorylation of kanosamine in the presence of citric acid for 24 h afforded kanosamine6P (Figure 18). AG-1 X8 anion exchanger resin (acetate form) was employed to separate inorganic and organic phosphates by passing a linear gradient of 0-2N acetic acid. The fractions testing positive for total phosphate and negative for inorganic phosphates were collected to yield white, crystalline kanosamine6P. The ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR of the isolated kanosamine6P can be seen in Figure 23, Figure 24 and Figure 25 respectively.

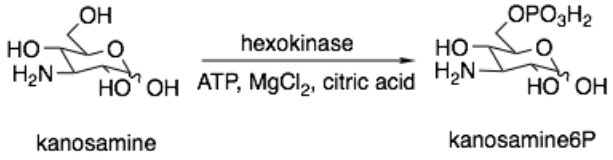


Figure 18. Hexokinase-catalyzed phosphorylation of bio-synthesized kanosamine.

2.4. Specific enzyme activities for NtdA and NtdC

To determine the specific activities of the *B. subtilis* enzymes expressed in *E. coli*, cell lysates obtained from DH5α were used. The proposed substrate for NtdA, 3-keto-glucose-6-phosphate is generated in situ and cannot be isolated. Hence, the best course of action would be a coupled NtdC-NtdA assay in which kanosamine6P and 2-ketoglutaric acid are the substrates and the decrease of absorbance of cofactor NADH is followed at 340 nm (Figure 19).[20]

Results from initial experiments have been inconsistent. The activity of NtdC was assayed by recording the increase in the formation of NADH at 340 nm (Figure 20).[20] However, the specific

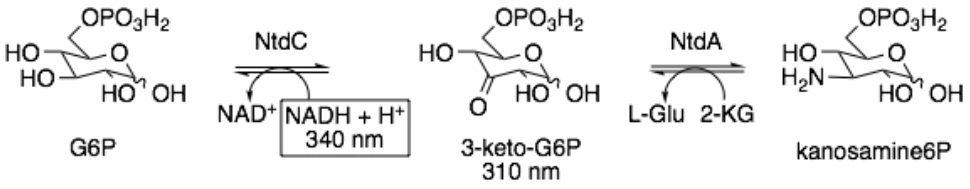


Figure 19. Strategy for NtdA assay by coupling it with NtdC starting with kanosamine6P to go in the reverse direction.

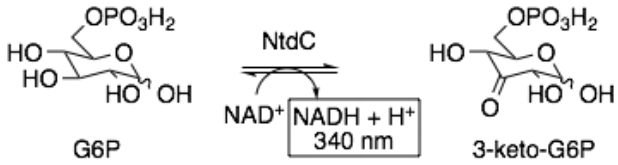


Figure 20. Strategy for NtdC assay to determine its specific activity.

activity calculated from the initial rate of increase of absorbance varies with different experiments even though the procedure does not change. One probable explanation would be the interference of another enzyme native to *E. coli* which is competing for the cofactor NAD^+. Purified enzyme instead of crude cell lysate can eliminate this problem. Moreover, the coupled NtdC-NtdA assay would need NtdC as a reagent, hence would require a standard concentration purified enzyme.

3. DISCUSSION

Biosynthesis of kanosamine in literature has been described by two pathways so far: 1. glucose to kanosamine via the intermediacy of UDP-glucose in *B. pumilus,*[8] *B. cereus,*[18] and *A. mediterranei;*[19] and 2. glucose to kanosamine in *B. subtilis.*[20] The first attempt to biosynthesize kanosamine was reported in 1967 by Umezawa et al. in a 300 L tank fermentation which yielded 2.6 g/L kanosamine.[2] This biosynthesis was reported to follow the first pathway listed above i.e., glucose to kanosamine via UDP-glucose. The culture medium used in these fermentations consisted of a ratio of 1 g glucose to 1.5 g soybean meal mixture along with 0.3% w/v sodium chloride. The second attempt at biosynthesizing kanosamine was reported in *B. cereus UW85* in a

series of batch fermentations to yield about 2.2 ± 0.5 μg/mL.[22] It was speculated but never substantiated that the kanosamine biosynthetic pathway in *B. cereus* UW85 followed the first kind where glucose was converted to kanosamine via UDP-glucose.[18] The biosynthetic pathway to kanosamine in *A. mediterranei* was found as part of the aminoshikimate pathway which produced AHBA, the precursor to Rifamycin B in the organism. Utilizing the results of *A. mediterranei* cell-free lysate reactions, Guo and Frost have shown that kanosamine was produced when UDP-glucose was taken as a substrate.[19] Guo and Frost sought to clone the genes responsible for biosynthesis of kanosamine from *B. pumilus* to construct the first half of the aminoshikimate pathway in *E. coli*. Their experimental results showed that kanosamine was biosynthesized from UDP-glucose via the intermediates UDP-3-keto-D-glucose and UDP-kanosamine. But the isolation of UDP-3-keto-D-glucose dehydrogenase proved to be difficult as the enzyme lost its activity with the loss of NAD^+-dependent coenzyme in the purification process.[24] Hence they opted to utilize kanosamine directly biosynthesized from *B. pumilus* shake flask experiments in which the highest yield was 4 g/L and fed-batch fermentations that have an impressive 25 g/L yield.[19] The heterogeneous culture medium consisted of glucose (30 g/L), soy flour (30 g/L) which was the nitrogen source, soytone (1 g/L) and sodium chloride (9 g/L).[19] However, being able to isolate and heterologously express the kanosamine biosynthetic operon in a microbe which could be easy to manipulate is still important. Recent reports of the *ntd* operon in the gram-positive bacteria *B. subtilis* provided the opportunity to experiment with the microbial synthesis of kanosamine.[20] The authors identified the complete set of genes required to biosynthesize kanosamine from glucose and proved that kanosamine was indeed biosynthesized from glucose without the intermediacy or requirement for UDP-glucose utilizing in vitro enzymatic reactions. In this chapter, the *ntd* genes were first cloned individually as standard Bglbrick parts, then cloned together in a specific order

following the *trc* promoter and appended with *serA* behind to yield plasmid pSN1.292. Expression of pSN1.292 in *E. coli* SP1.1 strain and fed-batch fermentations of the kanosamine-producer yielded a titer value of 8 g/L when both glucose rich and glucose minimal conditions were employed. This result established evidence for the second type of kanosamine biosynthetic pathway that was reported by Vetter et al. Furthermore, these titer values were comparable to the 10 g/L that we produced in this study by the fed-batch fermentations of *B. pumilus* following Guo's protocol. Additionally, this study adopted many techniques that could be advantageous in a synthetic biology perspective in the long run: 1. The culture medium is no longer heterogeneous and so, the cell mass can be monitored utilizing OD_{600}; 2. The Bglbrick strategy allows for rapid replacement of plasmid parts. For example, to examine the significance of kanosamine phosphatase (NtdB), a plasmid containing only *ntdC*, *ntdA* and *serA* was cloned in two days and the fed-batch fermentation demonstrated the clear lack of kanosamine in the fermentation broth indicating that kanosamine-6-phosphate cannot be hydrolyzed by a native *E. coli* enzyme.; 3. Having a standardized assembly of Bglbrick parts ensures that *trc* promoter can be substituted by another strong promoter, T7 in order to improve or tweak the titer value of kanosamine or pBbA1a, a medium copy number plasmid vector can be replaced by pBbA1k,[21] a high copy number vector or the order of the genes behind the promoter can be rapidly shuffled according to the measure of their enzyme activities. Few enzyme specific activity studies were attempted in this chapter for NtdA and NtdC but they need to be repeated with purified enzymes rather than cell-free lysate reactions.

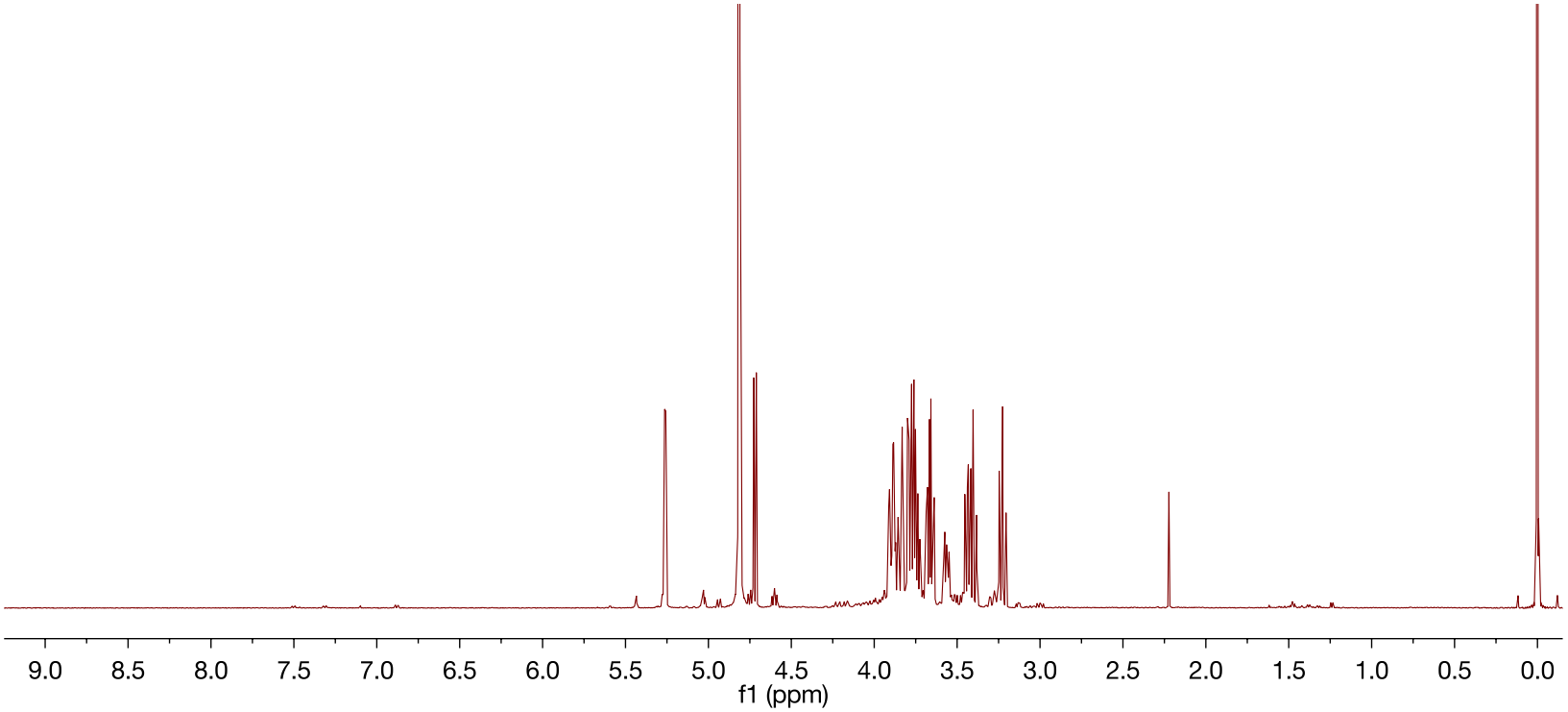


Figure 21. ^{1}H-NMR of kanosamine

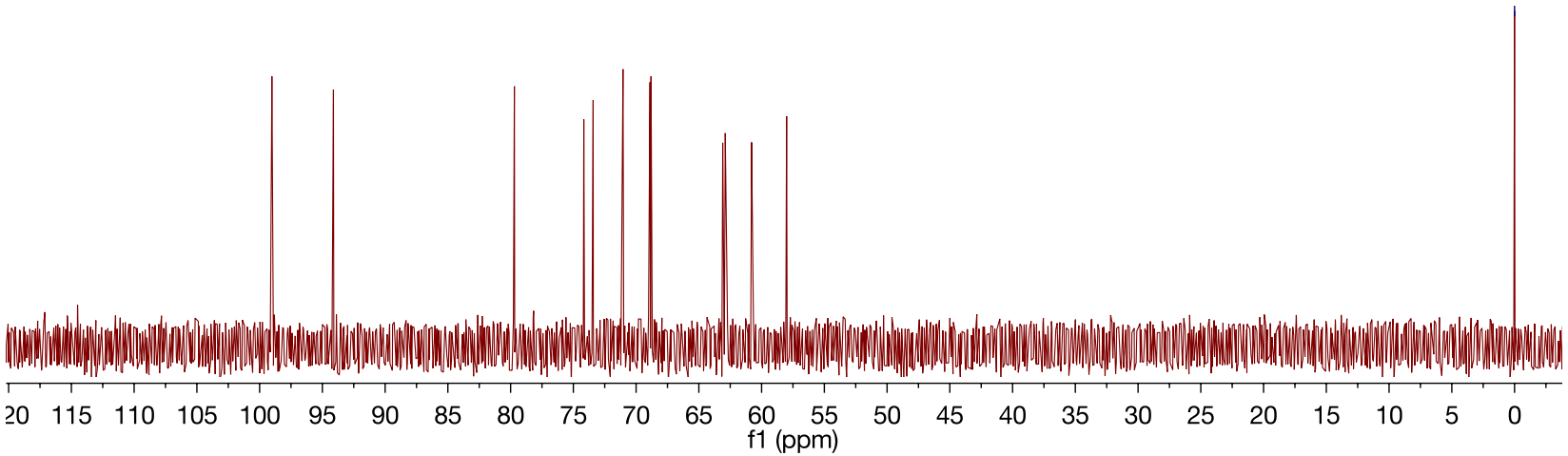


Figure 22. ^{13}C-NMR of kanosamine

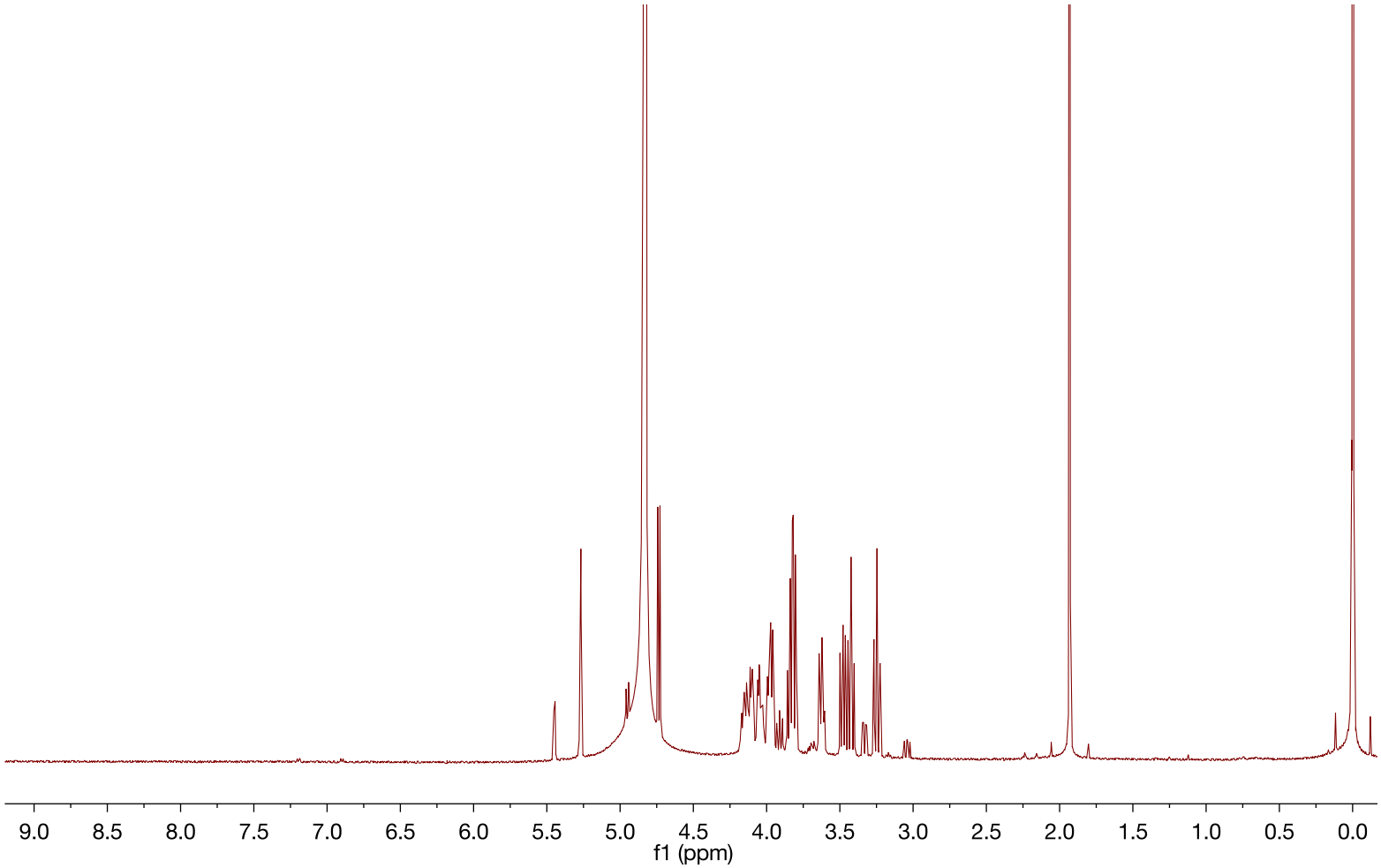


Figure 23. ^{1}H-NMR of enzymatically synthesized kanosamine 6-phosphate

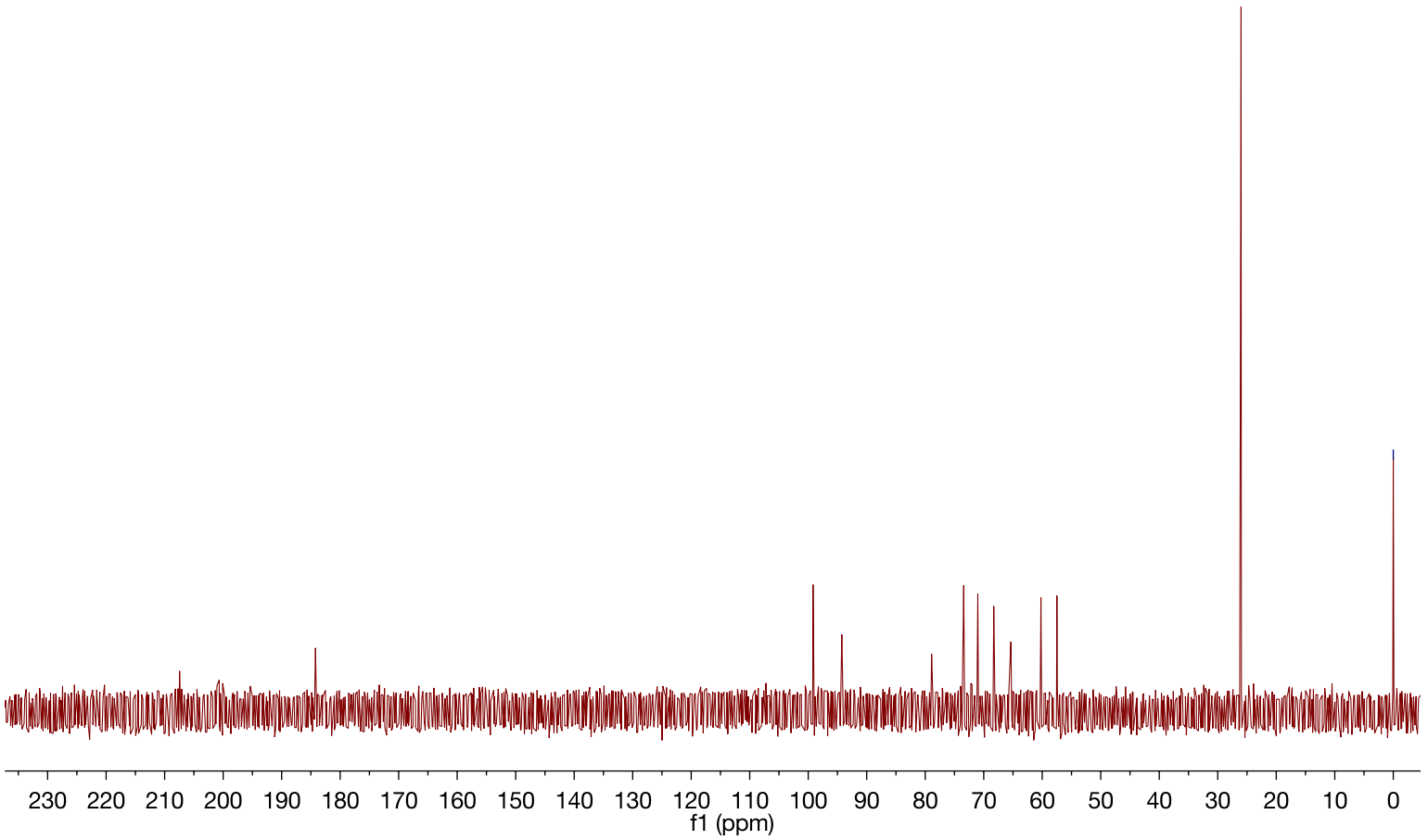


Figure 24. ^{13}C-NMR of enzymatically synthesized kanosamine-6-phosphate

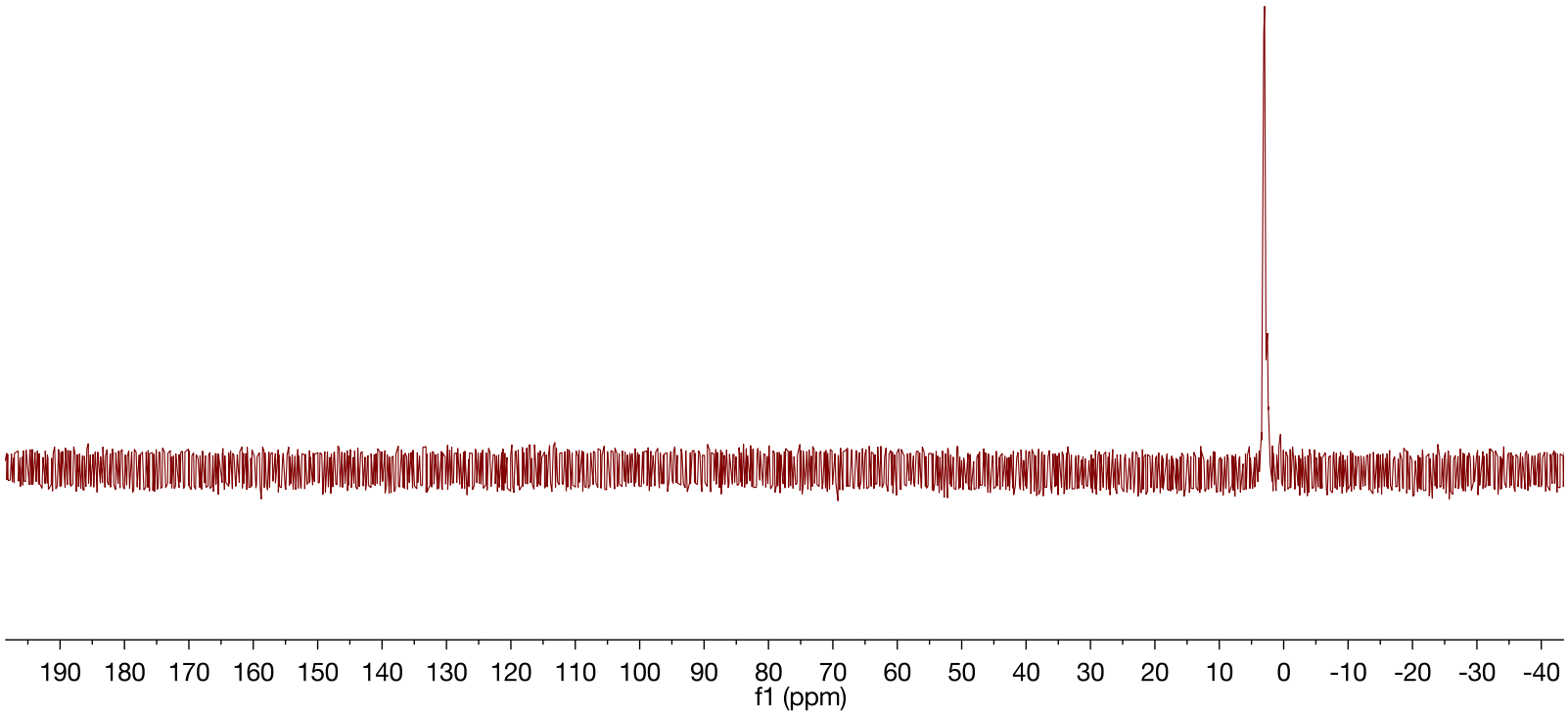


Figure 25. ^{31}P-NMR of enzymatically synthesized kanosamine-6-phosphate.

4. EXPERIMENTAL

4.1. General information

E. coli and *B. pumilus* cells were harvested at 6000 rpm for 15 min at 4 ℃. Cell lysis was achieved by two passages through a French pressure cell (SLM Aminco) at 18,000 psi. Cellular debris was removed from the lysate by centrifugation (20000 rpm, 30 min, 4 °C). Protein concentrations were determined using the Bradford dye-binding procedure using protein assay solution purchased from Bio-Rad. The assay solution was prepared by diluting 20 mL of the Bio-Rad concentrate to 100 mL with water followed by gravity filtration of the resulting solution. Assay solution (5 mL) was added to an aliquot of protein containing solution (diluted to 0.1 mL) and the sample was vortexed. After allowing the color to develop for 5 min, the absorbance at 595 nm of the solution was measured. Protein concentrations were determined by comparison to a standard curve prepared using bovine serum albumin. Q5 High-Fidelity DNA polymerase, Q5 Hotstart High Fidelity DNA polymerase Calf-alkaline phosphatase and, Q5 site-directed mutagenesis kit were purchased from NEB.

4.2. Spectroscopic measurements

^{1}H NMR spectra were recorded on a 500 MHz spectrometer. Chemical shifts for ^{1}H NMR spectra in D_2O is reported (in parts per million) relative to sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid, TSP (δ = 0.00 ppm). The following abbreviations are used to describe spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), m (unresolved multiplet), dd (doublet of doublets), br (broad).^{13}C NMR spectra were recorded at 125 MHz and chemical shifts for these spectra were reported (in parts per million) relative to sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid, TSP (δ = 0.00 ppm). Concentrations of fermentation and cell-free reaction products were determined by comparison of the integrals corresponding to each

compound with the integral corresponding to TSP (δ = 0.00 ppm) in the ^{1}H NMR. Kanosamine was quantified using the resonance at δ 5.27, d, 0.45 H and shikimic acid was quantified using the resonance δ 4.40, t, 1 H.

4.3. Chemical assays

4.3.1. Ninhydrin assay

The ninhydrin solution[26] for a total volume of 55 mL was prepared as follows: 20 mL of water and 14.5 mL of glacial acetic acid were added to 5.6 g of $LiOH{\cdot}H_2O$ in a beaker set up for continuous stirring. This is an exothermic reaction and once the temperature of the solution cools down, 1 g of ninhydrin and 20 mL of sulfolane were added. Solution was stirred for 15 min at the end of which 0.018 g of hydrindantin was added. The reagent must be freshly prepared and immediately used. An aliquot (25 μL) of the sample was added to a test tube (13 mm x 100 mm) containing 75 μL water and 900 μL of ninhydrin reagent.[26] The resulting mixture was heated at 100 °C for 5 min. A purple color develops if the sample contains free amino group. The concentration of amino group containing compound in a sample was quantified by comparing the absorbance at 570 nm of the sample to a standard curve that is prepared using glycine as the standard.

4.3.2. Organic and inorganic phosphate assay[27]

Reagents used to quantify both organic phosphate and inorganic phosphate include 10% $Mg(NO_3)_2$ (w/v, dissolved in ethanol), 0.5 M HCl, 10% ascorbic acid (w/v, dissolved in H_2O), and 0.42% $(NH_4)_2MoO_4$ (w/v, dissolved in 1 M H_2SO_4). The assay solution (freshly mixed) consists of one volume of 10% ascorbic acid and six volumes of 0.42% $(NH_4)_2MoO_4$.

To assay for organic phosphate, 100 μL of 10% $Mg(NO_3)_2$ was added to a test tube (13 mm x 100 mm) containing 100 μL of sample. The resulting mixture was then evaporated to dryness

over a flame, leaving a white solid. To this test tube was added 600 µL of 0.5 M HCl. After the white solid was dissolved, the solution was heated at 100 °C for 15 min in a boiling water bath. Assay solution (1400 µL, described above) was added to the cooled sample and the resulting mixture was kept at 45 °C for 20 min. If the original sample contains either inorganic phosphate or any organic phosphate, a blue color will develop and OD was measured at 820 nm.

To assay for inorganic phosphate, 600 µL of 0.5 M HCl and 1400 µL of assay solution were directly added to a test tube containing 100 µL of sample. The resulting reaction mixture was then heated at 45 °C for 20 min. Blue color is developed if sample contains inorganic phosphate.

4.4. Genetic Manipulation

4.4.1. Determination of DNA concentration

An aliquot (10 µL) of DNA solution was diluted to 100 µL with deionized distilled water and the absorbance at 260 nm was measured relative to water. An absorbance of 1.0 at 260 nm corresponds to 50 µg/mL of double stranded DNA.

4.4.2. Purification of plasmid DNA

Small, medium and large scale purifications of plasmid DNA was performed by using the Mini, Midi and Maxi kits (Qiagen) following the protocol provided by the manufacturers.

4.4.3. Restriction enzyme digestion of DNA

A typical digest (20 µL) contained less than 1000 ng of DNA, 2 µL of the appropriate NEB restriction enzyme buffer, 1 µL of restriction enzyme and dd water. Reactions were incubated at 37 ^{o}C for 1-2 h. For those reactions in which the two enzymes have different NEB buffers, the stepwise protocols provided by NEBcloner online tool have been used. Digests were terminated by addition of 10% v/v of Endostop solution (10X concentration) and subsequently analyzed by

agarose gel electrophoresis. DNA was isolated from agarose gel using Zymoclean Short Fragment Recovery Kit as per manufacturer's protocol (Zymo Research).

4.4.4. Agarose gel electrophoresis and Isolation of DNA from agarose

Agarose gels were run in TAE buffer containing 40 mM Tris-acetate and 2 mM EDTA (pH 8.0). Gels typically contained 0.7% agarose (w/v) in TAE buffer. Ethidium bromide (0.5 μg/mL) was added to the agarose to allow visualization of DNA fragments under a UV lamp. The size of the DNA fragments was determined by comparison to a 1:1:4 v/v/v solution of NEB 1 kb ladder, NEB purple gel loading dye and dd water. Zymoclean Short Fragment Recovery Kit was used to isolate DNA from agarose gel. The band of DNA was excised using a razor and transferred to a 1.5 mL microfuge tube. Three volumes of agarose dissolving buffer was added to each volume (100 μg = 100 μL) of agarose gel and this suspension was incubated at 55 ^{o}C with intermittent vortexing until the gel was completely dissolved. DNA from the resulting solution was purified using a Zymo-Spin Column.

4.4.5. Ligation of DNA

DNA ligation reactions were designed so that insert DNA to vector DNA molar ratio was 3:1. Quantification of DNA for this purpose was conducted on 0.7% TAE agarose gel where the corresponding restriction enzyme-digested DNA was compared with All Purpose Hi-Lo DNA mass ladder (Bionexus Hi-Lo™ DNA marker) to quantify the amount (ng) of DNA present in 1 μL. The components of ligation reactions (20 μL each) included 2 μL of 10X T4 ligation buffer, 1 μL of T4 DNA ligase, 3:1 pmol/μL ratio of insert DNA to vector DNA and dd water. A typical ligation was incubated at room temperature for a minimum of 2 h and then purified using Zymoclean DNA Clean and Concentrator kit (Zymo Research) as per manufacturer’s protocol.

4.4.6. Preparation and transformation of *E. coli* electrocompetent cells

Electrocompetent cells were prepared using the following procedure: A single colony was inoculated into 5 mL LB medium and shaken overnight at 37 ^{o}C. 2 mL of this inoculum was used to start a 500 mL 2xYT medium in a 2L baffled flask and was cultured at 37 ^{o}C at 250 to 300 rpm until OD_{600} equals 0.5 to 0.7. Then, the culture flask was chilled in ice for 10 min before being transferred to a chilled sterile 400 mL centrifuge bottle. The cells were harvested in a centrifuge at 4000 rpm and 4 ^{o}C for 5 min. Subsequently the cell pellet was exhaustively washed four times with 400 mL, 200 mL and 200 mL sterile cold dd water, and 100 mL ice cold 10% glycerol solution respectively. After each wash step, the cells were centrifuged for 10 min at 6000 rpm and 4 ^{o}C. Lastly, the cell pellet was suspended in 1.5 mL sterile cold 10% glycerol solution following which it was aliquoted (50 µL) into sterile 1.5 mL microfuge tubes kept on ice. The tubes were flash-frozen in liquid nitrogen and stored long term at -80 ^{o}C.

The first step in the transformation of electrocompetent cells was thawing them on ice for 5 min along with the electroporation cuvettes. Less than 5 µL of Zymoclean purified DNA was added to 50 µL cells. The electroporations (Bio-Rad Gene Pulser) were performed at field strength of 2.5 kV/cm, resistance of 200 ohms and capacitance of 25 µF, immediately after which cells were suspended in 1 mL SOC medium and shaken for 1 h at 250-300 rpm and 37 ^{o}C. If the cells were to be plated on rich medium, no wash step was needed. The cells were resuspended in 100 µL of appropriate medium and were plated in both high as well as low concentration to obtain single colonies. Conversely, if the cells were to be plated on minimal medium, the cells were washed three times in M9 medium via centrifugation and resuspension steps and then plated in high and low concentrations. The cells were incubated at 37 ^{o}C for 12-15 h for rich medium and 36-48h for minimal medium.

4.4.7. Bacterial Strains and Plasmids

4.4.7.1. BglBrick plasmid pBbA1a-ntdA

To make this 4.9 kb plasmid, *ntdA* was PCR amplified from pKD16.211 utilizing the following primers containing (a) BglII, BamHI, XhoI restriction sequences; (b) Shine Dalgarno ribosome binding sequence; (c) 20 nucleotides from the beginning of the gene for the forward and from the end for the reverse: 5' – AATAAGATCTAAAGGAGGCATCC ATGCAAAAACAGGTTAAGAT and 5'- TAATCTCGAGTCTGGATCC TTATACTCCGATTTCTTGCT. Both *ntdA* and pBbA1a-RFP were digested with BglII and XhoI, subsequently ligated to form pBbA1a-*ntdA*. The Bglbrick was sequenced and stored as a glycerol freeze at -80 ^{o}C.

4.4.7.2. Bglbrick plasmid pBbA1a-ntdB

PCR amplification of *ntdB* from pKD16.211 utilized primers containing (a) BglII, BamHI, XhoI restriction sequences; (b) Shine Dalgarno ribosome binding sequence; (c) 20 nucleotides from the beginning of the gene for the forward and from the end for the reverse: 5' - AATAAGATCTAAAGGAGGCATCCATGTTATTAAGCAAGAAATC and 5'- TAATCTCGAGTCT GGATCCTCATGATCCAATTAATTTTT**.** This 4.4 kb plasmid was assembled with ligation of BglII and XhoI-digested *ntdB* and pBbA1a-RFP, sequenced and stored at -80 ^{o}C.

4.4.7.3. Bglbrick plasmid pBbA1a-ntdC

Site-directed mutagenesis of the BglII site present in *ntdC* in the plasmid pKD16.211 yielded pSN1.052. The following silent mutation primers were designed through NEBasechanger online tool to substitute the serine-encoding TCT with TCA: 5'- TGGTTTTAGATCACAGGAAGTATTAG and 5'- TCACTAAAAGAAGGGATCTC. To make

this 4.6 kb Bglbrick plasmid, *ntdC* was PCR amplified from pSN1.052 utilizing the following primers containing (a) BglII, BamHI, XhoI restriction sequences; (b) Shine Dalgarno ribosome binding sequence; (c) 20 nucleotides from the beginning of the gene for the forward and from the end for the reverse: 5' – AATAAGATCTAAAGGAGGCATCC ATGAAGAAGATAGGCATCAT and 5'- TAATCTCGAGTCTGGATCC CTAGTTGACTGCTGAAACAG. Ligation of BglII/XhoI-digested *ntdC* and pBbA1a-RFP yielded pBbA1a-*ntdC* which was sequenced and stored as a glycerol freeze at -80 ^{o}C.

4.4.7.4. Plasmid pSN1.292

Digestion of pBbA1a-*ntdC* with EcoRI/BamHI yielded *ntdC*, which was inserted into EcoRI/BglII digested pBbA1a-*ntdA,* thus marking the junction between the two genes with BamHI-BglII scar. The 2.3 kb *ntdCntdA* cassette was inserted into pBbA1a-*ntdB* through the exact same procedure yielding pBbA1a-*ntdCntdAntdB.* The 8 kb plasmid pSN1.292 was assembled by inserting the 3 kb fragment *ntdCntdAntdB* into pBbA1a-*serA* in a similar fashion as above.

4.4.7.5. Plasmid pSN1.244

Digestion of pBbA1a-*ntdC* with EcoRI/BamHI yielded *ntdC*, which was inserted into EcoRI/BglII digested pBbA1a-*ntdA,* thus marking the junction between the two genes with BamHI-BglII scar. The 2.3kb *ntdCntdA* cassette was inserted into pBbA1a-*serA* through the exact same procedure yielding the 7.2 kb plasmid pSN1.244.

4.5. Culture medium

All solutions were prepared in distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g) Bacto yeast extract (5 g) and NaCl (10 g). M9 salts (1 L) contained Na_2HPO_4 (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g) and NaCl (0.5 g). M9 medium contained D-glucose (10 g), $MgSO_4$ (0.12 g) and thiamine (0.001 g) in 1 L of M9 salts. SSNG medium (1 L) contained soy flour (15

g), soytone (1 g), NaCl (6 g) and glucose (10 g). *E. coli* strains were grown on both LB/Ap and M9/glu/aros plates while *B. pumilus* grows on nutrient agar plates. The standard *E. coli* fermentation medium (1 L) contained K_2HPO_4 (7.5 g), ammonium iron (III) citrate (0.3 g), anhydrous citric acid monohydrate (1.98 g), and concentrated H_2SO_4 (1.2 mL). Concentrated NH_4OH (4.1 mL) was added to adjust pH of the medium to 7.0. Prior to initiating fermentation, the following supplements were added: D-glucose (20 g for glucose-minimal and 30 g for glucose-rich conditions); MgSO4 (0.24 g); aromatic amino acids including phenylalanine (0.7 g), tryptophan (0.35 g) and tyrosine (0.7 g); aromatic vitamins including *p*-hydroxybenzoic acid (0.010 g), potassium *p*-aminobenzoate (0.010 g), 2,3-dihydroxybenzoic acid (0.010 g); trace minerals including $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.0037 g), $ZnSO_4.7H_2O$ (0.0029 g), H_3BO_3 (0.0247 g), $CuSO_4.5H_2O$ (0.0025 g), and $MnCl_2.4H_2O$ (0.0158 g). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM where indicated. Glucose, $MgSO_4$ (1 M), and aromatic amino acids were autoclaved, while solutions of aromatic vitamins, IPTG and trace minerals were sterilized through 0.22-μm membranes. The standard *B. pumilus* fermentation medium (1 L) contained soy flour (30 g), soytone (1 g), NaCl (9 g) and glucose (30 g). Ampicillin was added where appropriate to the final concentration of 50 mg/mL.

4.6. Fed-batch fermentation conditions

4.6.1. General

A B. Braun BIOSTAT® B-DCU fermentor (2 L) connected to a DCU tower system was used for fed-batch fermentations at 1 L scale. Data acquisition utilized MFCS/win 3.0 software (Sartorius Stedim Systems), which was installed in a personal computer (Digilink) operated by Windows® 7 Professional. Temperature and pH were controlled with PID loop maintaining at 33

°C and pH 7.0±0.1 with 2N H_2SO_4 and concentrated NH_4OH. Impeller speed was varied between 50 and 1800 rpm to maintain dissolved oxygen (D.O.) levels at 10% air saturation. Airflow was increased from 0.06 L/min to 1 L/min to maintain dissolved oxygen (D.O.) levels at 10% air saturation. The concentration of glucose in the medium was maintained at glucose-rich culture conditions defined as a glucose concentration in the range of 25-30 g/L. Glucose levels were maintained throughout the run by addition of feed glucose solution (60% w/v). Glucose concentration was measured with GlucCell™ and glucose test strip (CESCO Bioengineering Co., Ltd.). Antifoam 204 (Sigma-Aldrich) was added as needed to mitigate foam accumulation. Inoculant was started by introduction of a single colony picked from an agar plate into 5 mL of M9 medium. The culture was grown at 37 °C with agitation at 250 rpm until turbid (24 h) and subsequently transferred to 100 mL of M9 medium. This 100 mL culture was grown at 37 °C and 250 rpm until the OD_{600} reached 1.5-2.5 (12 h). The fermentation vessel was inoculated with the 100 mL culture thus initiating the fermentation run (t = 0 h).

4.6.2. Fed-batch fermentation of *B. pumilus*

Growth of an inoculant was initiated by introduction of a single colony from a nutrient agar plate into 100 mL of SSNG medium in a 500 mL flask with baffles and grown at 30 ˚C with agitation. Since SSNG medium was a heterogeneous medium (a suspension of soy flour), cell growth could not be monitored by following OD_{600}. The pH of the culture supernatant was used to monitor the stage of the cell growth. The pH of the culture supernatant decreased from 7.0 to approximately 5.0 during the first 24 h of growth and increased from 5.0 to 8.5 thereafter due to the formation of kanosamine. When the pH of the culture supernatant reached 7.5 (30-48 h), the 100 mL culture was then transferred to the fermentation vessel. The maximum impeller speed was set at 750 rpm and airflow at 1.0 L/min to maintain the D.O. levels at 10% air saturation. The

airflow never reached its maximum, remaining at 0.06 L/min and impeller speed was around 550 rpm after the exponential phase which usually was about 3-6 h after inoculation. The fermenter runs were anywhere between 60-90h, and the glucose feed was started at t=19h. For ^{1}H-NMR quantification of kanosamine, the broth was concentrated to dryness under reduced pressure, concentrated to dryness one additional time from D_2O, and then dissolved in D_2O containing a known concentration of the sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid. The concentration of kanosamine was determined by comparison of integral corresponding to the resonance at 5.27 ppm (d, 0.45H) with integral corresponding to 10 mM TSP (δ = 0.00 ppm, s, 9 H) whereas for shikimic acid, the resonance at δ 4.40, t, 1 H.

4.6.3. Fed-batch fermentation of *E. coli* SP1.1

Inoculant was started by introduction of a single colony picked from an agar plate into 5 mL of M9 medium and incubated at 37 °C and 250 rpm overnight for at least 12 h. 4 mL of the overnight culture was added to 100 mL culture of M9 media and grown at 37 °C and 250 rpm until the OD_{600} reached 1.3-1.7 (7-8 h). The fermentation vessel was inoculated with this 100 mL culture thus initiating the fermentation run (t = 0 h). The dissolved oxygen (D.O.) levels were maintained at 10% air saturation. The cell growth reached stationary phase at t = 11 h. Approximately 150 g of glucose was consumed after 54-60 h. The broth volume increased gradually due to cell growth, product, addition of glucose solution, 2M H_2SO_4 and NH_4OH which was used to adjust pH. Final broth volume was approximately 1.2 L.

4.7. Product Purification from Fermentation Broth

4.7.1. Purification of kanosamine from fermentation broth of *B. pumilus*

After the completion of the fermentation, the broth is centrifuged 6000 rpm, 4 °C, 15 min). The supernatant was passed through six layers of cheesecloth and the remaining cells in the filtrate were removed by centrifugation (6000 rpm, 4 °C, 15 min). Activated charcoal (10 g/L) was then added to the supernatant and the mixture was shaken at 37°C and 250 rpm for 1h. A third step of centrifugation removes most of the charcoal and the supernatant is filtered through through Celite. The resulting solution was applied to Dowex 50WX8 (H^+, 2.5 cm x 51 cm, 300 mL) resin. The column was washed with H_2O (600 mL) and eluted with a linear gradient (750 mL + 750 mL, 0 to 1 M) of HCl. Fractions testing positive by the ninhydrin assay were collected and evaporated under reduced pressure to give kanosamine as a slightly brown solid (very hygroscopic). ^{1}H NMR (500 MHz, D_2O, TSP as reference): δ 5.27 (d, J = 3.5 Hz, 0.4 H), 4.72 (d, J = 8 Hz, 0.6 H), 4.39 (m, 1 H), 3.74-4.38 (m, 2 H), 3.67 (ddd, J = 10, 10, 4.5 Hz, 1 H), 3.57 (ddd, J = 7.5, 5.5, 2 Hz, 0.5 H), 3.45 (dd, J = 10, 8 Hz, 0.5 H), 3.41 (dd, J = 10, 10 Hz, 0.5 H), 3.25 (dd, J = 10, 10 Hz, 0.5 H). ^{13}C NMR (125 MHz, D_2O, TSP as reference): δ 99.0, 94.1, 79.7, 74.2, 73.4, 71.0, 68.9, 68.8, 63.2, 63.0, 60.8, 58.1.

4.7.2. Purification of kanosamine from fermentation broth of *E. coli* SP1.1

After the completion of the fermentation, cells were harvested by centrifugation (6000 rpm, 4 °C, 15 min). Activated charcoal (10 g/L) was then added to the supernatant and the mixture was shaken at 37°C and 250 rpm for 1 h. Centrifugation (6000 rpm, 4 °C, 15 min) removes most of the charcoal and the supernatant is filtered through through Celite. The resulting solution was applied to Dowex 50WX8 (H^+, 2.5 cm x 51 cm, 300 mL) resin. The column was washed with H_2O (600 mL) and eluted with a linear gradient (750 mL + 750 mL, 0 to 1 M) of HCl. Fractions testing

positive by the ninhydrin assay were collected and evaporated under reduced pressure to give kanosamine as a slightly brown solid (very hygroscopic). ^{1}H NMR (500 MHz, D_2O, TSP as reference): δ 5.27 (d, *J* = 3.5 Hz, 0.4 H), 4.72 (d, *J* = 8 Hz, 0.6 H), 4.39 (m, 1 H), 3.74-4.38 (m, 2 H), 3.67 (ddd, *J* = 10, 10, 4.5 Hz, 1 H), 3.57 (ddd, *J* = 7.5, 5.5, 2 Hz, 0.5 H), 3.45 (dd, *J* = 10, 8 Hz, 0.5 H), 3.41 (dd, *J* = 10, 10 Hz, 0.5 H), 3.25 (dd, *J* = 10, 10 Hz, 0.5 H). ^{13}C NMR (125 MHz, D_2O, TSP as reference): δ 99.0, 94.1, 79.7, 74.2, 73.4, 71.0, 68.9, 68.8, 63.2, 63.0, 60.8, 58.1.

4.8. Enzymology

4.8.1. Cell-free Lysate Purifications

The corresponding Bglbrick plasmid was streaked onto an LB/Ap plate and incubated overnight at 37 °C. A single colony was inoculated into a 100 mL LB/Ap culture in a 500 mL baffled flask and shaken overnight at 37 °C (at least 12h). A portion of the overnight culture was used to inoculate two 500 mL LB/Ap cultures so that the initial OD_{600} in the flask was 0.15 and the two cultures were shaken until OD_{600} equals 0.5 to 0.7. IPTG was added to one of them and the cultures shaken for four more hours after which they were harvested via centrifugation at 6000 rpm and 4 °C for 5 min. The cell pellets were resuspended in 250 mL 100 mM cold Tris-HCl, pH 8.0 separately and centrifuged 6000 rpm and 4 °C for 5 min. After the washing step, the cell pellets were resuspended in Tris-HCl according to their weight (2 mL per g of wet cell) and lysed with French Press (18000 psi). The cell debris was removed by centrifugation (20000 rpm, 4 °C, 30 min) and the supernatant was used directly in the cell-free reactions. Protein concentrations were determined by measuring OD_{595} in comparison to the protein calibration curve built using BIORAD Quickstart Bovine Serum Albumin Standard and BIORAD Bradford 1x dye reagent.

4.8.2. NtdC assay

Stock solutions of reaction components were prepared in 100 mM Tris-HCl buffer (pH 8.0). Solutions of 50 mM G6P and 50 mM NAD^+ were freshly prepared. Mixtures were prepared by combining G6P and NAD^+ in 100 mM Tris-HCl, pH 8.0, such that each component has a final concentration of 5 mM in a total volume of 1 mL. Spectrophotometer was blanked with 1 mL of the above mixture in a quartz cuvette (1 cm path length) and the assay was with initiated with addition of appropriate amount of NtdC into this mixture. Total assay time was 10 min.

4.8.3. NtdC-NtdA coupled assay

Stock solutions of reaction components were prepared in 100 mM Tris-HCl buffer (pH 8.0). Solutions of 50 mM K6P, 50 mM KG, 10 mM NADH were freshly prepared. Mixtures were prepared by combining K6P, and KG in 100 mM Tris-HCl, pH 8.0, such that the components have a final concentration of 5 mM in a total volume of 1 mL. NtdC was also added prior to blanking. Spectrophotometer was blanked with 1 mL of the above mixture in a quartz cuvette (1 cm path length) and the assay was initiated with addition of appropriate amount of NtdA, and NADH, whose final concentration should be 0.2 mM. Total assay time was 10 min.

4.9. Synthetic Procedures

4.9.1. Enzymatic synthesis of kanosamine-6-phosphate

In a 250 mL Erlenmeyer flask equipped with a stir bar were combined kanosamine (0.44 g, 2.04 mmol), ATP (sodium salt, 2.2 g, 4.0 mmol), $MgCl_2 \cdot 6H_2O$ (1.0 g, 4.9 mmol), citric acid$\cdot H_2O$ (0.4 g, 1.9 mmol), and 140 mL of deionized water. The pH of the solution was adjusted to 8.0 with aqueous NaOH, and the solution was deoxygenated with Ar for 5 minutes. Hexokinase (500 units) was added. The reaction mixture was stirred slowly at rt for 24 h and the mixture was maintained

between pH 7.5 and pH 8.0 by the addition of 1 N aqueous NaOH. The pH was adjusted every hour for six hours and then monitored every six hours until the end of the reaction. The crude reaction mixture was applied to AG-1 X8 anion exchange resin (2.5 cm x 16 cm, 80 mL, acetate form). The column was washed with distilled water (160 mL) and eluted with a linear gradient (200 mL + 200 mL, 0-2 M) of acetic acid. Column fractions containing inorganic phosphate and phosphate esters were identified by colorimetric assay. Fractions containing phosphate esters were combined and concentrated to a small volume (about 5 mL) and then lyophilized overnight to dryness. Kanosamine-6-phosphate was isolated as a white crystalline solid (0.1 g) ^{1}H NMR (D_2O): δ 5.27 (d, *J* = 3.5 Hz, 0.5 H), 4.74 (d, *J* = 7.5 Hz, 0.5 H), 4.02-4.17 (m, 2 H), 3.99 (br d, *J* = 9.5 Hz, 0.5 H), 3.8 (m, 1.5 H), 3.67 (br d, *J* = 9.5 Hz, 0.5 H), 3.48 (dd, *J* = 10.5, 7.5 Hz, 0.5 H), 3.43 (dd, *J* = 10.5, 10.5 Hz, 0.5 H), 3.25 (dd, *J* = 10.5, 10.5 Hz, 0.5 H). ^{13}C NMR (D_2O): δ 99.1, 94.2, 78.5, 73.3, 73.2, 70.9, 68.4, 68.3, 66.2, 60.5, 57.7. ^{31}P NMR (D_2O): δ 2.99.

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CHAPTER TWO

MICROBIAL SYNTHESIS OF VALUE-ADDED CHEMICAL SHIKIMIC ACID

1. INTRODUCTION

Starch- and sugar-based feedstocks have traditionally been employed as the carbon source for the commercial production of biobased fuels and chemicals. Starch-based feedstocks which include grains such as corn, wheat, rye, barley; and tubers such as sweet potatoes and cassava,[28] contain long complex chains of sugar molecules which are easy to extract and convert into fermentable sugars whereas sugar-based feedstocks like sugar beet, sugarcane, sweet sorghum,[29] composed of a large proportion of simple sugars are even easier to utilize for fermentation. Initially, the raw material undergoes crushing in the case of sugar-based feedstocks and wet or dry milling in case of starch-based feedstocks to reduce the particle size distribution to facilitate maximum contact between enzymes and starch molecules in the subsequent steps. While the sugar-based feedstocks are directly fed into the fermentation after this step, starch-based feedstocks are dismantled into low molecular sugar units called dextrins by the action of α-amylase. In the next step, dextrins undergo saccharification into fermentable sugars by means of gluco-amylase.[30]

55 million tons of commodity chemicals are produced by fermentation annually,[31] and much of these processes utilize starch- and sugar-based feedstocks, thus increasing the risk of diverting farmland and crops for biofuel and chemical production to the detriment of food supply, resulting in a food versus fuel dilemma. Food-based feedstocks must be replaced by non-food-based feedstocks such as energy crops, agricultural biomass and residues that amount to a total of 534 million dry tons produced annually in the Unites States. Despite the high volume of extremely useful biomass available for fermentation purposes, only a small portion is being employed in fuel and chemical production. Feedstock handling and conversion is the primary barrier that is

hindering the biorefining industry from exploiting non-food-based feedstocks. By their nature, agricultural biomass and residues contain significant quantities of non-pristine compounds, necessitating feedstock-specific handling methods that would lead to logistical and transportation difficulties. Furthermore, the energy- and chemical-intensive pretreatments to release monosaccharides from the agricultural biomass and residues often raises process efficiency and environmental concerns. New technologies are urgently required to address these issues and to enable efficient biomass handling and environmentally friendly conversion of non-food-based feedstocks into value-added fuels and chemicals.

In this chapter, an overview of a self-sustaining feedstock handling system, developed by Liao and coworkers,[2] that processes lignocellulosic feedstocks to prepare homogeneous cellulosic material for value-added chemical production will be given. This study will show the successful utilization of the monosaccharides acquired from the above feedstock handling system to produce a value-added chemical, shikimic acid through fermentation of the shikimic acid-producer *E. coli* SP1.1/pKD12.138. Comparison of the production of shikimic acid through shake-flask experiments with both pure sugars and lignocellulosic biomass-derived sugars demonstrate that the amount of product produced in both cases is the same, about 1 g/L titer of shikimic acid. Additionally, fed-batch fermentations utilizing a mixture of a 5:3 molar ratio of glucose and xylose to approximate the composition of the anaerobic digestate (AD) hydrolysate were studied to estimate the amount of shikimic acid produced and to develop a fed-batch fermentation method that is suitable for the low sugar concentrations in the AD hydrolysate.

1.1. Anaerobic digestion of lignocellulosic feedstock

Lignocellulosic feedstock consists of three carbon-based polymers - cellulose, hemicellulose and lignin (Figure 26). Cellulose is a high molecular weight linear polymer of β-

1,4-linked D-glucose units which can appear as highly crystalline material.[1] Hemicellulose is a branched polysaccharide consisting of pentoses D-xylose, L-arabinose, and the hexoses D-mannose, D-glucose, D-galactose and uronic acids.[1] Lignin is an aromatic polymer composed of three major phenolic components, namely *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S), and is synthesized by polymerization of these monomers in various ratios depending on the source from which it is found.[1] Lignins are divided into two classes, guaiacyl lignins and guaiacyl-syringyl lignins, differing in the substituents of the phenylpolypropanoid skeleton.[1] Guiacyl lignins have a methoxy group in the 3-carbon position whereas syringyl-lignins have a methoxy group in both the 3-carbon and 5-carbon positions.[1]

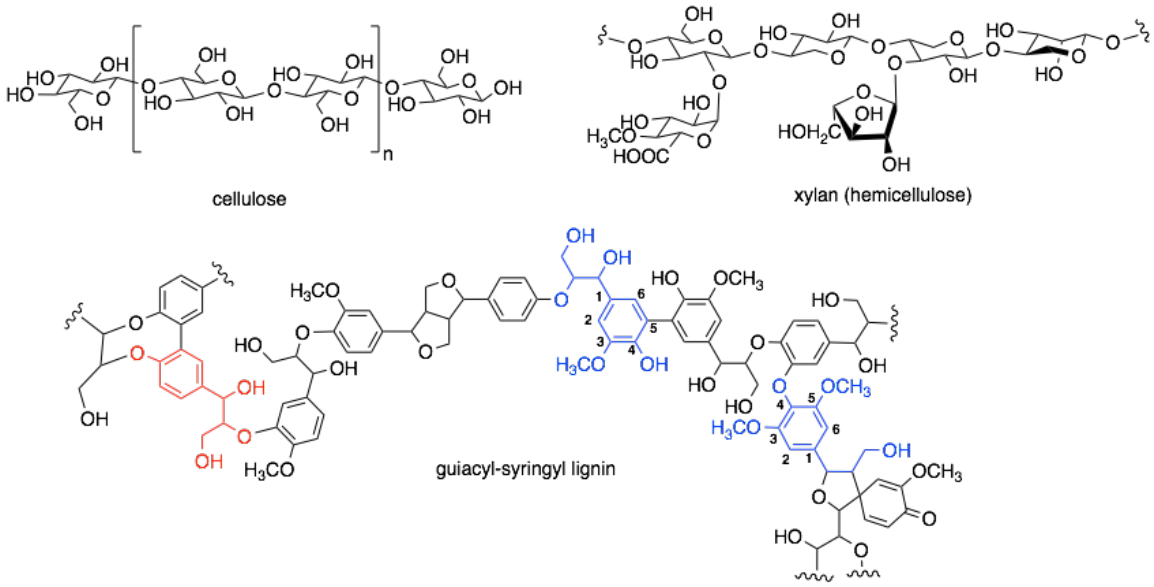

Figure 26. Structures of cellulose, xylan (an example of hemicellulose)**, guiacyl-syringyl lignin** (coumaryl alcohol is depicted in red; coniferyl alcohol which has 3-methoxy group and sinapyl alcohol which has 3- and 5-methoxy groups are drawn in blue)**.**

Lignocellulosic biomass is first fed into the anaerobic digester to produce methane biogas and solid digestate.[2] Anaerobic digestion (AD) has been traditionally used for greenhouse gas reduction, odor control and bioenergy production. AD processing uses acetogenic and acidogenic bacteria of the *Clostridia* species as well as methanogens of the anaerobic *Archaea* species to

convert organic compounds into methane and other products. In this AD metabolic cascade, organic materials are degraded to hydrogen and volatile fatty acids by acidogenic bacteria. The volatile fatty acids are then treated with acetogenic bacteria to give acetate and other volatile fatty acids, which are fed to methanogens to produce methane, liquid effluent and solid digestate. Unlike the conventional AD process which focuses on reduction of solid residues and enhancement of methane production, the current AD process requires the simultaneous generation of methane and homogeneous cellulose- and hemicellulose-rich solid residue. Unpublished results by Liao and colleagues indicate that the quality of the solid residues is significantly improved when considering the following factors: 1. Chemical composition and physical properties such as good hemicellulose-cellulose contents and water impregnated fiber; 2. Hydrolyzibility that comes from mild pretreatment conditions; 3. Fermentation performance.

1.2. Pretreatment and saccharification of solid digestate

Following the anaerobic digestion, the solid digestate needs to be treated and converted into fermentable sugars of sufficient purity to support microbial synthesis of value-added chemicals. Many treatment methods can be used in this stage including biological pretreatment like utilizing fungi;[3] physical pretreatment such as ball milling, microwave and steam expansion;[4–6] physical-chemical pretreatment like dilute acid treatment,[7–10] ethanol-alkaline wet explosion,[11] ammonia fiber expansion,[12] one-step extrusion/NaOH,[13] and aqueous ethanol.[14] For this study, as the AD fiber is alkaline, dilute alkali pretreatment was performed with 2% (w/w) sodium hydroxide in an autoclave for 3 h at a temperature of 130 ^{o}C. [2,15] The resulting slurry was treated with cellulase and xylanase, and shaken at 50 ^{o}C and 150 rpm for 72 h to convert cellulose and xylan into fermentable mono sugars.[2] After enzymatic hydrolysis, the liquid hydrolysate consisting of glucose, xylose, acetate and various non-pristine compounds such as furfural, 5-hydroxymethyl

furfural, coumaric acid and ferulic acid was collected as a supernatant following a centrifugation step (Figure 27).[2]

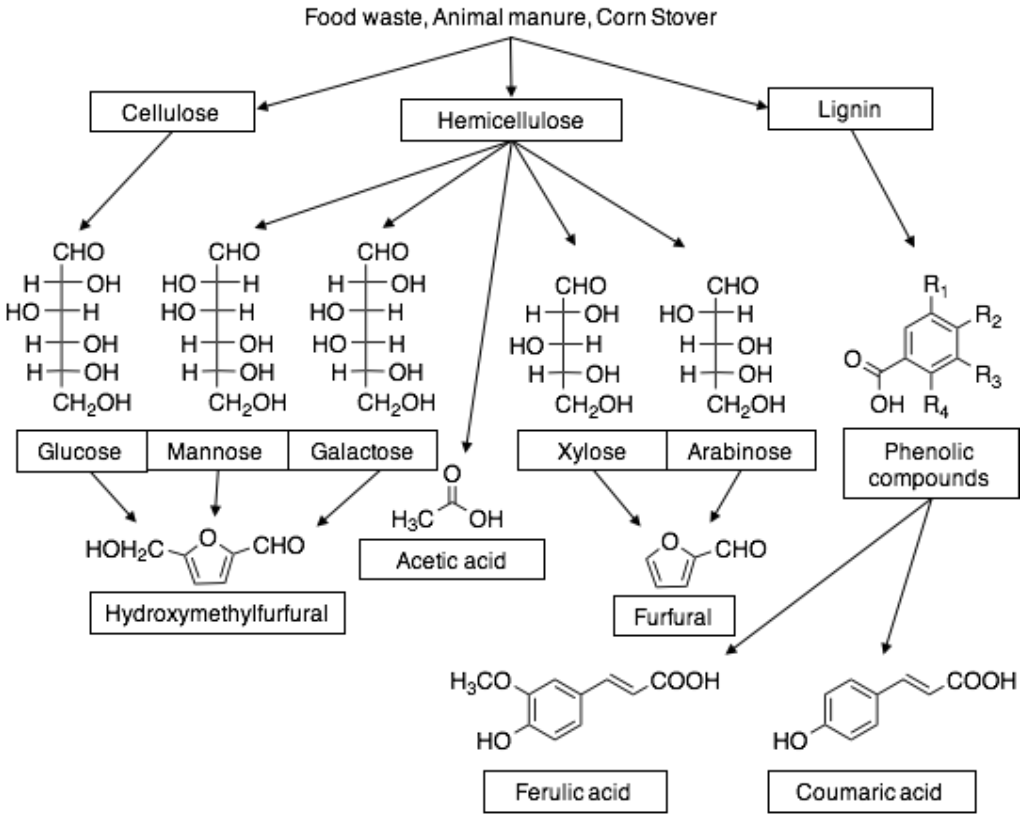

Figure 27. Reactions occurring during hydrolysis of lignocellulosic materials.[21]

1.3. Shikimic acid

Shikimic acid is a biosynthetic metabolite in the aromatic amino acid pathway (Figure 28), possessing a six-membered carbocyclic ring which makes for a valuable chiral synthon currently used as the starting material for the manufacture of anti-influenza drug Tamiflu® (Figure 29).[16]

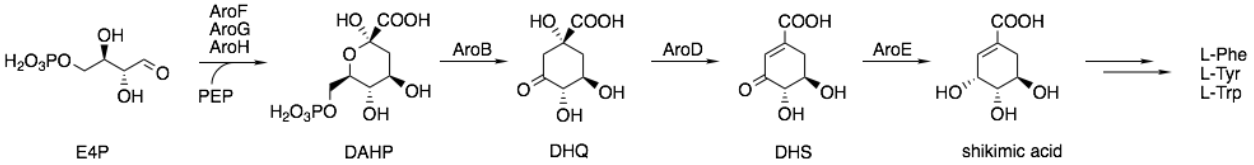

Figure 28. Shikimate pathway. (a) Encoding gene, enzyme: *aroF*, *aroG*, *aroH*, DAHP synthase; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase. (b) Abbreviations: E4P, D-erythrose-4-phosphate; PEP: phosphoenolpyruvate; DAHP, 3-deoxy-D-*arabino*-heptulosonIc acid 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimic acid.

Shikimic acid is commercially available from two sources: 1. The star-shaped pericarp from *Illicium verum*, a bush that grows in limited regions of Vietnam and China;[16] 2. Microbial synthesis from glucose under fed-batch fermenter-controlled conditions using an engineered *E. coli* strain. [17–20]

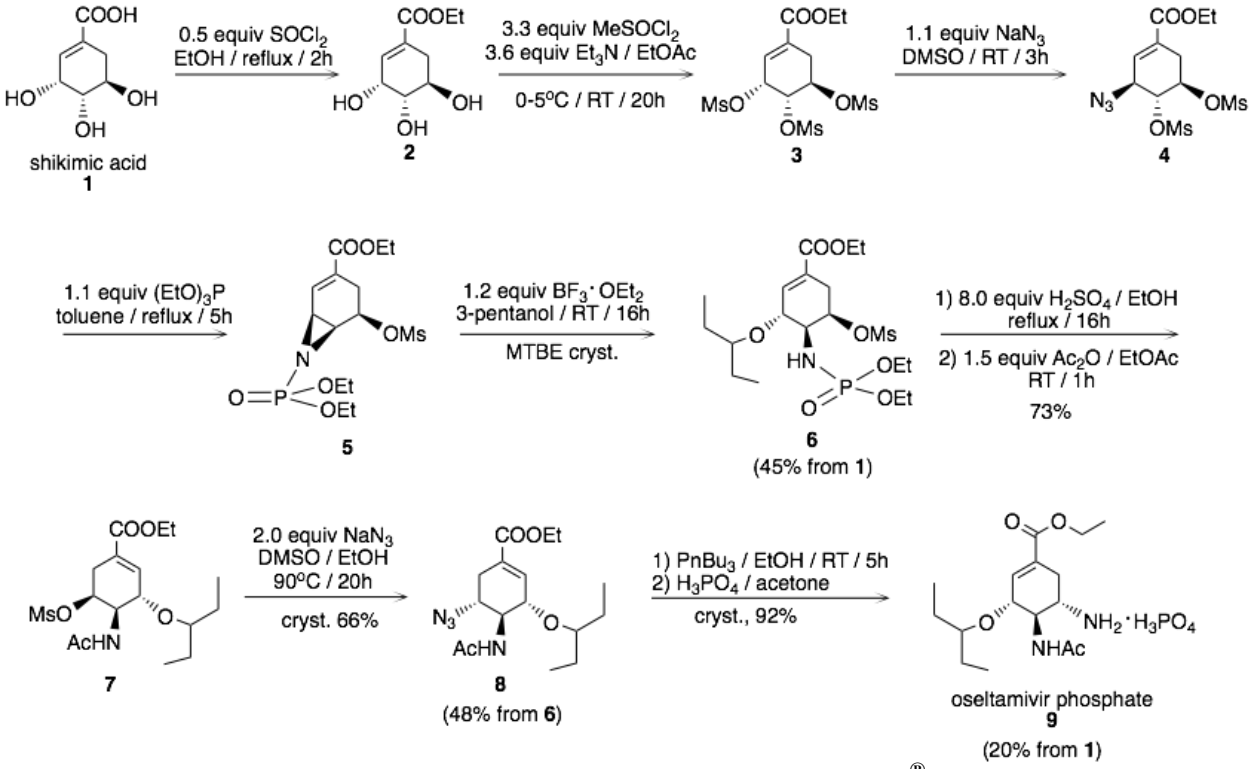

Figure 29. Eight-step Synthesis of Oseltamivir Phosphate (Tamiflu®) from Shikimic acid.

As shikimic acid is present in low concentrations in *Illicium verum* and its production is dependent on seasonal uncertainties, to meet the growing demand for its availability as the synthetic precursor to Tamiflu®, Frost, Draths and co-workers developed a series of biocatalysts that synthesize shikimic acid up to concentrations of 80 g/L in 33% yield from glucose at a 1 L scale.[17–20]

A larger volume of shikimic acid might be used in the future after the publication of the single-step aromatization of shikimic acid into *p*-hydroxybenzoic acid (pHBA) by Frost and collaborators.[27] The largest market for pHBA is a class of specialty polymers called liquid crystal

polymers (LCP), that possess low liquid phase viscosity enabling them to fill intricate molds, and are dimensionally stable once solidified, even at elevated temperatures. Due to these unique combination of properties, LCPs are used to manufacture electronic and automobile parts, flexible circuitry film, high-end barrier film, and high strength fiber. All commercial LCPs contain a minimum of 50% by weight pHBA leading to its market consumption of 9000-11,000 metric tons annually. The other pHBA market is for the manufacture of parabens i.e., antibacterial agents used as preservatives in cosmetics and toiletry products, which necessitates 4500-7000 metric tons of pHBA annually.

Originally developed in 1860, [22,23] Hock-style oxidation of cumene gives the toxic phenol, whose potassium salt is reacted with carbon dioxide at high temperatures and pressures to synthesize the potassium salt of pHBA, which when acidified with sulfuric acid affords pHBA. Over the past 50 years, this process has been optimized and nevertheless, the overall synthesis cannot escape from certain key disadvantages which include the utilization of expensive potassium hydroxide instead of sodium hydroxide[24] and production of stoichiometric amounts of salt as byproduct. The single step aromatization of shikimic acid to produce pHBA could potentially be an environmentally friendly alternative to the current industrial process.

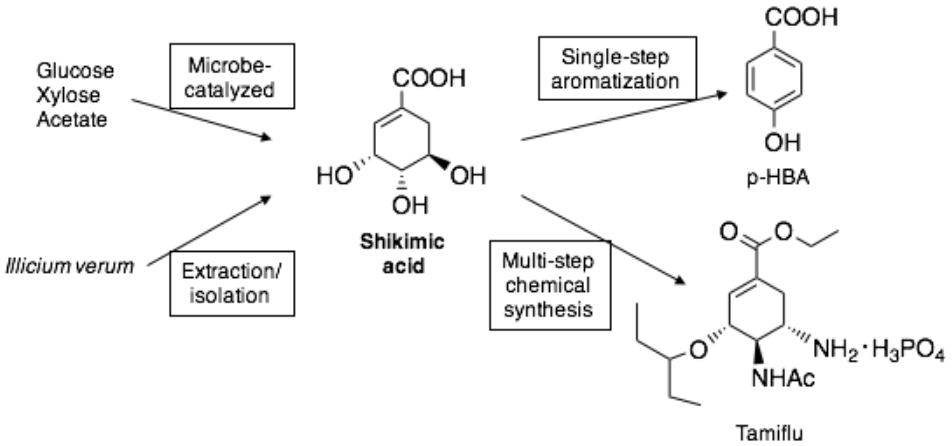

Figure 30. Shikimic acid and its derivatives.

Shikimic acid that is required for the aromatization reaction is produced through the microbial synthesis of *E. coli* SP1.1/pKD15.071 in fed-batch fermentation conditions in a 30 L fermenter using glucose as the carbon source.[27] A titer of 74 g/L shikimic acid is achieved in 60 h, which then undergoes hollow fiber tangential flow filtration to remove biomass and extraneous protein.[27] Cell-free broth is then concentrated four-fold by boiling at atmospheric pressure after which pH is adjusted to 2.5 by adding sulfuric acid.[27] Shikimic acid is extracted into 2.4 volumes of 90% aqueous isopropanol using countercurrent extraction method to afford a 94% recovery.[27] After concentrating the extract to dryness and redissolving in aqueous *n*-butanol, shikimic acid is isolated by evaporative crystallization.[27] Shikimic acid recovery over the extraction and crystallization steps is 65% and provides material in the range of 95-99+% pure, depending on conditions.[27] Chemical aromatization of shikimic acid to pHBA in the presence of the ionic liquid 1-butyl-3-methylimidazolium bromide (Figure 31) yields 76% pHBA along with byproduct *m*-hydroxybenzoic acid (mHBA).[27] Interestingly, it has been found that the yield of the reaction is not dependent on extremely pure shikimic acid samples.[27]

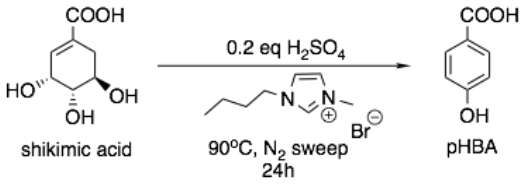

Figure 31. Chemical aromatization of shikimic acid into pHBA. Abbreviations: pHBA, *para* hydroxybenzoic acid.

Thus, the significance of shikimic acid (Figure 30) indicates that its microbial synthesis using the AD hydrolysate produced from lignocellulosic biomass would be an ideal example to demonstrate that with the right technology, non-food-based feedstocks can and should replace food-based feedstocks in value-added chemical production.

2. RESULTS

2.1. Overview

AD hydrolysate, which is acquired from lignocellulosic feedstocks, contains various non-pristine compounds such as furfural, 5-hydroxymethyl furfural, coumaric acid and ferulic acid along with glucose, xylose and acetate. The first step of lignocellulosic feedstock treatment is anaerobic digestion where the food wastes and animal manure are fed to acidogenic and acetogenic bacteria that result in hydrogen and volatile fatty acids (VFAs). In the second step, acetogenic bacteria reduce the volatile fatty acids into acetate and other volatile fatty acids, following which methanogens act on the remaining volatile fatty acids in the third step to yield methane and lignocellulosic solid digestate. As part of the saccharification process, the solid digestate is autoclaved with sodium hydroxide in the chosen chemical pretreatment method due to its alkaline nature, in order to enhance the enzymatic digestibility of the solid residues.[2] If corn stover were used instead of food waste and animal manure, dilute acid pretreatment is employed instead of alkali pretreatment.[2] Use of high temperature under acidic or alkaline conditions in this step leads to formation of various non-pristine compounds, which can be toxic to the microbial catalysts. Moreover, the concentration of sugars in the AD hydrolysate i.e., 4% (w/v) sugar is much lower than the concentration generally employed for both shake flask experiments and fed-batch fermentations i.e., 20% (w/v) and 60% (w/v) sugar solution respectively. New fermentation methodology was developed in this study to overcome this challenge and to demonstrate that the shikimate producer *E. coli* SP1.1/pKD12.138 can indeed grow on the hydrolysate obtained from lignocellulosic biomass despite containing certain inhibitory compounds. An additional challenge to surmount is that AD hydrolysate which consists of a mixture of glucose and xylose, would need

a different fed-batch fermentation strategy modelled after the published microbial synthesis of 3-dehydroshikimic acid synthesized from glucose-xylose mixtures by Li et al.[25]

2.2. Shake flask experiments of the shikimic acid producer *E. coli* SP1.1/pKD12.138

Over the years, Draths, Frost and colleagues have developed multiple *E. coli* strains and plasmids that synthesize shikimic acid,[17–20] and several patents of the same have been issued.[20] Hoffman-LaRoche holds a license to some of these strains as microbial synthesis of shikimic acid plays a large role in the synthesis of Tamiflu, thus satisfying the global demand for the anti-influenza drug.[16] The shikimate pathway is shown in Figure 32. It begins with the transportation of glucose into the microbial cytoplasm through the phosphoenolpyruvate: carbohydrate

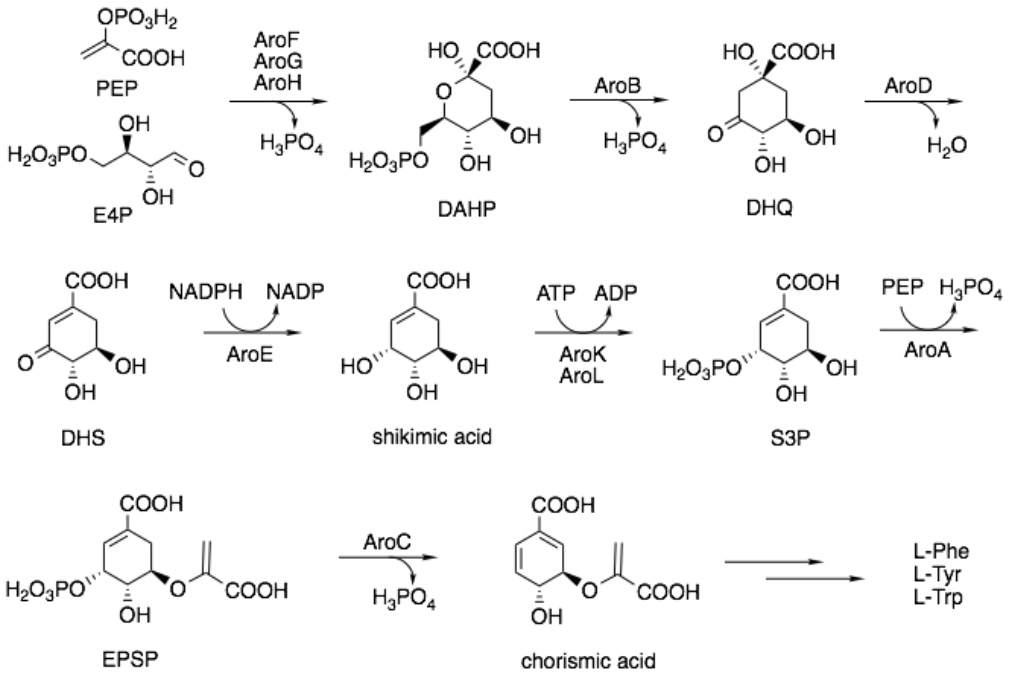

Figure 32. The common pathway of aromatic amino acid and aromatic vitamin biosynthesis. (a) Encoding gene, enzyme: *aroF*, *aroG*, *aroH*, DAHP synthase; *aroB*, DHQ synthase; *aroD*, DHQ dehydratase; *aroE*, shikimate dehydrogenase; *aroK*, *aroL*, shikimate kinase; *aroA*, EPSP synthase; *aroC*, chorismate synthase. (b) Abbreviations: E4P, D-erythrose-4-phosphate; PEP: phosphoenolpyruvate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimic acid; S3P, shikimate-3-phosphate; EPSP, 5-enolpyruvylshikimate 3-phosphate.

phosphotransferase (PTS) system. For each molecule of glucose that is converted into glucose-6-phosphate, one molecule of phosphoenolpyruvate is converted into pyruvic acid. Following that, transketolase plays a significant role in siphoning the D-glucose equivalents from glycolysis into the pentose phosphate pathway with the conversion of D-fructose-6-phosphate into D-erythrose-4-phosphate.[26]

The first step of the common aromatic amino acid biosynthetic pathway is the condensation of D-erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to form 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) by the action of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase. Three isozymes of DAHP synthase - AroF, AroG, and AroH - exist in *E. coli*, each of them feedback inhibited by one of the three aromatic amino acids tyrosine, phenylalanine, and tryptophan respectively. DAHP is then converted to 3-dehydroquinic acid (DHQ) by AroB which is the DHQ synthase. Dehydration of DHQ is the next step in the shikimate pathway where DHQ is converted into 3-dehydroshikimic acid (DHS) by the action of AroD, the DHQ dehydratase. Subsequent reduction of DHS by AroE, the shikimate dehydrogenase affords shikimic acid (SA). Two isozymes of shikimate kinase, AroL and AroK catalyze the ATP aided phosphorylation of SA to generate shikimate-3-phosphate. In subsequent steps, 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, AroA catalyzes the condensation of PEP with shikimate-3-phosphate, after which the final metabolite in the pathway, chorismic acid is formed from the removal of inorganic phosphate from EPSP by chorismate synthase, AroC.

SP1.1/pKD12.138 and SP1.1/pKD15.071 are the two finely-tuned, significant shikimic acid producing *E. coli* strains that are developed by the Draths and Frost research group to achieve high titer values of the product in fed-batch fermentations (Figure 33). The genomic portion, SP1.1 was constructed from RB791 by the disruption of *serA* by the insertion of *aroB* into its locus, and

successive P1 phage-mediated transductions of *aroL478*::Tn*10* and *aroK17*::Cm^R into the *aroL* and *aroK* loci.[17] These mutations in the genome will enable: 1. The accumulation of shikimic acid due to the absence of the *aroL*- and *aroK*- encoded isozymes for shikimate kinase; 2. The increase in the catalytic activity of DHQ synthase, AroB owing to the second copy of the gene; 3. The disruption of *serA*-encoded phosphoglycerate dehydrogenase, an important enzyme in the L-serine biosynthesis ensuring that the *serA*-encoding plasmid is retained by the cell.[17] The 9.9-kb plasmid pKD12.138 is a pSU18-derived plasmid that encodes feedback-insensitive DAHP synthase *aroF*^{FBR}*,* shikimate dehydrogenase *aroE,* transketolase *tktA,* plasmid selection marker *serA*, and antibiotic resistance marker Ap^R.[18] The 11.9-kb plasmid pKD15.071 was assembled by replacing 1.0-kb β-*lac* gene that encodes for antibiotic resistance with a plasmid-localized copy of phosphoenolpyruvate synthase *ppsA* in pKD12.138.[19] The intention was to increase shikimic acid concentrations by increasing regeneration of PEP from pyruvic acid and ATP, thus enhancing the availability of PEP for the first step in the shikimate pathway.[19]

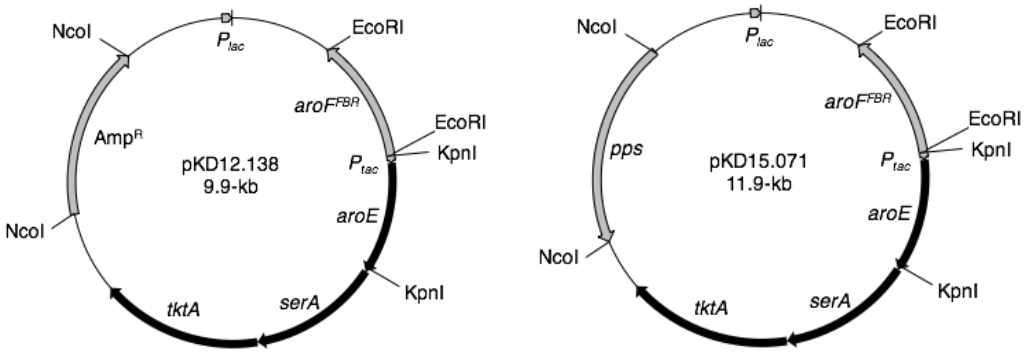

Figure 33. Plasmid maps of pKD12.138 and pKD15.071.

In this study, preliminary experiments on the shikimate producers SP1.1/pKD12.138 and SP1.1/pKD15.071 were performed in shake flasks. These experiments helped in evaluation of cell growth and shikimic acid production when the AD hydrolysate containing a mixture of glucose, xylose and acetate is used as the sole carbon source. For comparison, samples were also run using

comparable concentrations of pure glucose and xylose as the carbon source. AD hydrolysate contains approximately 25 g/L glucose and 15 g/L xylose concentrations which equals 4% (w/v) sugar concentration. In regular shake-flask experiments, 20% (w/v) glucose solution is used along with M9 culture medium, which consists of Na_2HPO_4, KH_2PO_4, NH_4Cl and NaCl salts supplemented with glucose and aromatic vitamins, termed as M9/glu/aros. In these experiments, the final sugar concentration in the shake flask will be 1% (w/v). To achieve a 1% (w/v) sugar culture media using a dilute 4% (w/v) sugar stock solution, the necessary concentration of the M9 stock solution was calculated to be twice the normal composition i.e., 2xM9. For instance, in the 20 mL shake-flask experiments containing 1% sugar concentration, each reaction included 5 mL 4% (w/v) sugar solution, 10 mL 2xM9 salts and 5 mL dd water. To prepare the above reaction mixture, 2xM9 salt solution and 4% (w/v) glucose, xylose and AD hydrolysate solutions were first autoclaved. Then, all the separate solutions were mixed together. Similarly, 3% (w/v) shake-flask experiments were also assembled by replacing 2xM9 medium with 4xM9 medium and in this case, each reaction contained 15 mL 4% (w/v) sugar solution and 5 mL 4xM9 salts.

In the first step of the shake-flask experiment, 5 mL M9/glu/aro cultures were incubated for 15 h at 37 ^{o}C and 250 rpm. 10% of this overnight inoculum was pelleted out, washed with M9 salts and resuspended in 1 mL M9 medium, which was then added to the 20 mL M9/carbohydrate culture media in 125 mL baffled flasks, and incubated at 37 ^{o}C and 250 rpm for 48 h. Cell growth was periodically evaluated by measuring OD_{600} and shikimic acid was quantified in cell-free broth by ^{1}H-NMR. The concentrations of shikimic acid formed from both the shikimate-producers SP1.1/pKD12.138 and SP1.1/pKD15.071 were comparable. Additionally, pKD15.071 does not contain antibiotic resistance that was needed for resuspension shake-flask experiments discussed

later in this chapter. For both reasons, pKD12.138 was chosen as the sole shikimate-producing microbial catalyst for further experiments.

Table 2. OD_{600} values and shikimic acid concentrations in SP1.1/pKD12.138 shake-flask experiments at 24h and 48h

Entry	Carbon source	OD_{600} 24 h	OD_{600} 48 h	[SA][a] 24 h	[SA][a] 48 h
1	1% glucose (56 mM)	3.93	4.55	1.8 mM	unchanged
2	1% xylose (66 mM)	3.08	3.32	2.5 mM	unchanged
3	1% AD hydrolysate	5.53	5.41	3.6 mM	unchanged
4	3% glucose (166 mM)	4.05	4.57	3.7 mM	unchanged
5	3% xylose (200 mM)	2.9	3.99	4.4 mM	unchanged
6	3% AD hydrolysate	2.94	3.00	3.7 mM	SA can't be seen

[a]Abbreviations: SA, shikimic acid; [b] concentrations are calculated using the SA ^{1}H-NMR resonance peak at δ 4.40, t, 1 H by comparing with the integral corresponding to 1mM TSP (δ = 0.00 ppm)

From the results in Table 2, there is no change in the shikimic acid production at 1% or 3% sugar concentrations. Moreover, shikimic acid production was complete at 24 h, and this could be since both microbial cell growth and metabolite production were dependent entirely on the sole carbon source employed in that experiment. One way to improve the shikimic acid titer values was to grow the cells in a rich or minimal medium through their logarithmic phase, transfer the cell pellet to an M9/carbohydrate culture medium during their stationary phase at which stage the metabolite production takes place.

In the case of resuspension experiments involving rich medium (Table 3), 5 mL LB/Ap seed cultures were inoculated with freshly transformed SP1.1/pKD12.138 and shaken for 10 h at 37 ^oC and 250 rpm. 2 mL of this inoculum was then transferred to 50 mL LB/Ap cultures in 250 mL baffled flasks for 5h at 37 ^oC and 250 rpm. The cells were centrifuged and washed with M9

salts before being re-suspended in 5 mL M9 salts and added to 50 mL M9/carbohydrate cultures in 500 mL baffled flasks to be shaken for 56 h.

Table 3. Final OD_{600} values and shikimic acid concentrations in SP1.1/pKD12.138 rich to M9/carbohydrate resuspension shake-flask experiments

Entry	Carbon source	Final OD_{600}	Final [SA][b]
1	1% glucose (56 mM)	4.45	8.5 mM
2	1% xylose (66 mM)	2.26	9 mM
3	1% AD hydrolysate	8.18	8.4 mM

[a]Abbreviations: SA, shikimic acid; [b] concentrations are calculated using the SA ^{1}H-NMR resonance peak at δ 4.40, t, 1 H by comparing with the integral corresponding to 1 mM TSP (δ = 0.00 ppm)

For resuspension experiments involving minimal medium only (Table 4), 5 mL M9/glu/aro seed cultures were inoculated with freshly transformed SP1.1/pKD12.138 and shaken for 16 h at 37 ^oC and 250 rpm. 2 mL of this inoculum was then transferred to 50 mL M9/glu/aro cultures in 250 mL baffled flasks for 14 h at 37 ^oC and 250 rpm. The cells were centrifuged and washed with M9 salts before being re-suspended in 5 mL M9 salts and added to 50 mL M9/carbohydrate cultures in 500 mL baffled flasks to be shaken for 51 h.

Table 4. Final OD_{600} values and shikimic acid concentrations in SP1.1/pKD12.138 minimal to M9/carbohydrate resuspension shake-flask experiments

Entry	Carbon source	Final OD_{600}	Final [SA][b]
1	1% glucose (56 mM)	2.18	8.0 mM
2	1% xylose (66 mM)	2.95	9.7 mM
3	1% AD hydrolysate	1.92	1.3 mM

[a]Abbreviations: SA, shikimic acid; [b] concentrations are calculated using the SA ^{1}H-NMR resonance peak at δ 4.40, t, 1 H by comparing with the integral corresponding to 1 mM TSP (δ = 0.00 ppm)

Based on the results in Table 3, it is notable that SP1.1/pKD12.138 grew to higher cell densities on AD hydrolysate than on glucose or xylose in the rich to M9/carbohydrate resuspension

experiments; nevertheless, all three reactions produced an almost equal amount of shikimic acid i.e., approximately 8 mM (entries 1, 2, and 3, Table 3). This result demonstrates that AD hydrolysate derived from lignocellulosic feedstock can indeed replace food-based feedstocks in the production of value-added chemicals. On the other hand, the minimal resuspension experiments in the case of synthetically derived glucose and xylose (entries 1 and 2, Table 4) behaved similarly to the their rich to M9/carbohydrate resuspension experiment counterparts (entries 1 and 2, Table 3). Even though rich resuspension experiments were successful in providing proof of concept for the hypothesis of this study, shake-flask experiments are not ideal for large scale value-added chemical production due to limited oxygen availability, lower cell densities and limited carbon source availability.

Since regular fed-batch fermentation conditions would require 60% (w/v) sugar solution as substrate feed, some reaction engineering must be performed to overcome the challenge of utilizing 4% (w/v) sugar concentrations in the AD hydrolysate. The fact that wild-type *E. coli* is unable to simultaneously utilize both glucose and xylose present in the AD hydrolysate poses an added challenge in developing new fed-batch fermentation conditions.

2.3. Microbial synthesis of shikimic acid utilizing a model AD hydrolysate system

E. coli does not simultaneously utilize glucose and xylose due to a phenomenon called carbon catabolite repression (CCR).[32] Transportation of glucose via the phosphoenolpyruvate phosphotransferase (PTS) system is connected to the concentration levels of unphosphorylated glucose transporter EII^{Glc} in the microbial cytoplasm.[32] Glucose is converted into glucose-6-phosphate by the desphosphorylation of EII^{Glc} and when glucose levels are abundant, the unphosphorylated EII^{Glc} directly inhibits the levels of cyclic adenosine monophosphate (cAMP), thus blocking the complex formation between cAMP and the catabolite activator protein.[32] As a

result, expression of enzymes that catabolize other sugars is repressed.[32] Once all the glucose is consumed, *E. coli* begins to catabolize the second preferred carbon source.[32] This phenomenon leads to diauxic growth where rapid growth by glucose consumption is followed by a lag phase when observable cell growth stops until the catabolic genes for the second carbon source are activated.[32] Therefore, the goal is to study fed-batch fermentations growing on a 4% substrate feed of a 5:3 molar ratio of glucose/xylose mixture that mimics the AD hydrolysate without inhibitors and to fine-tune the fermentation conditions.[32]

Fermentations were performed under both sugar-minimal and sugar-rich conditions. Variables in the fed-batch fermenter runs – temperature (33 ^{o}C), pH (7.0), Dissolved oxygen (D.O.) levels (10% saturation) and 5:3 molar ratio glucose/xylose feed (4 % w/v) - were maintained by PID control loops. Eight 5 mL cultures of M9 salts supplemented with glucose and aromatic vitamins (M9/glu/aros) were grown for 15 h overnight at 37 ^{o}C with agitation at 250-300 rpm until they were turbid. 80% of four of these cultures were transferred into fresh 100 mL M9/glu/aros cultures, which were grown at 37 ^{o}C with agitation until the OD_{600} reached 1.0-1.5 i.e., the exponential phase of the cells. One of the cultures, picked at random, was added to the fermenter vessel. Because the sugar feed was so dilute, initial culture volume was reduced to 600 mL from 1 L in the 2 L B. Braun BIOSTAT® B-DCU fermenter vessel. The standard *E. coli* fermentation culture medium at 1 L scale contained K_2HPO_4 (7.5 g), ammonium iron (III) citrate (0.3 g), anhydrous citric acid monohydrate (1.98 g), and concentrated H_2SO_4 (1.2 mL). Concentrated NH_4OH (4.1 mL) was added to adjust pH of the medium to 7.0. However, due to the changes in initial volume, the new 600 mL scale culture medium contained K_2HPO_4 (4.5 g), ammonium iron (III) citrate (0.18 g), anhydrous citric acid monohydrate (1.15 g), and concentrated H_2SO_4 (0.72 mL). Concentrated NH_4OH (2.46 mL) was added to adjust pH of the medium to 7.0.

Initial glucose/xylose mixture of 10.8 g was added with appropriately scaled supplements (see the experimental section of this chapter). The D.O. level was maintained at 10% in three stages. In the first stage, the airflow was kept constant at 0.06 L/min and the impeller speed increased from 50 rpm to 750 rpm. During the second stage, once the impeller speed reached 750 rpm, airflow was increased from 0.06 L/min to 1.0 L/min. Around 12 h, all the glucose was consumed which was marked by a sharp dip in the impeller speed and airflow. Xylose consumption began at that time, and maximum impeller speed and airflow were regained in the next 2 h. These two stages usually occur during the exponential phase of the microbial growth. At stage three, the impeller speed and airflow reached their set maxima, 750 rpm and 1.0 L/min at which point the D.O. level dropped to zero. Oxygen sensor control of the 4% (w/v) substrate feed was started. Because of the dilute nature of the sugar solution, effective oxygen sensor control was not observed and D.O levels were not maintained at 10% saturation as intended. Over 48 h, all 900 mL of 4% (w/v) substrate feed was added to the fermenter vessel and the fermentation was stopped. The results for this fermentation can be seen in Figure 34. Apart from the model AD hydrolysate fermentation, 4% (w/v) substrate feed glucose- and xylose-minimal fermentation conditions were done as the controls (Figures 35 and 36).

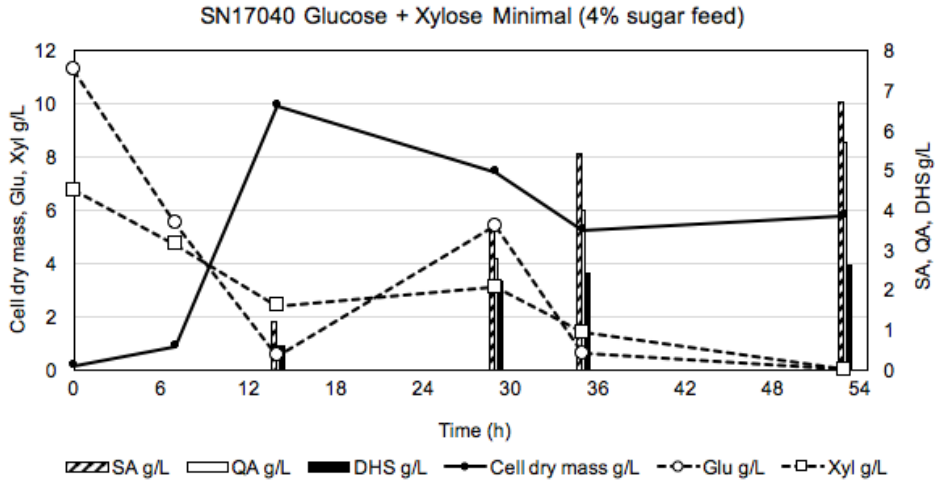

Figure 34. Model AD hydrolysate 5:3 glucose/xylose minimal fermentation of *E. coli* SP1.1/pKD12.138.

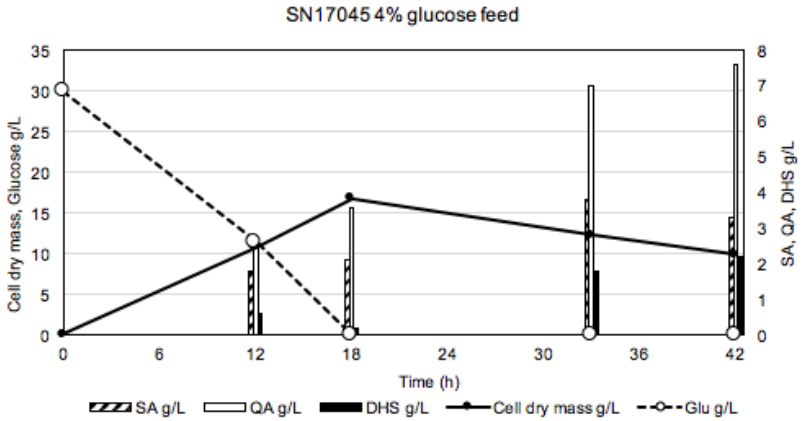

Figure 35. Glucose minimal fermentation of *E. coli* SP1.1/pKD12.138

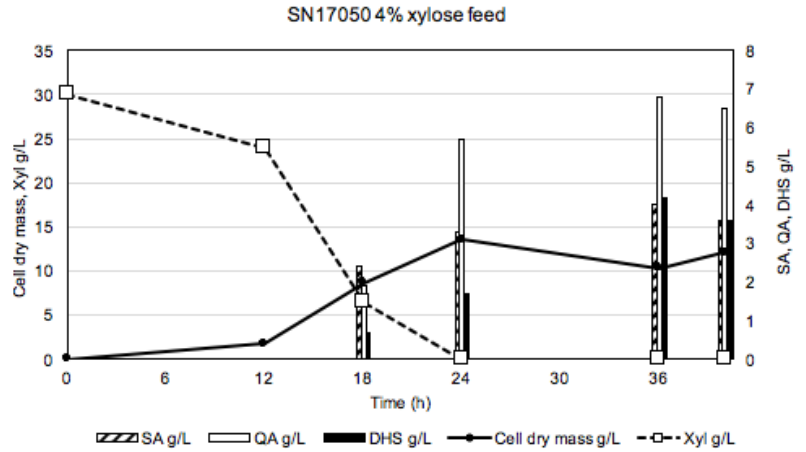

Figure 36. Xylose minimal fermentation of *E. coli* SP1.1/pKD12.138.

Table 5. Final concentrations of SA, QA and DHS in sugar minimal fed-batch fermentations.

Entry	Substrate 4% (w/v)	Cell dry mass g/L	SA[a] g/L	QA[a] g/L	DHS[a] g/L
1	Glucose/xylose	5.81	6.7	5.7	2.6
2	Glucose	9.9	3.3	7.6	2.2
3	Xylose	12.1	3.6	6.5	3.6

[a]Abbreviations: SA, shikimic acid, QA, quinic acid, DHS, 3-dehydroshikimic acid; [b] concentrations are calculated using the ^{1}H-NMR resonance peaks for SA at δ 4.40, t, 1 H, for QA at δ 4.13-4.17, q, 1 H, and for DHS at δ 2.6-2.68, ddd, 1 H or δ 3.03-3.08, dd, 1 H by comparing with the integral corresponding to 10 mM TSP (δ = 0.00 ppm)

It is evident from the entries 1, 2 and 3 in Table 5 that quinic acid (QA) and 3-dehydroshikimic acid (DHS) are major byproducts in all these fermentations, which was expected according to Knop et al.[18] Shikimic acid and quinic acid were hypothesized to be in equilibrium through two possible mechanisms: 1. DHQ might be reduced to QA by *aroE*-encoded shikimate

dehydrogenase before it could be converted to DHS by *aroD*-encoded DHQ dehydratase; 2. The final product SA present in the culture supernatant might be transported back into the cell, reversing the direction of product formation wherein SA would be converted to DHS by *aroE*-encoded shikimate dehydrogenase, which subsequently would be converted to DHQ by *aroD*-encoded DHQ dehydratase, finally to QA by *aroE*-encoded shikimate dehydrogenase.[18]

One method that has been used in the past to prevent the equilibrium between SA and QA production was to run the fermentation in glucose-rich conditions.[18] However, this method is not feasible due to the dilute sugar concentration and the mixture of glucose and xylose in the AD hydrolysate. Therefore, new fermenter conditions where glucose concentrations were maintained between 2 to 8 g/L were attempted. The first two stages of oxygen sensor control were the same as for sugar-minimal conditions described previously in this chapter. In the first stage, the airflow was kept constant at 0.06 L/min and the impeller speed increased from 50 rpm to 750 rpm. During the second stage, once the impeller speed reached 750 rpm, airflow was increased from 0.06 L/min to 1.0 L/min. These two stages usually occur during the exponential phase of the microbial growth. At stage three, the impeller speed and airflow reached their set maxima, 750 rpm and 1.0 L/min at which point the D.O. level dropped to zero. Unlike the sugar-minimal fermenter conditions, oxygen sensor control was not connected to the substrate feed. D.O. levels were maintained at 10% by a steady addition of 4% (w/v) glucose feed over the course of six hours between 12 h to 18 h on the fermentation timeline. Fermentation was stopped 3 h after all the glucose has been added to the vessel.

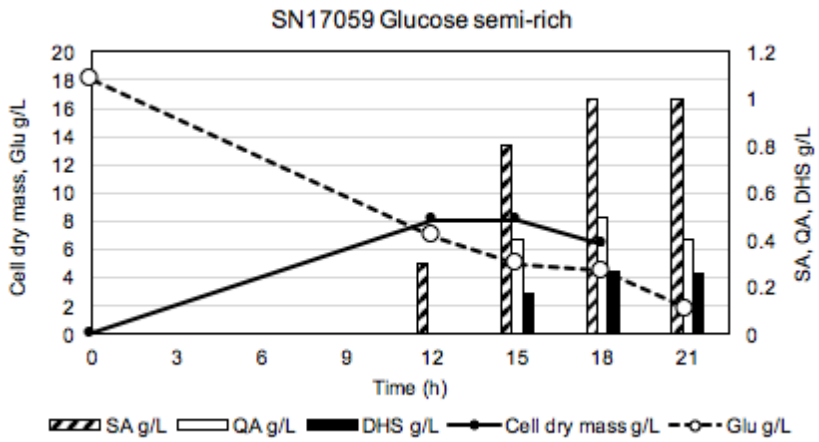

Figure 37. Fermentation of *E. coli* SP1.1/pKD12.138 in which glucose concentrations were maintained at 2-8 g/L

As seen in the Figure 37, this strategy was successful in reducing the production of quinic acid relative to shikimic acid, but the overall yield was affected. Titer values achieved for SA, QA and DHS were 1 g/L, 0.4 g/L and 0.26 g/L respectively. Further exploration in this avenue involving glucose/xylose mixtures is required and other strategies such as metabolic engineering of SP1.1 to inactivate catabolic repression of xylose or upregulation of xylose operon may be pursued. Ultimately, the goal is to use the AD hydrolysate directly in fed-batch fermentations to see its effect on shikimic acid production.

3. DISCUSSION

In 2015, bio-based chemicals constituted about 12% of the total market share in global chemical sales, and this number is projected to increase to 22% by 2025. Most of these industrial fermentation processes that produce bio-based chemicals utilize starch- and sugar-based feedstocks, which lead to the food versus fuel dilemma. On the other hand, 534 billion dry tons of lignocellulosic biomass is produced annually in the United States. Therefore, food-based feedstocks must be replaced by non-food-based feedstocks. Lack of study and research into new technologies to enable efficient biomass handling for second-generation biobased chemical production limits the massive potential held by lignocellulosic biomass. Dr. Wei Liao's research

group at Michigan State University aims to close this gap by developing environmentally benign integrated systems to process lignocellulosic biomass obtained from municipal and agricultural residues for value-added chemical production. Food waste and animal manure are first anaerobically digested by the action of acidogenic and acetogenic bacteria, and methanogens to produce lignocellulosic biomass, which is then pretreated by alkali at high temperatures before being subjected to enzymatic hydrolysis to yield a mixture of glucose, xylose, acetate and various non-pristine compounds. The technology is still in its nascent stages and Dr. Liao's research group is currently working on modifying the AD process such that both the production of solid residues and methane are enhanced. Currently, it takes approximately three weeks to obtain 600-900 mL of AD hydrolysate which was utilized in this study. Hence, there are two challenges associated with this study: 1. limited sugar solution to utilize for value-added chemical production through microbial synthesis; 2. dilute concentrations of sugar i.e., 4% w/v in the AD hydrolysate (Figure 43 shows ^{1}H-NMR). The goal of this project is to provide proof-of-principle that the sugars generated from lignocellulosic biomass, despite containing non-pristine compounds, could indeed be consumed by *E. coli* biocatalysts to synthesize shikimic acid. The shikimate producer SP1.1/pKD12.138 was selected to perform shake flask experiments with three sole carbon sources - AD hydrolysate, pure glucose and pure xylose (Figures 41 and 42 show ^{1}H-NMRs). Shikimic acid was successfully produced to the concentration of 3.6 mM in both 1% and 3% AD hydrolysate conditions (entries 1 and 4, Table 2), thus demonstrating that *E. coli* microbial catalysts can grow on AD hydrolysate as its sole carbon source. However, the disadvantages of shake flask experiments include minimal amounts of carbon source, restricted supply of oxygen to facilitate cell growth and the inability to maintain the optimum pH levels. On the other hand, a limited quantity of AD hydrolysate and insufficient testing of the non-pristine compounds inherent to it

would not allow for a fed-batch fermentation. Consequently, two types of resuspension shake flask experiments were conducted with the shikimate producer SP1.1/pKD12.128. The first kind was rich medium to M9/carbohydrate medium resuspension experiment in which cells were initially grown through their exponential phase in rich medium, and were then transferred into M9/carbohydrate medium in their stationary phase. The second kind was the transfer of fully grown cells from minimal medium to M9/carbohydrate medium. The rich medium resuspension experiments yielded a favorable increase in the shikimic acid titer values (Table 3) compared to regular shake-flask experiments (Table 2). The shikimic acid titer values were approximately 8 mM i.e., 1 g/L no matter which carbon source was used carbon sources, either AD hydrolysate or synthetic glucose or xylose (entries 1, 2 and 3, Table 3). This positive result can be further augmented by fed-batch fermentation conditions but owing to quantitative restrictions for the AD hydrolysate, it was substituted by an approximated glucose/xylose sugar solution to develop new fermentation methodology. In these minimal fed-batch fermentations (Table 5), the final shikimate concentrations calculated by ^{1}H-NMR in 5:3 glucose/xylose, glucose and xylose mixtures were 6.7, 3.3 and 3.6 g/L respectively. It is also interesting to note that quinic acid was a significant byproduct whose final concentrations in the above three fermentation conditions were 5.7, 7.6 and 6.5 g/L (Table 5). In both pure glucose and pure xylose fermentations, the amount of quinic acid formed was almost two times the amount of shikimic acid formed. This result can be explained by the fact that QA and SA are in equilibrium when glucose is not abundant.[18] A proven method to prevent the accumulation of QA was to have a glucose abundant environment,[18] which was applied to the current methodology involving dilute sugar solutions. In these new fermentation conditions, glucose concentration in the fermentation vessel was maintained above 2 g/L always (Figure 37). This strategy certainly circumnavigated the problem of QA accumulation but due to the shortening

of the fermentation run time, the overall yield was affected as the concentration of shikimic acid dropped to 1 g/L. Since glucose/xylose mixtures have given higher titer values of SA than QA in the 4% w/v minimal fermentation conditions, it would be interesting to study the results of semi-rich fermentation in comparison to the above glucose semi-rich fermentations. Apart from reaction engineering strategies that were employed in this chapter, other essential ideas to exploit would be the upregulation of the xylose operon to double the consumption of xylose and deactivation of catabolite repression genes in *E. coli* SP1.1 so that xylose could be simultaneously utilized along with glucose.

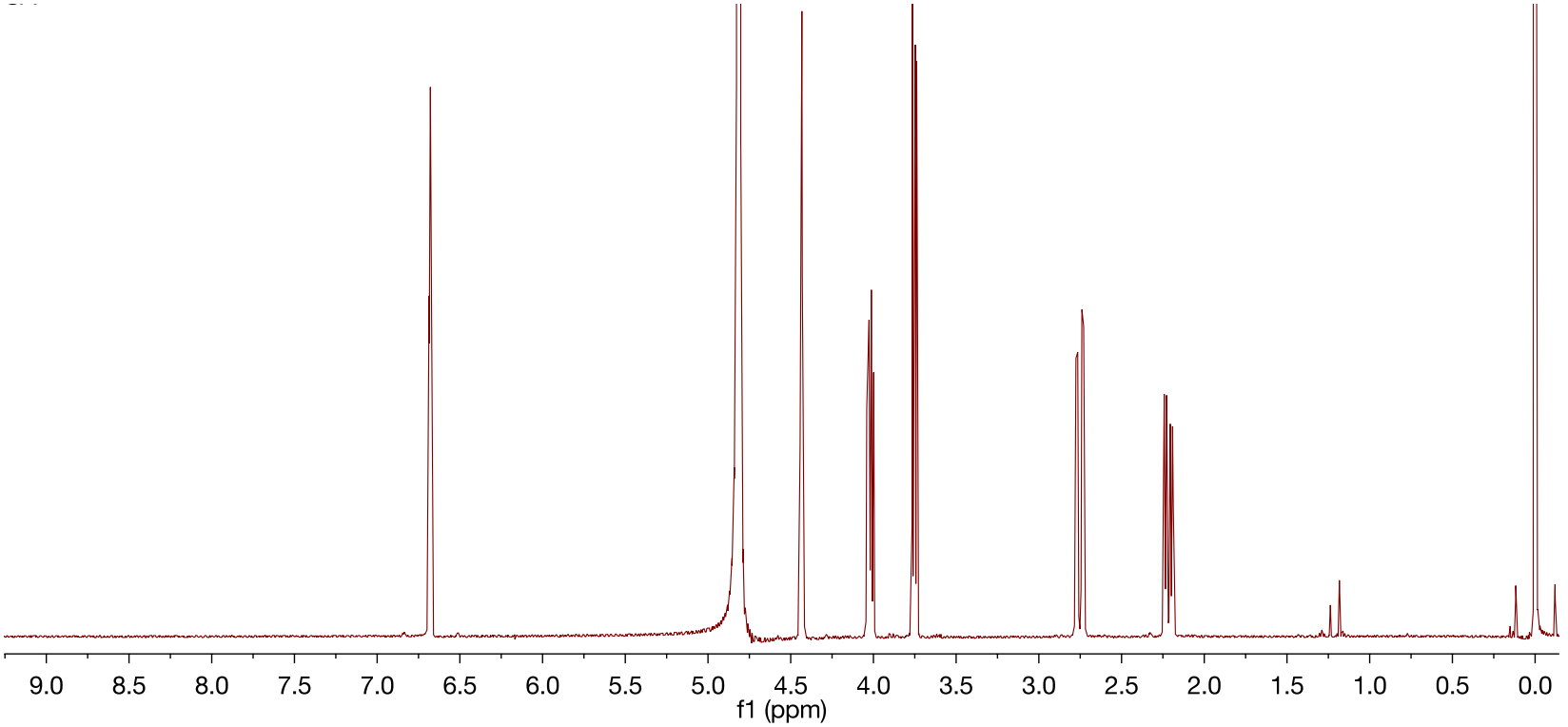

Figure 38. ^{1}H-NMR of Shikimic acid.

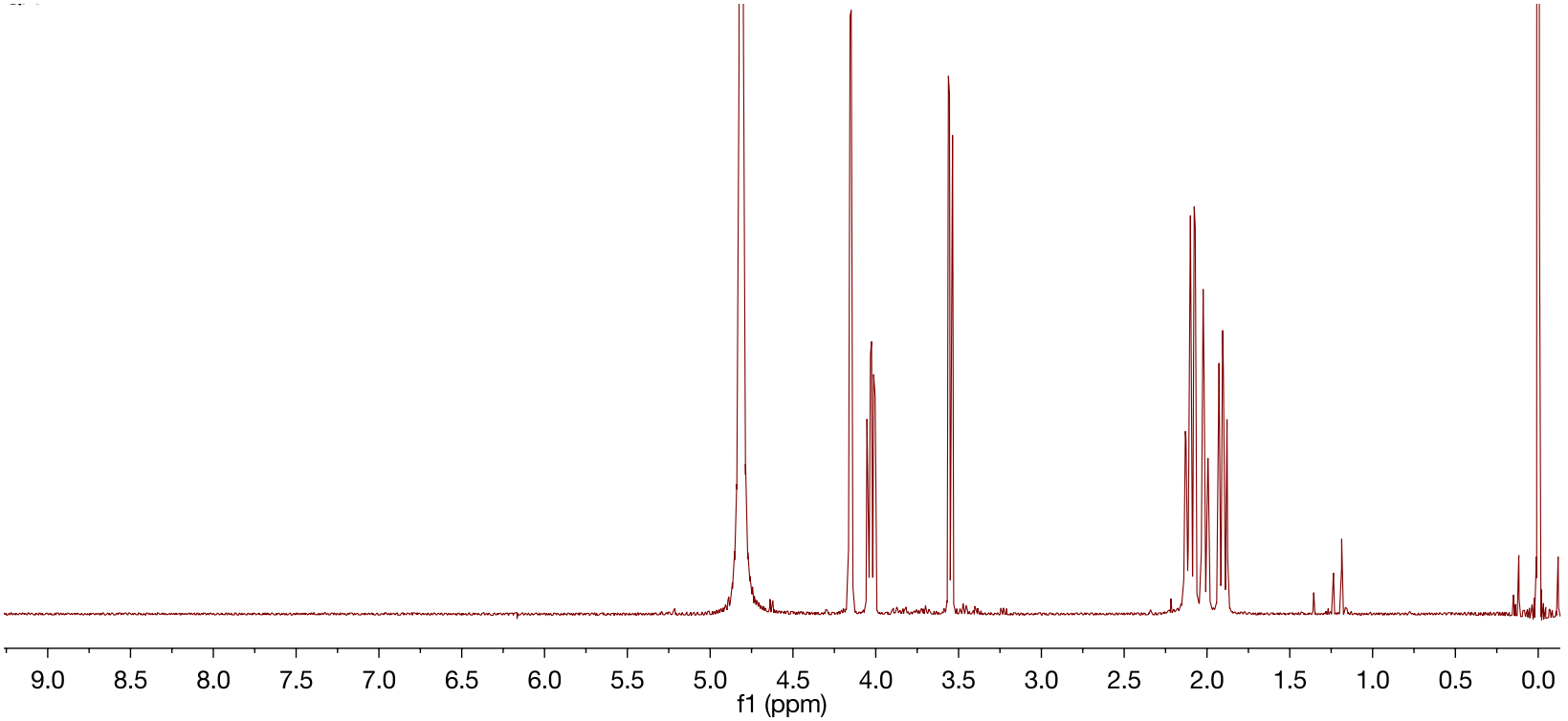

Figure 39. ^{1}H-NMR of Quinic acid.

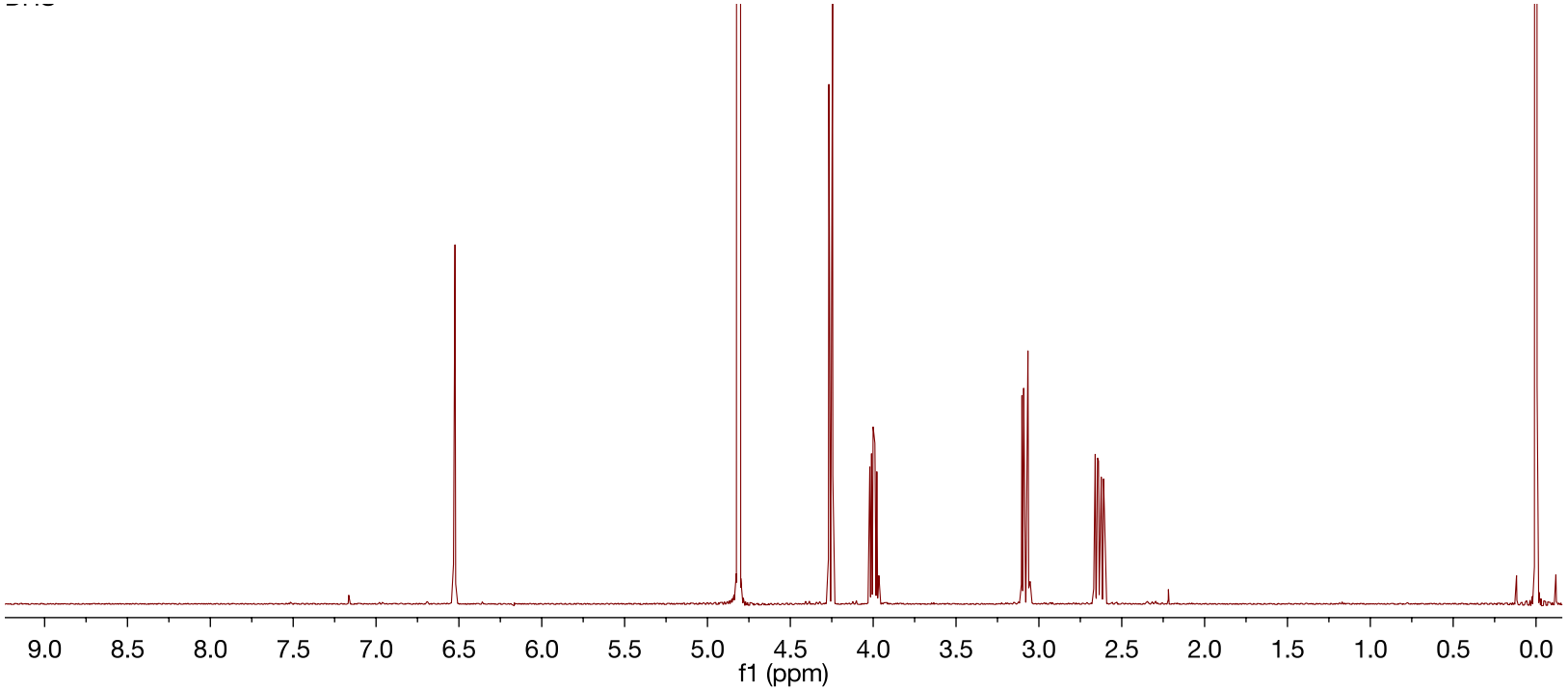

Figure 40. ^{1}H-NMR of 3-dehydroshikimic acid.

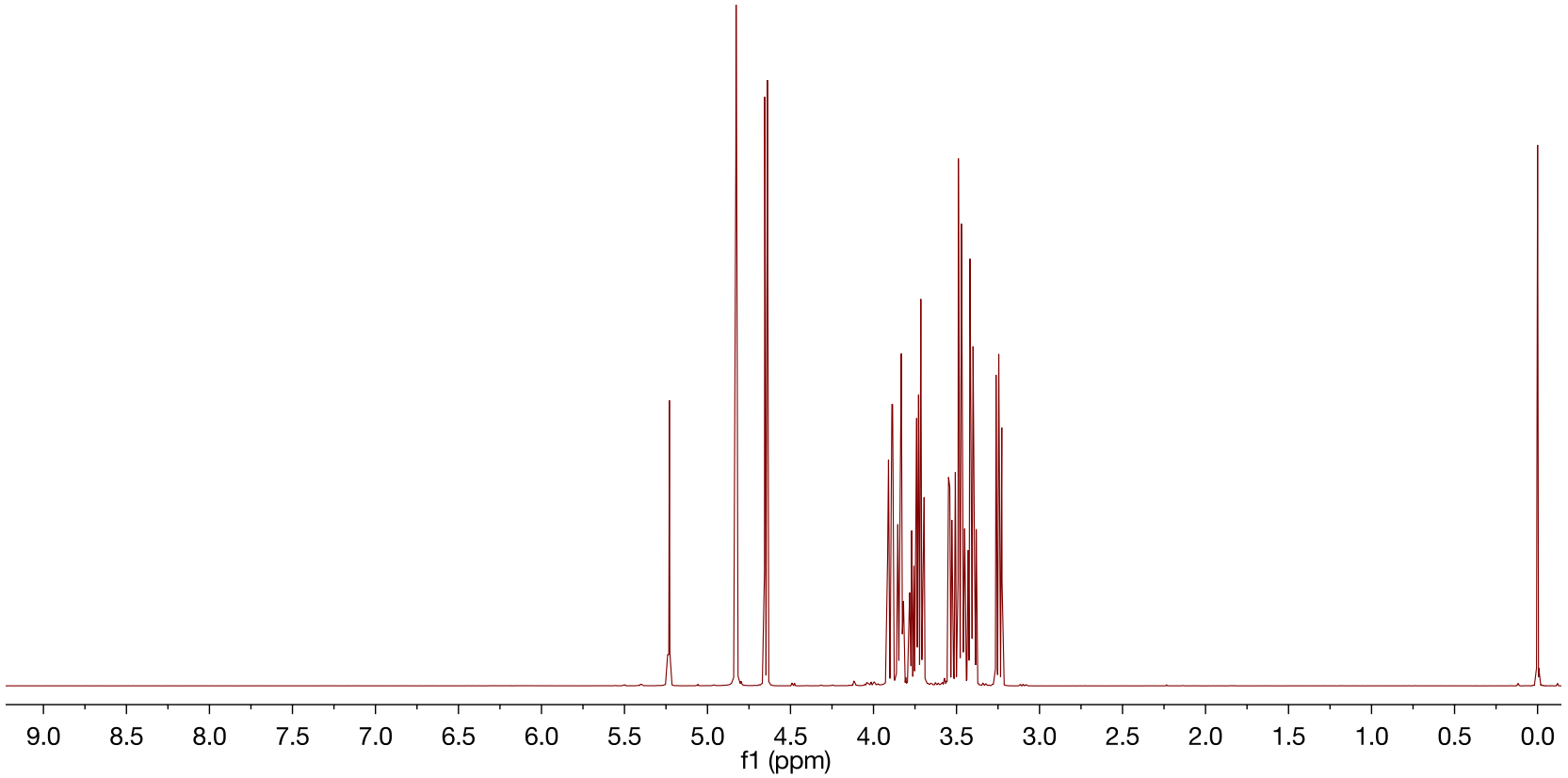

Figure 41. ^{1}H-NMR of glucose.

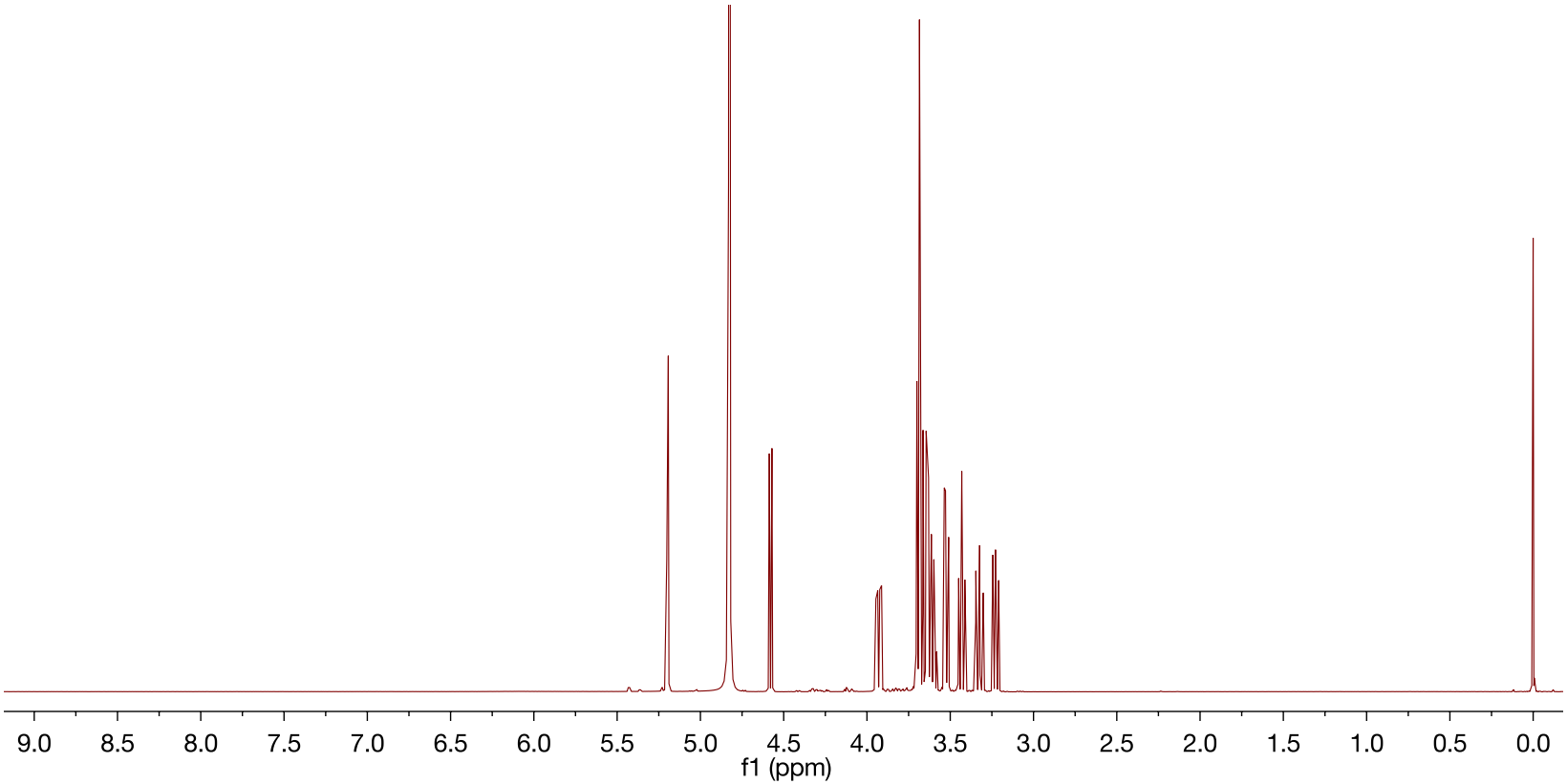

Figure 42. ^{1}H-NMR of xylose.

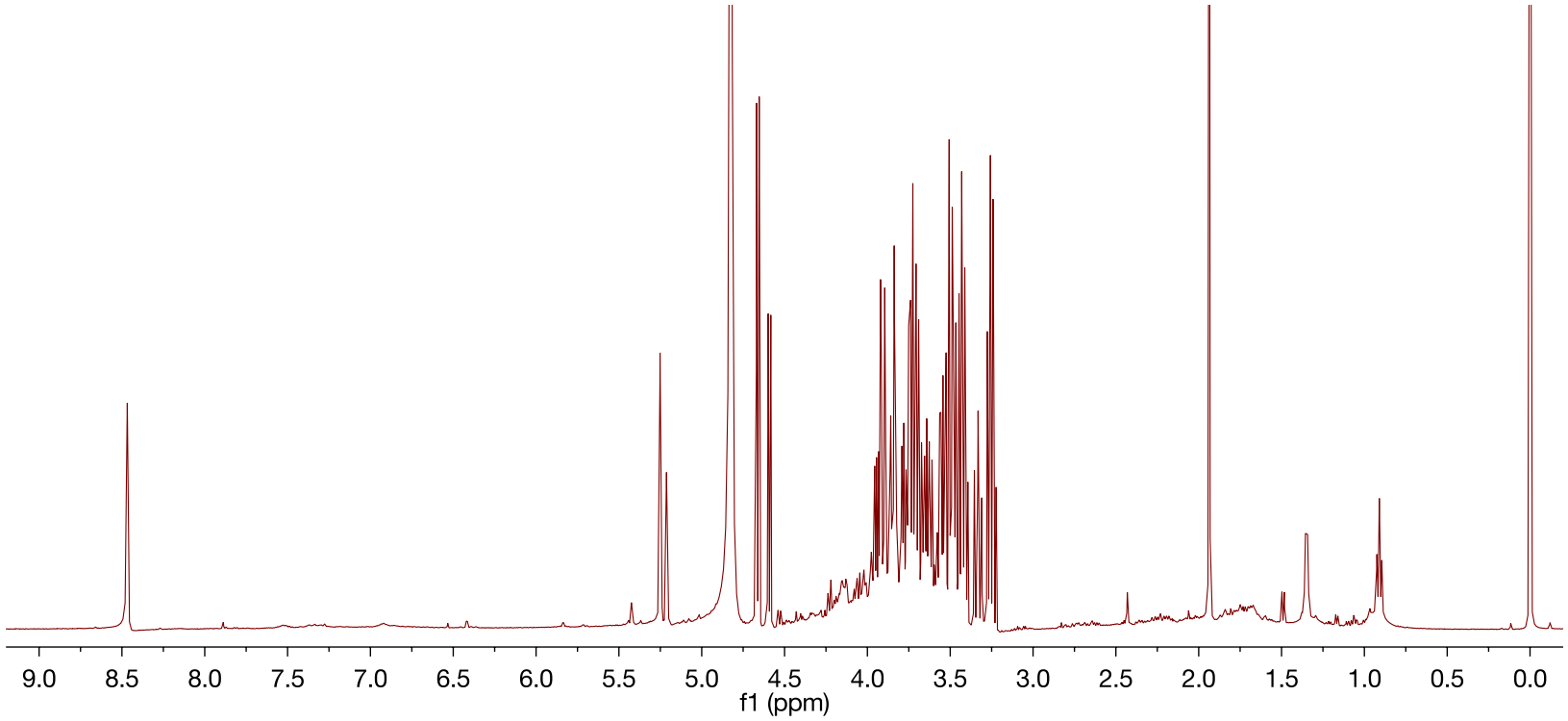

Figure 43. ^{1}H-NMR of AD hydrolysate.

4. EXPERIMENTAL

4.1. Spectroscopic measurements

^{1}H NMR spectra were recorded on a 500 MHz spectrometer. Chemical shifts for ^{1}H NMR spectra in D_2O is reported (in parts per million) relative to sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid, TSP (δ = 0.00 ppm). The following abbreviations are used to describe spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), m (unresolved multiplet), dd (doublet of doublets), br (broad).^{13}C NMR spectra were recorded at 125 MHz and chemical shifts for these spectra were reported (in parts per million) relative to sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid, TSP (δ = 0.00 ppm). Concentrations of fermentation and cell-free reaction products were determined by comparison of the integrals corresponding to each compound with the integral corresponding to TSP (δ = 0.00 ppm) in the ^{1}H NMR. Compounds were quantified using the following resonances: shikimic acid (Figure 38, δ 4.40, t, 1 H), quinic acid (Figure 39, δ 4.13-4.17, q, 1 H), 3-dehydroshikimic acid (Figure 40, δ 2.6-2.68, ddd, 1 H or δ 3.03-3.08, dd, 1 H). The precise concentrations of SA, QA and DHS were calculated by application of the following formulas derived from calibration curves: [SA (g/L)]actual = 0.96 x [SA (g/L)]NMR - 0.15; [QA (g/L)]actual = 1.08 x [QA (g/L)]NMR + 0.11; [DHS (g/L)]actual = 1.32 x [DHS (g/L)]NMR - 0.02. The procedure followed for obtaining the above equations is described here: At least five standard solutions of each compound were prepared using dd water with concentrations ranging from 0.375 g/L to 30 g/L depending on the general titer values for each of them. 1 mL of each of these standard solutions was concentrated under reduced pressure and redissolved in 1 mL of D_2O containing 10 mM TSP for SA, and 1 mM TSP for QA and DHS, after which ^{1}H NMR spectra were recorded. The solute concentration in each sample estimated from

^{1}H NMR was plotted against the original concentration for that sample resulting in the calibration curve.

4.2. Preparation and transformation of *E. coli* electrocompetent cells

Electrocompetent cells were prepared using the following procedure: A single colony was inoculated into 5 mL LB medium and shaken overnight at 37 ^{o}C. 2 mL of this inoculum was used to start a 500 mL 2xYT medium in a 2L baffled flask and was cultured at 37 ^{o}C at 250 to 300 rpm until OD_{600} equals 0.5 to 0.7. Then, the culture flask was chilled in ice for 10 min before being transferred to a chilled sterile 400 mL centrifuge bottle. The cells were harvested in a centrifuge at 4000 rpm and 4 ^{o}C for 5 min. Subsequently the cell pellet was exhaustively washed four times with 400 mL, 200 mL and 200 mL sterile cold dd water, and 100 mL ice cold 10% glycerol solution respectively. After each wash step, the cells were centrifuged for 10 min at 6000 rpm and 4 ^{o}C. Lastly, the cell pellet was suspended in 1.5 mL sterile cold 10% glycerol solution following which it was aliquoted (50 µL) into sterile 1.5 mL microfuge tubes kept on ice. The tubes were flash-frozen in liquid nitrogen and stored long term at -80 ^{o}C.

The first step in the transformation of electrocompetent cells was thawing them on ice for 5 min along with the electroporation cuvettes. Less than 5 µL of purified DNA was added to 50 µL cells. The electroporations (Bio-Rad Gene Pulser) were performed at field strength of 2.5 kV/cm, resistance of 200 ohms and capacitance of 25 µF, immediately after which cells were suspended in 1 mL SOC medium and shaken for 1 h at 250-300 rpm and 37 ^{o}C. If the cells were to be plated on rich medium, no wash step was needed. The cells were resuspended in 100 µL of appropriate medium and were plated in both high as well as low concentration to obtain single colonies. Conversely, if the cells were to be plated on minimal medium, the cells were washed three times in M9 medium via centrifugation and resuspension steps and then plated in high and

low concentrations. The cells were incubated at 37 ^{o}C for 12-15h for rich medium and 36-48h for minimal medium.

4.3. Culture medium

All solutions were prepared in distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g) Bacto yeast extract (5 g) and NaCl (10 g). M9 salts (1 L) contained Na_2HPO_4, (6 g), KH_2PO_4 (3 g), NH_4Cl (1 g) and NaCl (0.5 g). M9 medium contained D-glucose (10 g), $MgSO_4$ (0.12 g) and thiamine (0.001 g) in 1 L of M9 salts. M9/glu/aros medium (100 mL) contained M9 medium with supplemented aromatic vitamins including *p*-hydroxybenzoic acid (0.010 g), potassium *p*-aminobenzoate (0.010 g), 2,3-dihydroxybenzoic acid (0.010 g). *E. coli* strains were grown on both LB/Ap and M9/glu/aros plates. Initial volume for an *E. coli* SP1.1 fermentation was 600 mL. Each fermenter vessel contained 400 mL dd water, K_2HPO_4 (4.5 g), ammonium iron (III) citrate (0.18 g), anhydrous citric acid monohydrate (1.15 g), and concentrated H_2SO_4 (0.72 mL). Concentrated NH_4OH (2.46 mL) was added to adjust pH of the medium to 7.0. Prior to initiating fermentation with 100 mL of inoculant, the following supplements were added: 100 mL of appropriate sugar solution (10.8 g); $MgSO_4$ (0.24 g); aromatic amino acids including phenylalanine (0.42 g), tryptophan (0.21 g) and tyrosine (0.42 g); aromatic vitamins including *p*-hydroxybenzoic acid (0.010 g), potassium *p*-aminobenzoate (0.010 g), 2,3-dihydroxybenzoic acid (0.010 g); trace minerals including $(NH_4)_6(Mo_7O_{24}).4H_2O$ (0.0037 g), $ZnSO_4.7H_2O$ (0.0029 g), H_3BO_3 (0.0247 g), $CuSO_4.5H_2O$ (0.0025 g), and $MnCl_2.4H_2O$ (0.0158 g). Sugar solution, $MgSO_4$ (1 M), and aromatic amino acids were autoclaved, while solutions of aromatic vitamins and trace minerals were sterilized through 0.22-μm membranes. 900 mL 4% (w/v) substrate feed was prepared that contained 36 g either glucose/xylose mixture or glucose or xylose depending on the fermentation.

4.4. Shake flask experiments

4.4.1. Regular shake flask experiments

5 mL M9/glu/aro cultures were inoculated with freshly transformed SP1.1/pKD12.138, and incubated for 15 h at 37 ^{o}C and 250-300 rpm. 2 mL of this overnight culture was pelleted via centrifugation and washed with M9 salts. The resuspended cells in 1 mL M9 salts were transferred to 20 mL M9/carbohydrate cultures in 125 mL baffled flasks and incubated for 48 h at 37 ^{o}C and 250-300 rpm. Two different concentrations of M9/carbohydrate solutions were used in these experiments: 1. 1% sugar solutions contained 5 mL of the 4% w/v sugar (glucose, xylose or AD hydrolysate), 10 mL of 2xM9 salt solution, 5 mL dd water to make up 20 ml of culture media; 2. 3% sugar solutions contained 15 mL of the 4% w/v sugar (glucose, xylose or AD hydrolysate) and 5 mL of 4xM9 salt solution to make up 20 ml of culture media. 2xM9 salts, 4xM9 salts, dd water and sugar solutions were all separately autoclaved and combined in a sterile environment.

4.4.2. Resuspension shake flask experiments

In the case of rich to M9/carbohydrate resuspension experiments, three 5 mL LB/Ap cultures were inoculated with freshly transformed SP1.1/pKD12.138 and incubated for 10 h at 37 ^{o}C and 250-300 rpm. 2 mL of this inoculum was added to three 50 mL LB/Ap cultures in 250 mL baffled flasks and incubated for 5 h at 37 ^{o}C and 250-300 rpm until the cells reached upto halfway through their exponential phase. Then, the cells were pelleted and washed with M9 salts. Cells were resuspended in 5 mL M9 salts, added to 50 mL M9/carbohydrate cultures in 500 mL baffled flasks and incubated for 48-56 h at 37 ^{o}C and 250-300 rpm. 2 mL samples were taken every 6 h to monitor cell growth by measuring OD_{600} and to run ^{1}H-NMR to calculate SA production.

Minimal to M9/carbohydrate resuspension experiments follow the same procedure as above except for changes in times of incubation. Three 5 mL M9/glu/aros cultures were inoculated with freshly transformed SP1.1/pKD12.138 and incubated for 16h at 37 ^{o}C and 250-300 rpm. 2 mL of this inoculum was added to three 50 mL M9/glu/aros cultures in 250 mL baffled flasks and incubated for 14h at 37 ^{o}C and 250-300 rpm until the cells reached upto halfway through their exponential phase. Then, the cells were pelleted and washed with M9 salts. Cells were resuspended in 5 mL M9 salts, added to 50 mL M9/carbohydrate cultures in 500 mL baffled flasks and incubated for 48-51 h at 37 ^{o}C and 250-300 rpm. 2 mL samples were taken every 6 h to monitor cell growth by measuring OD_{600} and to run ^{1}H-NMR to calculate SA production.

4.5. Fed-batch fermentation conditions

4.5.1. General

A B. Braun BIOSTAT® B-DCU fermentor (2 L) connected to a DCU tower system was used for fed-batch fermentations at 1 L scale. Data acquisition utilized MFCS/win 3.0 software (Sartorius Stedim Systems), which was installed in a personal computer (Digilink) operated by Windows® 7 Professional. Temperature and pH were controlled with PID loop maintaining at 33 °C and pH 7.0±0.1 with 2N H_2SO_4 and concentrated NH_4OH. Impeller speed was varied between 50 and 1800 rpm to maintain dissolved oxygen (D.O.) levels at 10% air saturation. Airflow was increased from 0.06 L/min to 1 L/min to maintain dissolved oxygen (D.O.) levels at 10% air saturation. Sugar levels were maintained throughout the run by addition of feed glucose solution (4% w/v). Antifoam 204 (Sigma-Aldrich) was added as needed to mitigate foam accumulation. Inoculant was started by introduction of a single colony picked from an agar plate into 5 mL of M9/glu/aros medium. The culture was grown at 37 °C with agitation at 250 rpm until turbid (24 h) and subsequently transferred to 100 mL of M9/glu/aros medium. This 100 mL culture was

grown at 37 °C and 250 rpm until the OD_{600} reached 1.0-1.5 (12 h). The 600 mL of *E. coli* fermentation media in the fermenter vessel were inoculated with the 100 mL culture.

4.5.2. Fed-batch fermentation of *E. coli* SP1.1

Inoculant was started by introduction of a single colony picked from an agar plate into 5 mL of M9 medium. The culture was grown at 37 °C with agitation at 250 rpm until turbid (24 h) and subsequently transferred to 100 mL of M9/glu/aros medium. This 100 mL culture was grown at 37 °C and 250 rpm until the OD_{600} reached 1.0-1.5 (12 h). The 600 mL of *E. coli* fermentation media in the fermenter vessel were inoculated with the 100 mL culture. The dissolved oxygen (D.O.) levels were maintained at 10% air saturation. The cell growth reached stationary phase around 11-14 h. 36 g of sugar was consumed after 48h. The broth volume increased gradually upto 1.5 L due to cell growth, product, addition of glucose solution, 2M H_2SO_4 and NH_4OH which was used to adjust pH. Samples were collected every 6 h and were concentrated to dryness under reduced pressure twice, the second time from D_2O, and then dissolved in D_2O containing a known concentration of the sodium salt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid. The concentrations of SA, QA and DHS were calculated using the following resonances: shikimic acid (δ 4.40, t, 1 H), quinic acid (δ 4.13-4.17, q, 1 H), 3-dehydroshikimic acid (δ 2.6-2.68, ddd, 1 H or δ 3.03-3.08, dd, 1 H).

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