Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Revalorization of agar from red seaweed as microbial feedstock for *Corynebacterium glutamicum*

Master's thesis in Chemical engineering and biotechnology
Supervisor: Dr. Fernando Pérez-García
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Preface

This master's thesis marks my fulfillment of a five-year M.Sc. program in Chemical Engineering and Biotechnology at the Faculty of Natural Science, Norwegian University of Science and Technology (NTNU). It was completed in the spring of 2023 as part of the Cell Factory group, under the guidance of Postdoctoral Fellow Fernando Pérez-García and Professor Trygve Brautaset, who serves as the principal investigator of the group. The practical research work has been conducted at the Microbial Biotechnology Division within the Department of Biotechnology and Food Science.

First, I would like to thank my fellow colleagues in the laboratory Rosa Jodalen Rudberg and Monica Frøystad, all the members of the Cell Factories group as well as my friends at the MTKJ study program for creating a friendly and pleasant working atmosphere, making each day joyful.

Furthermore, a special thanks to my supervisor Dr. Fernando Pérez-García for your invaluable mentor ship, assistance and patience, providing me with the skills needed for the completion of this thesis. Thank you for always supporting me, motivating me and answering my questions. It has been a true pleasure being one of your students.

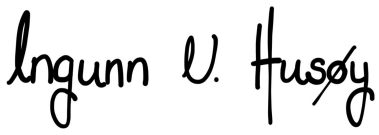
Lastly, I would like to extend my heartfelt gratitude to my father and sister. You have always been my unwavering support team throughout my years in school. It is safe to say that I would not be where I am today without you.

"Believe in yourself"

Declaration of complience

I, Ingunn U. Husøy, hereby declare that this is independent work according to the exam regulations of the Norwegian University of Science and Technology (NTNU).

Signature:



Place and Date: Trondheim - Gløshaugen, July 2023

Abstract

Today, the predominant reliance on sugar-based fermentation poses limitations in terms of sustainability and carbon source availability across various industries. In this regard, red seaweed biomass is abundant and rich in diverse polysaccharides that can potentially be fermented as sustainable alternative microbial feedstock, as for instance agar. However, the utilization of agar as carbon source in biotechnology remains unestablished. This master's thesis aimed to lay the foundation of agar utilizing cell-factories. Hence, the biotechnology workhorse *Corynebacterium glutamicum* was chosen for the production of secreted agarases. A toxicity test was conducted to assess the robustness of *C. glutamicum* as an expression host for agar degradating enzymes; results revealed that a concentration of 0.4 g/L agar was unsuitable for growth among the tested conditions. Next, *C. glutamicum* was engineered via traditional molecular techniques to establish production and secretion of three agarases of interest, namely AhgA from *Zobellia galactanivorans*, as well as DagA and DagB from *Streptomyces coelicolor*. Microbial growth, protein production, and agar degradation were evaluated, concluding that the supernatant from the *C. glutamicum* strain overexpressing ahgA and dagA, namely CgAAopt, exhibited 4.5-fold increase in secreted protein concentration and achieved successful agar degradation after 24 hours of incubation. Additionally, the strain CgAAopt was further engineered to enable galactose uptake and utilization by overexpression of the *galETKM* operon along with the galactose permease gene *galP* from *Escherichia coli*, yielding the strain CgGalAAopt. However, when testing CgGalAAopt with agar as sole carbon source no growth was observed. The qualitative results of this work suggested low efficiency of agar degradation and, hence, no agar-derived galactose was formed. Nevertheless, agar degradation by these strains was visually confirmed. Concluding, the outcomes of this study pave the way for further research to optimize agar degradation and utilization strategies, ultimately establishing red seaweed alternative and competent microbial feedstock.

Sammendrag

I dag er mikrobiell fermentering hovedsakelig basert på bruk av sukker som karbonkilde, hvilket kan virke begrensende når det kommer til bærekraft og konkurrerende bruk av sukker som kilde for menneske- og dyrenæring. Biomasse fra rødt sjøgress har et rikt og mangfoldig innhold av polysakkarider, som for eksempel agar, med potensiale for utnyttelse som et bærekraftig alternativ til mikrobielt råstoff. Imidlertid har potensialet for å bruke agar som karbonkilde innen bioteknologi ennå ikke blitt realisert. Denne masteroppgaven hadde som mål å legge grunnlaget for å utvikle cellefabrikker som kan utnytte agar. Derfor ble bakterien *Corynebacterium glutamicum*, som er mye brukt innen bioteknologi, valgt for produksjon og utskillelse av agar-nedbrytende enzymer. En toksisitetstest ble utført for å vurdere *C. glutamicum*s evne som vertsbakterie for agaraser. Resultatene viste at en konsentrasjon på 0.4 g/L agar var uegnet for vekst under de testede forholdene. Videre ble *C. glutamicum* modifisert ved hjelp av tradisjonelle molekylære teknikker for å etablere produksjon og utskillelse av de tre utvalgte agarasene: AhgA fra *Zobellia galactanivorans* samt DagA og DagB fra *Streptomyces coelicolor*. Mikrobiell vekst, produksjon av proteiner og nedbrytning av agar ble evaluert. Resultatene viste at supernatanten fra *C. glutamicum*-stammen som overuttrykte ahgA og dagA, kjent som CgAAopt, viste økning på 4.5 i konsentrasjonen av utskilte proteiner, og oppnådde vellykket nedbrytning av agar etter 24 timers inkubasjon. Dermed ble stammen CgAAopt videre modifisert for å kunne ta opp og utnytte galaktose ved å overuttrykke *galETKM*-operonet sammen med genet *galP* for galaktosepermease fra *Escherichia coli*. Dette resulterte i stammen ved navn CgGalAAopt. Likevel ble det ikke observert vekst når CgGalAAopt ble testet med agar som eneste karbonkilde. De kvalitative resultatene fra dette arbeidet antydet lav effektivitet ved nedbrytning av agar og dermed ingen dannelse av agar-avledet galaktose. Derimot ble nedbrytning av agar av de konstruerte stammene visuelt bekreftet. Oppsummert legger resultatene fra denne studien grunnlaget for videre forskning for å optimalisere strategier for nedbrytning og utnyttelse av agar, og dermed etablering av rødt sjøgress som et alternativt og kompetent mikrobielt råstoff.

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Abbreviations

Table 0.1: Explanation of the abbreviations used in this work.

Abbreviation	Explanation
ACI	3,6-anhydrogalactonate cycloisomeras
AhgA	α-1,3-(3,6-anhydro)-L-galactosidase from *Zobellia galactanivorans*
ALG	Anhydro-L-galactose
BHI	Brain heart infusion
BSA	Bovine serum albumin
DagA	β-agarase from *Streptomyces coelicolor*
DagB	β-agarase from *Streptomyces coelicolor*
EPB	Electroporation buffer
G	D-galactose
GABA	Gamma-aminobutyric acid
GH	Glycoside hydrolases
GRAS	Generally Recognized As Safe
HiFi	High fidelity
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan^R	Resistance to kanamycin
KDGal	2-keto-3-deoxy-galactonate
K_i	Inhibitory konstant
L-AHGA	L-3,6-anhydrogalactonate
MQ	Milli-Q water
MS	Mass spectrometry
NABH	α-neoagarobiose hydrolase
ORF	Open reading frame
P	Phosphate
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate
PTS	Phosphotransferase system
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec-pathway	General Secretory pathway
Tat-pathway	Twin-arginine translocation pathway
Tet^R	Resistance to tetracycline
TLC	Thin-layer chromatography
UPD	Uridine diphosphate
5HMF	5-hydroxymethylfurfural

1 Introduction

1.1 New substrates

The rapid advancement of technology worldwide is posing a challenge to the pursuit of sustainable production, as well as the exploration and commercialization of innovative products derived from biological sources[3]. As a result of unsustainable production processes and the inefficient origin of ingredients in current products, research efforts have been directed towards developing alternative methods for producing ecologically-safe products from sustainable processes[4]. Consequently, there is a significant need to thoroughly investigate new and more environmentally friendly production lines for synthesizing biological-based products both in the laboratory and at an industrial scale. Microbial production has gained increasing attention in recent years due to its potential for sustainable and efficient manufacturing processes[5]. However, the use of traditional carbon substrates, such as sugars and starches, for industrial production can be a limiting factor in terms of product diversity, cost, and environmental impact[6]. Today, the biotechnological production of valuable compounds, such as pharmaceuticals and bulk chemicals, primarily depends on the use of glucose[7]. Even though the production of glucose from starch crops is well developed, the utilization of glucose in industrial manufacturing competes with the usage of land for the production of human and animal nutrition, forcing a progressive increase in the prices[8]. Additionally, studies show that the agricultural phase of glucose production has an overall negative climate impact due to the use of synthetic fertilizer and non-renewable fuels in field operations[9]. Thus, the search for alternative feedstocks for microbial production has become a research focus[7]. Finding novel substrates that are cheaper and more readily available can not only provide environmental benefits but also reduce production costs and enhance the accessibility of microbial products to consumers. For instance, using substrates e.g., food- and animal waste can help conserve natural resources while reducing waste[10;11]. Furthermore, the exploitation of new microbial substrates can accommodate the exploration of novel and diverse products that have not been previously discovered due to limitations by existing substrate sources[12]. In addition, the identification of new substrates that can be metabolized by microorganisms allows researchers to design novel fermentation processes that could lead to better production yields[13].

1.2 Seaweed

Marine macroalgae, widely known as “seaweed,” is an abundant and promising alternative feedstock for industrial microbial-based production of value-added compounds, owing to its high growth rate, and chemical and bioactive properties[14;15]. Moreover, seaweeds have a lower lignin content compared to terrestrial biomass as they do not need the same level of rigidity to maintain their structure in water. This can provide benefits for biorefinery usage as intricate processes like lignin removals and detoxification of lignin-originated inhibiting compounds are not needed[16]. Seaweed cultivation does not require arable land or freshwater and has a higher production yield than land-based energy crops[17]. Therefore, the use of seaweed as a feedstock for biomanufacturing has the potential to provide cost-effective and

sustainable solutions for meeting the worlds growing demand for production[14]. Algae cultivation exhibits a distinct advantage as it does not necessitate the use of fertilizers, pesticides, or freshwater resources that could pose environmental risks[18]. Furthermore, the conservation and restoration of coastal habitats for seaweed cultivation possess significant potential for climate change adaptation and mitigation[18]. Industrial utilization of seaweed can be optimized considering composition-specific polysaccharides or bioactive compounds depending on the intended application. To extract these compounds, various methods such as acid hydrolysis, enzymatic digestion, and solvent extraction can be employed[19]. The extracted compounds can serve directly as feedstocks for microbial production or as functional ingredients in the food and pharmaceutical industries[20]. The by-products of seaweed processing, including protein-rich biomass and mineral-rich ash, are even more importantly suitable as feedstocks for microbial production[20].

1.2.1 Composition of seaweeds

Seaweeds are complex and multifarious organisms that contain diverse carbohydrates, proteins, lipids, vitamins, and volatile compounds. The composition varies depending on the species and growth conditions, and based on pigmentation and structural characteristics, seaweed is commonly classified into three groups, i.e., green, red, and brown[21;22]. Furthermore, seaweed harbors an array of bioactive compounds, such as antioxidants (phenolic compounds[23]), pigments (phycocyanin, phycoerythrin[19] and chlorophyll[23]), and phycotoxins (okadaic acid, gonyautoxins and yessotoxins[24]), that possess potential biomedical applications in the health and cosmetics sectors. Seaweed is particularly rich in polysaccharides, which exhibit unique rheological properties and are extensively used in the food and pharmaceutical industries[25]. The most relevant polysaccharides of significance include alginate and laminarin, which are present in brown seaweed, and carrageenan and agar, found in red seaweed[25]. Alginate, the major polysaccharide synthesized by brown seaweed, has become one of the most widely utilized polysaccharides by various types of industries. For instance, alginate is extensively applied in the food industry due to exhibiting exceptional qualities as a thickening and stabilizing agent in processed foods[26]. The composition of alginates varies depending on the species, ranging from 20% to 60% dry matter, but commonly exist in the form of gels consisting of a mixture of magnesium, calcium, sodium, and barium ions[27;27]. Laminarin, the primary glucan storage polysaccharide in algae[28], has been found to exhibit various activities such as antioxidative, anti-tumorigenic, anticoagulant, antiviral, and antibacterial activities[29]. It can account for up to approximately 20% of the weight of brown seaweed at maturity[30] and up to levels of 35% on a dry basis[31]. Research has shown that feeding laminarin-rich extracts to animals is promising, suggesting that it can be a viable functional ingredient for use in food applications[31]. Carrageenan refers to a group of naturally occurring water-soluble and sulphated galactans that are found in the cell wall and intercellular matrix of the plant tissue of red seaweeds[32;33]. Seaweeds used in commercial applications usually contain 30-60% carrageenan dry weight, with some reaching up to 70-80%[34]. Carrageenans serve as gelling, thickening, and stabilizing agents in a variety of commercial applications,

particularly in food products, in addition to having applications within experimental medicine, pharmaceutical and formulation[33]. Agar is an easily accessible polysaccharide found in several families of red seaweed such as *Gracilariaceae* and *Gelidiaceae*[35], initially employed in the food industry, biotechnology, and pharmaceuticals besides being utilized as a raw material for manufacturing bioplastic film[36]. Additionally, agar has applications in microbiology and molecular biology as the large thermal hysteresis of agar gel makes it suitable for the growth of culture media for bacteriological use[37]. Furthermore, it can be used as an encapsulating agent for microorganisms and cells[38]. This polysaccharide is a major component of the cell walls of red algae and can account for up to 57% of biomass dry weight[39;40].

1.3 Red Seaweed

Red seaweed, or Rhodophyta, is a group of marine algae that has gained increasing attention as a competitive feedstock for microbial fermentation over the last decade due to its remarkable characteristics that include the absence of lignin, high carbon dioxide fixation properties as well as high substantial amounts of carbohydrates such as galactan and glucan[41;42;43]. Additionally, complex polysaccharides are abundant in red seaweed, with the most commercialized ones being the galactans agar and carrageenan, both of which have a wide range of biomedical and industrial applications[36]. Furthermore, red seaweed contains unique bioactive compounds, including phycobiliproteins and polyphenols, which have shown applications as potential antiviral, anticoagulant, and anti-inflammatory agents[39;44]. The red color of Rhodophyta is expressed due to the predominance of the pigments phycocyanin and phycoerythrin[19]. Moreover, red algae have the added advantage of a high growth rate, which can be enhanced by elevating the CO_2 in the surrounding atmosphere, the nutrient-enriched conditions in the seawater and increasing the availability of nitrogen[45]. Red seaweeds exhibit a highly diverse nutrient composition across different species, with the composition being dependent on environmental conditions, and the majority of them are found in cold areas at greater depths than green and brown seaweed[46]. Additionally, Rhodophyta has the potential as an efficient feedstock for biofuel production. Studies have reported successful bioconversion of *Gelidium amansii* to bioethanol[47]. Due to their remarkable properties, red seaweeds represent an economically important resource accounting for 61% of global seaweed production[48]. Other polysaccharides from red seaweed, such as porphyran, have also gained attention for potential applications in various industries, including exhibiting anti-cancer activities[49]. Agar and carrageenan are the major constituents of red alga and have different chemical compositions and physical properties, which affect their suitability for various applications, including their use as microbial feedstock[16]. Both are deemed sustainable sources of biomass suitable as feedstock for manufacturing several fermentation products[50]. However, it is crucial to consider the specific properties of each substance, as well as the specific requirements of the microorganism and the desired end product, to determine which is more promising as a microbial feedstock for a particular application.

1.4 Agar

The natural polymer agar consists of two types of polysaccharides, namely agarose and agaro pectin. Agarose is a neutral linear polysaccharide, primarily composed of 1,3-linked β-D-galactose (G) and 1,4-linked 3,6-anhydro-α-L-galactose (ALG) units, with only a few of the hydroxyl groups being sulfated. This makes it suitable for gel formation. In contrast, agaropectin is a more complex polysaccharide that is build up by the same backbone as agarose, but in addition contains D-gluconic acid, pyruvic acid, methyl esters and a significantly higher proportion of sulfate ester groups[51;52]. Hence, the complete digestion of agar results in the formation of D-galactose and 3,6-anhydro-L-galactose[53] whose chemical structure is illustrated in Figure 1.1[51].

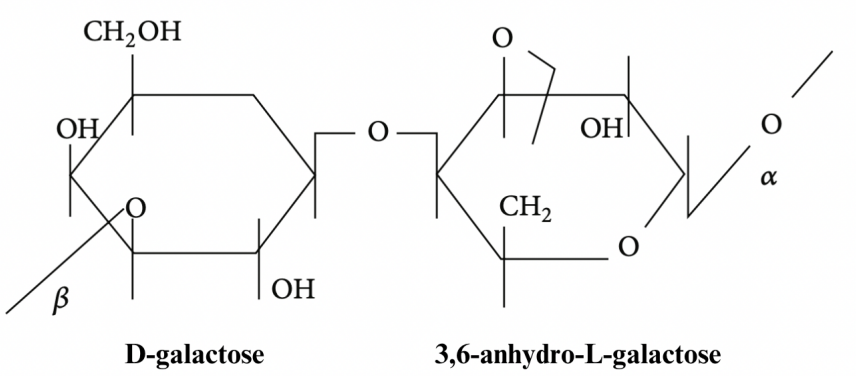


Figure 1.1: Chemical structure of the subunits D-galactose and 3,6-anhydro-L-galactose of agar. Picture was modified from Varshosaz et al. (2015)[51]

Agar is a major component of the cell wall of red seaweeds and is primarily derived from the species *Gracilaria*, *Gelidium*, and *Pterocladia*[16;54]. The polysaccharide exhibits some remarkable physical properties that include insolubility in cold water but solubility in boiling water. Its gelation temperature ranges from 31°C to 36°C and exposed to temperatures below this, thermally reversible hydrogel is formed. This hydrogel will exist in its gel form and does not undergo a reversible gel-to-sol transition below its melting point (65-85°C)[51]. Extraction of agar usually happens by solubilization of red seaweed in hot water, for some species following an acid/heat or alkali treatment. Due to a multi-step extraction process, producing agar is a slow and intricate procedure[55]. Optimization of the extraction parameters including temperature, extraction time, and alkali concentration is necessary for each individual seaweed species[19]. Hence, other approaches, such as degradation by enzymatic activity or other chemical treatments, is important areas for exploration as this can provide a more sustainable and effective alternative[53].

1.4.1 Enzymatic degradation

The conversion of carbohydrates from seaweed into sugars by microorganisms for use as feedstock in biotechnology encompasses important research areas, given the variety of carbohydrates present in macroalgae[16]. Although degradation of agar can be achieved by the use of chemical treatments involving high temperatures and pH changes, enzymatic processes are favored as a more sustainable solution that generates fewer toxic by-products[53]. An enzymatic approach offers the advantage of preserving the bioactive properties of the oligosaccharide produced, rendering them valuable for a variety of applications[53]. However, general research utilizing macroalgae-specific enzymes such as alginate lyases, laminarinase and agarase to saccharify macroalgal biomass is still in its early stages[16], and studies have reached different outcomes regarding hydrolysis efficiency[56]. Recently, oligosaccharides derived from agar by the use of different agarases have gained more attention due to their promising biological activities[54]. Agarases have shown practical applications in industries for saccharification of agar for example when it comes to the production of neoagarooligosaccharides or agarooligosaccharides[54]. These oligosaccharides - especially neoagarotetraose and neoagarohexaose - have demonstrated notable antioxidative properties by effectively scavenging hydroxyl free radicals and superoxide anion radicals, as well as inhibiting lipid peroxidation[57]. Therefore, novel and more advanced methods for the degradation of agar are sought to enhance saccharification efficiency and product yield. Suggested methods to explore are for instance additional pretreatment of the algal biomass, the use of multi-enzyme complexes, or genetically engineering microbial workhorses in biotechnology[56].

1.4.2 Agarases

Due to the complex nature of agar as a polysaccharide, the complete enzymatic hydrolysis of agar into its constituent monomers, namely β-D-galactose (G) and anhydro-α-L-galactose (ALG), needs the involvement of multiple enzymes with different catalytic activities[40]. Agarases are glycoside hydrolases (GH) that catalyze the hydrolysis of agarose and besides being used in the preparation of agar-derived oligosaccharides[54], agarases have been employed to recover DNA bands from gels made of agarose[40]. Generally, agarases are classified into two groups, i.e., α-agarase and β-agarase, on the basis of their mode of action. This classification is due to their ability to recognize and target different repeating units present in agar[54]. β-agarases specifically recognize and target the (G-ALG) repeating units of agar and cleave the β-(1,4) glycosidic bonds. This enzymatic depolymerization of agar leads to the production of neoagaro-oligosaccharides, with G residues present at the reducing ends[40]. In contrast, α-agarases hydrolyze the α-(1,3) glycosidic linkages in the (ALG-G) repetition moieties of agarose, resulting in the generation of agaro-oligosaccharides with ALG residues at the reducing ends[40;54]. The expression and activity of agarases are specifically observed in numerous microorganisms that utilize agar as sole carbon source, which indicates simultaneous gene expression and the presence of common regulatory mechanisms[58]. A schematic representation of α-agarose (to the right) and β-agarose (to the left) hydrolytic pathways is illustrated in Figure 1.2[40].

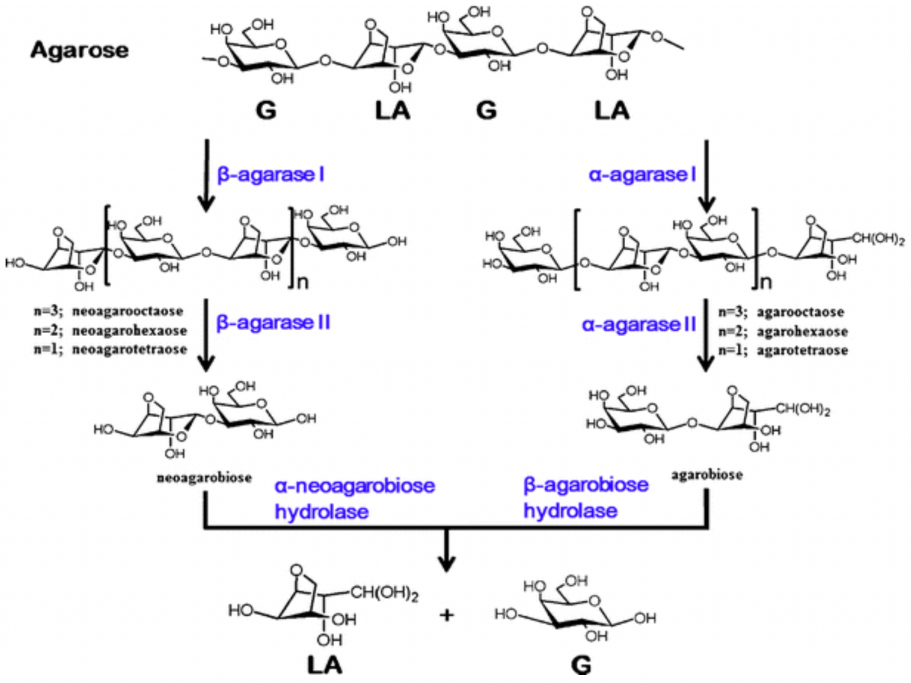


Figure 1.2: Illustration of the the hydrolytic pathways of α-agarose (on the right) and β-agarose (on the left). Agarose oligomer is cleaved by either α-agarase or β-agarase producing agarotetraose or neoagarotetraose, respectively. These saccharides are further broken down to agarobiose or neoagarobiose. Finally, agarobiose or neoagarobiose is cleaved to produce G or ALG, respectively, by β-agarobiose hydrolase or α-neoagarobiose hydrolase. The picture was taken from Chi et al. (2012)[40]

1.5 Agarolytic microorganisms

Considerable progress has been made through extensive research on isolating agarolytic microorganisms and conducting biochemical investigations on agarases. Acquiring a comprehensive understanding of the agar-degrading system of microorganisms is of great significance for effective utilization of biomass from red seaweed. Despite concerted efforts, several gaps in our knowledge of the agar-hydrolyzing pathway warrant further research[40]. Agarases have been successfully isolated from diverse sources including sea- and freshwater, marine sediments, algae, and soil, demonstrating a wide distribution of agar-degrading enzymes across different environments[59]. However, it appears that the production of these agarases is primarily attributed to microorganisms that rely on agar as a carbon and energy source. Thus, the majority of agarolytic organisms have been found in marine habitats[40]. Agarase activity has been documented in a diverse array of microorganisms such as *Bacillus*[60], *Pseudomonas*[61], *Agarivorans*[62], *Vibrio*[63], *Pseudoalteromonas*[64], *Cytophaga*[65], *Thalassomonas*[66], and *Acinetobacter*[67], amongst others. While the majority of agarases are produced by Gram-negative bacteria which predominantly secrete the enzymes into the extracellular environment[53], some agarases are produced intracellularly[60;61].

1.5.1 *Zobellia galactanivorans*

Zobellia galactanivorans is a Gram-negative, non-spore-forming marine bacterium belonging to the family *Flavobacteriacea*. It is isolated from the red alga *Delesseria sanguinea* and recognized for its proficient degradation of algal polysaccharides in various marine environments, both in the open ocean and in coastal regions[68;69]. This bacterium produces a repertoire of agar-degrading enzymes, including both α- and β-agarases, all belonging to the glycoside hydrolase family[70]. This enables it to effectively hydrolyze agarose, allowing it to grow in minimal medium with agar as its sole carbon source[69]. The study of *Z. galactanivorans* and its agar-degrading properties has given important insight when it comes to the enzymatic mechanisms of agar degradation and has contributed to enhancing our understanding of the microbial utilization of complex polysaccharides in marine environments[68]. For instance, the two agarases AgaA and AgaB isolated and purified from *Z. galactanivorans* have been utilized in developing crystals for X-ray diffraction studies[71].

1.5.2 The novel AhgA agarase from *Z. galactinovorans*

The α-1,3-(3,6-anhydro)-L-galactosidase AhgA is a β-agarase that is involved in the terminal step of agar degradation as it cleaves short oligosaccharides releasing individual monosaccharides, particularly 3,6-anhydro-L-galactose, from the non-reducing end of neoagaro- oligosaccharides[40;70]. AhgA (coded by Zg4663) is a part of the agar degradation system in *Z. galactanivorans* belonging to the GH-117 family[68]. Its predicted ribbon structure is shown in Figure 1.3 A. 3D structural analysis of the enzyme has suggested that the transcriptional product of the *ahgA* gene adopts a six-bladed β-propeller fold, which creates a distinctive active site with a funnel-like shape[40]. This finding endorses the theory that AhgA likely hydrolyzes the external ends of the substrate molecules[40]. Its catalytic activity is zinc-dependent[72]. AhgA contains a Sec signal peptide with characteristics of lipoprotein which in combination with its biochemical properties supports the suggestion that it is located in the periplasmic region of the bacterium[72]. AhgA represents a novel family of glycoside hydrolases that is particularly prevalent in coastal waters[72].

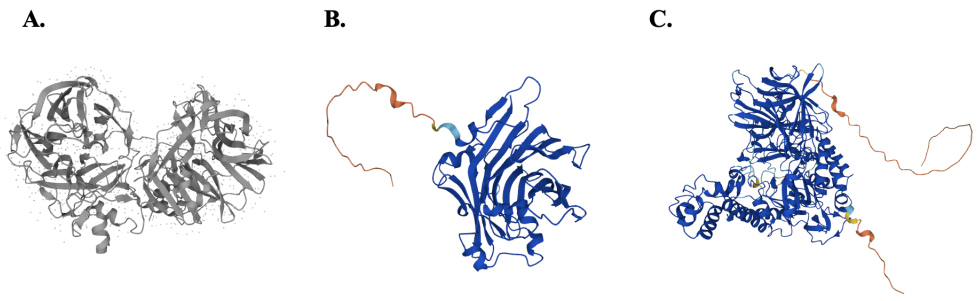


Figure 1.3: Representative ribbon structures of agar-specific polysaccharidases. A. α-1,3-(3,6-anhydro)-L-galactosidase AhgA (ZG4663) from *Z. galactanivorans*. B. β-agarase DagA (SCO3471) from *S. coelicolor*. C. β-agarase DagB (SCO3487) from *Streptomyces coelicolor*. The β-strands and the α-helices are represented by arrows and ribbons, respectively. Source of figures: UniProt[73].

1.5.3 *Streptomyces coelicolor*

Streptomyces coelicolor is a Gram-positive soil bacterium that due to unique characteristics, such as the ability to undergo complex morphological and physiological differentiation, produces a variety of secondary metabolites with important applications in medicine and biotechnology[40]. It is especially known for its large-scale production of a majority of the natural antibiotics used in human and veterinary medicine[74]. *S. coelicolor* has a high GC content in its genome and has the ability of spore formation[40]. Owing to its importance in medicine and industrial applications, extensive research has been conducted on the transcriptional regulation of the primary and secondary metabolism in this bacterium[75]. Based on the analysis of genome sequence data, it is predicted that approximately 10.5% of *S. coelicolor* A3(2)' total protein content is secreted proteins that likely are involved in the utilization of diverse nutrients present in the soil[74]. The bacterium has developed the metabolic versatility to metabolize a variety of chemicals due to gradual adaptation to soil environment, as a consequence of rapid fluctuations in nutrition, physical conditions, and biological factors[40]. This ability allows *S. coelicolor* to efficiently degrade complex organic compounds, including lignocellulose and chitin, which makes it an important contributor to carbon recycling processes in the environment[40]. In addition, *S. coelicolor* is one of few bacterial species capable of degrading agar and utilizing it as sole carbon source[53]. Its most abundant agarases belong to the glycoside hydrolase family GH-16[71]. This indicates that the agarases originate from a common ancestor and have diverged substantially in their primary sequence. Hence, the agarases from *S. coelicolor* share a similar fold and identical molecular mechanism[71].

1.5.4 Extracellular agarases from *S. coelicolor*

DagA (coded by SCO3471) is an endo-type extracellular β-agarase that can be isolated from *S. coelicolor*. This enzyme belongs to the GH-16 family and catalyzes the degradation of agar into two neoagaro-oligosaccharides, namely neoagarotetraose and neoagarohexaose[40;76]. Transcription of the *dagA* gene has a primary translation product of 279 amino acids, in addition to a 30-amino acid Sec signal peptide which is cleaved at the N-terminus[53]. Figure 1.3 B. illustrates its ribbon structure with the signal peptide colored in orange. *In vitro* reconstitution experiments have revealed that the transcription is regulated by multiple promoters that is recognized by at least three distinct holoenzymes of the RNA polymerase[53]. Moreover, the transcription of DagA is induced by agar and repressed by glucose[77]. The β-agarase is observed to be the most enzymatic active at pH 7.0 and 40°C, and its major reaction product is neoagarotetraose[53].

Another extracellular β-agarase that also is a part of the agarolytic gene cluster in *S. coelicolor* is the putative hydrolase DagB (coded by SCO3487) which together with DagA plays a crucial role in agar degradation[40]. The *dagB* gene product DagB encodes a primary translation product of 798 amino acids that catalyzes the hydrolysis of agarose, neoagarotetraose and neoagarohexaose, converting agar into neoagarobiose[76]. DagB is a bifunctional agarase that has two catalytic domains and is the only known agarase to generate neoagarobiose as its

major product[78]. Figure 1.3 C. illustrates its ribbon structure. DagB exhibits both exolytic and endolytic activities as it cleaves the non-reducing ends of the polysaccharide chain in addition to cleaving within the chain[40;79]. It shows similarities to GH-50 β-agarases and carries a 45 amino acid Sec signal peptide[40]. DagB is observed to be the most enzymatic active at pH 7.0 and 40°C[67].

1.6 Traditional industrial workhorses

As described, *Z. galactanivorans* and *S. coelicolor* are microorganisms with well-developed metabolic systems for proficient saccharification of agar, a polysaccharide with promising potential for utilization as microbial feedstock owing to its unique biological activities[54]. Consequently, these bacteria have potential for industrial applications as they produce a variety of agarases that can provide sustainably favored enzymatic approaches for the degradation of agar[68;53]. However, it is worth considering that there may be certain limitations and challenges when using marine and soil bacteria for industrial implementations. For instance, their slow growth rates compared to other microbial workhorses such as *Bacillus subtilis*, *Escherichia coli* and *Corynebacterium glutamicum* pose impediments for large-scale industrial production, where high throughput and rapid turnaround times are essential[80;81]. Moreover, implementation in extensive industrial manufacturing will require special equipment as these bacteria are not considered model organisms and are more difficult to cultivate compared to mesophilic workhorses. Hence, a more cost-effective and sustainable solution to harness their unique properties would be to utilize genetically engineered mesophilic organisms for which industrial equipment and technological advancements initially were developed[82]. In general, metabolic engineering aims to establish efficient cell-factories and optimize metabolic pathways and genetic regulatory elements in order to enhance production, focusing on yield and productivity[83]. The advancement of recombinant DNA technology has enabled the use of industrial organisms as biocatalysts, offering a potential for the development of novel and optimized bioprocesses. Thus, metabolic engineering, in combination with the utilization of renewable carbon sources as for instance seaweed hydrolysates, can offer a more sus tainable approach to industrial microbial production[83].

1.6.1 *Corynebacterium glutamicum*

Ever since its discovery in Japan in 1956, the soil bacterium *Corynebacterium glutamicum* has been an important workhorse in industrial biotechnology[84]. It has shown a strong potential for protein manufacturing and is today primarily utilized for fermentative million-ton-scale production of L-glutamate and L-lysine due to its native ability to secrete amino acids under adapted conditions[85]. The interest in the fast-growing bacterium has constantly increased over the years as such has led to the complete sequencing of its genome as well as extensive study of its biochemistry and physiology[86;87]. The genomic analyses of *C. glutamicum* has revealed an extensive repertoire of pathways, indicating favorable traits for usage in industrial production[88]. *C. glutamicum* is a facultative anaerobe Gram-positive rod-shaped bacterium within the phylum *Actinobacteria* with a high guanine and cytosine content[89]. A deeper un-

derstanding of the bacterium combined with the use of novel genome editing techniques and synthetic biology tools has largely expanded its areas of application[84]. Successful engineering of the versatile *C. glutamicum* has made it a suitable host for the production of value-added chemicals including diamines, carotenoids, organic acids (e.g., lactate and succinate)[85], non-proteinogenic amino acids, poly(3-hydroxybutyrate), isopropanol and the compatible solutes ectoine, hydroxyextoine, and mannosylglycerate[90;91;88]. Previous studies have shown that whenever exposed to stress, the bacterium possesses the remarkable property of remaining metabolic active by ceasing growth or secreting amino acids[85]. Additionally, *C. glutamicum* has shown to be especially robust when subjected to fluctuations in oxygen and substrate supply in large-scale fermentation[92]. On top of that, *C. glutamicum* is considered a GRAS organism (Generally Recognized As Safe). Hence, it is safe for both genetic engineering and laboratory work[92]. The extensive study of *C. glutamicum* has enabled the development of toolkit of genetic engineering techniques. Therefore, metabolic engineering of *C. glutamicum* holds important potential for achieving a promising cell factory for agarase production[88].

1.6.2 Alternative and sustainable carbon sources for *C. glutamicum*

Today, *C. glutamicum* primarily utilizes the main sugars derived from agricultural crops i.e., glucose, fructose, and sucrose, as feedstocks for industrial amino acid fermentation[93]. However, in recent years, various corynebacterial strains have exhibited capabilities that extend beyond the traditional production of amino acids, as it was discovered that these strains can be genetically engineered to efficiently utilize alternative carbon sources[94;95]. For instance, *C. glutamicum* has successfully been modified to utilize alternative substrates such as lignocellulosic pentoses like xylose and arabinose by integration of the *xylA* and *araBAD* genes from *Xanthomonas campestris* and *E. coli*, respectively[7;88]. Additionally, *C. glutamicum* has a relaxed carbon catabolite repression system, which distinguishes this bacterium from other commonly used biotechnological organisms such as *E. coli* and *B. subtilis*[96]. Unlike these microorganisms, *C. glutamicum* is capable of utilizing secondary carbon sources even in the presence of glucose[96]. The bacterium uses the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) to take up and phosphorylate glucose, fructose, and sucrose[93]. A study by Lindner S. (2011) found that *C. glutamicum* has the ability to use glucose even in the absence of the PTS by utilizing inositol permeases to import glucose and endogenous glucokinases for the phosphorylation of glucose[97].

1.6.3 Galactose utilization

Galactose is a natural aldohexose that commonly exist in its D-configuration found in seaweed such as carrageenan and agar[98;99]. While *C. glutamicum* lacks the native capability to use galactose as carbon source, the introduction of galactose-utilizing genes from *E. coli* or *Lactococcus cremoris* can enable this ability[7;100;101;102;103]. For *E. coli*, galactose can serve as sole carbon source by the utilization of the *galETKM* operon in collaboration with the *galP* gene that encodes a symporter responsible for the transport of β-D-galactose and protons[7;104]. The conversion of β-D-galactose into UPD-glucose is controlled by the four genes of the operon:

galM, which codes for an epimerase that converts β-D-galactose to α-D-galactose; *galK*, which codes for a galactokinase that phosphorylates α-D-galactose; *galT*, which codes for a uridylyltransferase that converts galactose-1-P and UDP-glucose into UDP-galactose and glucose-1-P; and *galE*, which codes for an epimerase that converts UDP-galactose into UDP-glucose[30]. Subsequently, UDP-glucose can participate in various metabolic pathways within *E. coli*[30]. An illustration of the pathway for galactose utilization in *E. coli* by the use of the galactose operon *galMKTE* and the galactose symporter gene *galP* is provided in Figure 1.4[105]. Comparably, *L. cremoris* exhibit similar genes for galactose utilization as described for *E. coli*[103].

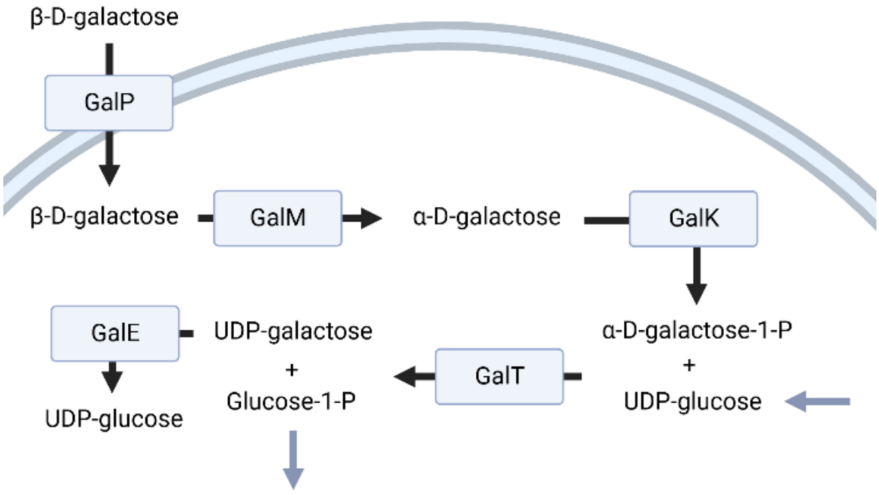


Figure 1.4: Pathway for galactose utilization in *E. coli* by the use of the galactose operon *galMKTE* and the galactose symporter gene *galP*. The blue boxes represent the gene products with respective enzymatic activity indicated by a black arrow. Blue arrows represent substrate movement. UDP: uridine diphosphate; P: represents phosphate. Picture was taken from the master thesis of Frøystad M. (2023)[105]

1.7 The objective of this work

This work seeks to establish recombinant bacterial degradation of agar combined with the utilization of agar-derived galactose as carbon source utilizing microbial strain engineering technologies to modify the bacterium *C. glutamicum* (Figure 1.5).

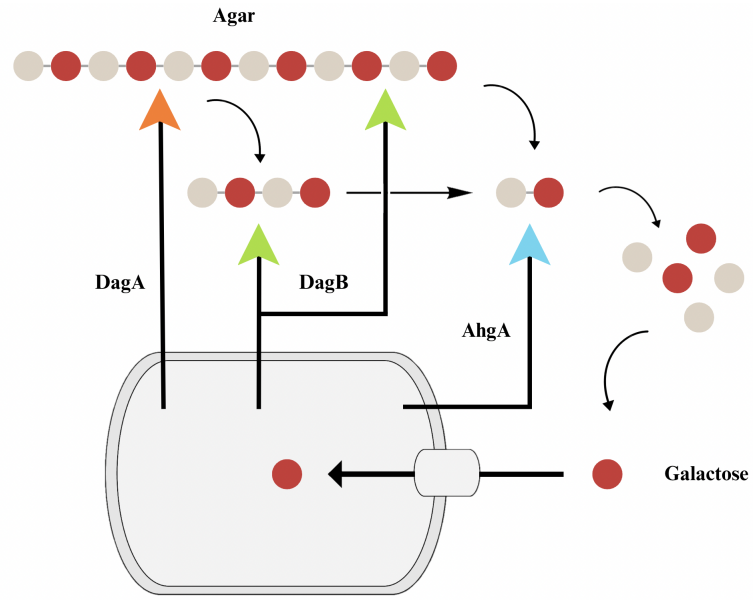


Figure 1.5: Illustration of *C. glutamicum* expressiong the genes *ahgA* from *Z. galactanivorans* and *dagA* and *dagB* from *S. coelicolor* to enable secretion of the agarases AhgA, DagA and DagB, respectively, for degradation of agar[40].

Finally, the goal of this thesis will be reached by fulfilling the following objectives:

- Assessing the specific concentration of agar that inhibits growth of *C. glutamicum*, thereby establishing a benchmark for future experiments
- Establishing production and secretion of the three agarases AhgA, DagA and DagB in *C. glutamicum*
- Monitoring growth characteristics, extracellular protein secretion patterns and enzymatic activity of the constructed strains
- Establishing consumption of galactose derived from external agar degradation in *C. glutamicum*

By achieving these objectives, this thesis seeks to contribute to the understanding of *C. glutamicums*' metabolic capabilities and shed light on the utilization of agar, and ultimately agar containing red seaweed biomass, as a sustainable carbon source. The findings of this research could offer practical value for the development of more efficient and eco-friendly bioprocesses in various industries, including food, pharmaceuticals, and biofuels.

2 Materials and methods

The material and methods used during this project will be described in the following section. Specific used methods will be indicated in the corresponding result section.

2.1 Media and buffers

Recipes and procedures for the preparation of all media used in this work are given in Appendix A. All the chemical components that were used were purchased at Sigma-Aldrich.

2.2 Biological material

Various expression plasmids for different combinations of the genes *dagA, dagB* and *ahgA* encoding the three agarases were constructed. *E. coli* DH5α was used as a general cloning host for the construction of plasmids while *C. glutamicum* ATCC 13032 was used as the expression host. For the PCR amplification of genes, genomic DNA from *S. coelicolor* and codon optimized synthetic DNA from *S. coelicolor* and *Z. galactanivorans* was used as template. The gene *dagA* was amplified from both genetic and synthetic DNA from *S. coelicolor*. The *dagB* gene was amplified from two synthetic sequences of codon optimized DNA from *S. coelicolor* because cloning from genomic DNA was unsuccessful. The *ahgA* gene was amplified from codon optimized synthetic DNA from *Z. galactanivorans*. The plasmids and bacterial strains used and constructed in this study are listed in Table 2.1 and Table 2.2, respectively. The DNA was obtained from IDT (Integrated DNA Technologies).

Table 2.1: Plasmids constructed in this work. Tet^R: tetracycline resistance

Name of plasmid	Description	Source
pECXT99a	Tet^R, *C. glutamicum*/*E. coli* shuttle plasmid (Ptrc, *lac*I, pGA1, OriVCg)	Kirchner and Tauch 2003 [106]
pECXT99a-*dagA*	Tet^R, pECXT99a plasmid carrying the *dagA* gene from *S. coelicolor*	This work
pECXT99a-*dagAopt*	Tet^R, pECXT99a plasmid carrying *C. glutamicum* codon optimized *dagA* gene from *S. coelicolor*	This work
pECXT99a-*dagB*	Tet^R, pECXT99a plasmid carrying the *dagB* gene from *S. coelicolor*	This work
pECXT99a-*dagA-ahgA*	Tet^R, pECXT99a plasmid carrying *dagA* gene from *S. coelicolor* and *C. glutamicum* codon optimized *ahgA* gene from *Z. galactanivorans*	This work
pECXT99a-*dagAopt-ahgA*	Tet^R, pECXT99a plasmid carrying *C. glutamicum* codon optimized *dagA* gene from *S. coelicolor* and *ahgA* gene from *Z. galactanivorans*	This work
pECXT99a-*dagAopt-ahgA-dagB*	Tet^R, pECXT99a plasmid carrying *C. glutamicum* codon optimized *dagA* and *dagB* gene from *S. coelicolor* and *ahgA* gene from *Z. galactanivorans*	This work

Table 2.2: Name of bacterial strains constructed in this work

Name of bacterial strain	Description	Source
Corynebacterium glutamicum	wild type strain ATCC 13032, auxotrophic for biotin	Abe *et al.*, 1967[107]
Escherichia coli DH5α	Δ*lac*U169 (ϕ80*lacZ* ΔM15), *sup*E44, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1	Hanahan, 1983[108]
Corynebacterium glutamicum (pVWEx1-*galETKM-galP*)	*C. glutamicum* carrying the plasmid (pVWEx1-*galETKM-galP*) which enables galactose uptake and utilization	Frøystad, 2023[105]
EcEmpty	*E. coli* (pECXT99a)	This work
EcA	*E. coli* (pECXT99a-*dagA*)	This work
EcAopt	*E. coli* (pECXT99a-*dagAopt*)	This work
EcB	*E. coli* (pECXT99a-*dagB*)	This work
EcAA	*E. coli* (pECXT99a-*dagA-ahgA*)	This work
EcAAopt	*E. coli* (pECXT99a-*dagAopt-ahgA*)	This work
EcAABopt	*E. coli* (pECXT99a-*dagAopt-ahgA-dagB*)	This work
CgEmpty	*C. glutamicum* (pECXT99a)	This work
CgA	*C. glutamicum* (pECXT99a-*dagA*)	This work
CgAopt	*C. glutamicum* (pECXT99a-*dagAopt*)	This work
CgB	*C. glutamicum* (pECXT99a-*dagB*)	This work
CgAA	*C. glutamicum* (pECXT99a-*dagA-ahgA*)	This work
CgAAopt	*C. glutamicum* (pECXT99a-*dagAopt-ahgA*)	This work
CgAABopt	*C. glutamicum* (pECXT99a-*dagAopt-ahgA-dagB*)	This work
CgGalEmpty	*C. glutamicum* (pVWEx1-*galETKM-galP*)(pECXT99a)	This work
CgGalAAopt	*C. glutamicum* (pVWEx1-*galETKM-galP*)(pECXT99a-*dagAopt-ahgA*)	This work
CgGalAABopt	*C. glutamicum* (pVWEx1-*galETKM-galP*)(pECXT99a-*dagAopt-ahgA-dagB*)	This work

2.3 Primers

Table 2.3: Overview of the origin of the genes and the sizes of the fragments along with the primers used to construct the plasmids of this work. cDNA: codon optimized DNA; gDNA: genomic DNA.

Gene	Template DNA	Length PCR product	Plasmid	Annealing temperature [°C]	Primer name
dagA	*S. coelicolor* gDNA	1001	pECXT99a-*dagA*	59	dagAFw1
				59	dagARv1
dagAopt	*S. coelicolor* cDNA	1001	pECXT99a-*dagAopt*	59	dagAoptFw1
				59	dagAoptRv1
dagB1	*S. coelicolor* cDNA	1272	pECXT99a-*dagB*	62	dagB1
				62	dagB2
dagB2	*S. coelicolor* cDNA	1252		60	dagB3
				60	dagB4
dagA	*S. coelicolor* gDNA	1001	pECXT99a-*dagA-ahgA*	59	dagAFw2
				59	dagARv2
ahgA	*Z. galactanivorans* cDNA	1306		56	ahgAFw1
				56	ahgARv1
dagAopt	*S. coelicolor* cDNA	1001	pECXT99a-*dagAopt-ahgA*	59	dagAoptFw2
				59	dagAoptRv2
ahgA	*Z. galactanivorans* cDNA	1306		56	ahgAFw3
				56	ahgARv3
dagAopt-ahgA	pECXT99a-*dagAopt-ahgA*	2229	pECXT99a-*dagAopt-ahgA-dagB*	62	dagoptAahgAFw1
				62	dagoptAahgARv1
dagB	pECXT99a-*dagB*	2488		63	dagBoptFw1
				63	dagBoptRv1

Primers were used for amplification of genes via PCR. An overview of the amplification templates, the sizes of the amplified fragments, annealing temperatures along with the name of the primers used in this work to construct the plasmids is listed in Table 2.3. The name of the primers together with their respective sequence from 5' to 3' are listed in Table 2.4. The upmost primers in the table were used for HiFi PCR and the bottommost, for colony PCR. The different colors are used as a tool to distinguish the purpose of the different parts of the primer sequence. The black regions are the plasmid overlapping sequence, the red regions are the ribosomal binding site, the blue regions are the annealing sequence, the green regions are the linker sequence when multiple genes are cloned, and the brown regions are parts that overlap with another gene. All primers were ordered from Invitrogen by ThermoFisher Scientific and

resuspended with ionized water to gain a concentration of 100 μM followed by a 1:10 dilution before use. Clone Manager V9 (Bioinformatics tool, Scientific & Educational Software) was used for primer design.

Table 2.4: The sequence (5' $\longrightarrow$ 3') of the primes used for HiFi PCR (top) and for colony PCR (bottom) in this work given with their respective annealing temperature. Black region: the plasmid overlapping sequence. Red region: the ribosomal binding site. Blue region: the annealing sequence. Green region: linker sequence between genes. Brown region: overlapping sequence between two genes.

Name	Sequence (5' $\longrightarrow$ 3')
dagAFw1	ATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGGTCAACCGACGTGATCTCATC
dagARv1	GATCCTCTAGAGTCGACCTGCAGGCATGCTCACCTGTTGAGCACCAGAAG
dagAoptFw1	ATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGGTGAACCGCCGCGATCTGATC
dagAoptRv1	GATCCTCTAGAGTCGACCTGCAGGCATGCCTACACTGCCTGGTAGGTGCGCAC
dagAFw2	ATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGGTCAACCGACGTGATCTCATC
dagARv2	AACTGGGAGTACTTGTTCATCTGAAGGGCCTCCTTTCGGGGCGTTCGAA CTACACGGCCTGATACGTCCTGAC
ahgAFw1	GGACGTATCAGGCCGTGTAGTTCGAACGCCCCGAAAGGAGGCCCTTCAG ATGAACAAGTACTCCCAGTTCCT
aghARv1	TGCATGCCTGCAGGTCGACTCTAGAGGATCCTACTGCTTCTTCACGCCCTTTGC
dagAoptFw2	ATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGGTGAACCGCCGCGATCTGATC
dagAoptRv2	AACTGGGAGTACTTGTTCATCTGAAGGGCCTCCTTTCGGGGCGTTCGAA CTACACTGCCTGGTAGGTGCGCAC
ahgAFw3	GCACCTACCAGGCAGTGTAGTTCGAACGCCCCGAAAGGAGGCCCTTCAG ATGAACAAGTACTCCCAGTTCCTG
ahgARv3	TGCATGCCTGCAGGTCGACTCTAGAGGATCCTACTGCTTCTTCACGCCCTTTGC
dagB1	ATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGACCGTGCACAAGCGCGCATGC
dagB2	CTGTGCCACTGGGCCTGCCAGGGTATCTGGTGCGTAGCCGAAGCCTTCGCCTG
dagB3	ACCCGCGCAGGCGAAGGCTTCGGCTACGCACCAGATACCCTGGCAGGCCCAG
dagB4	GCCTGCAGGTCGACTCTAGAGGATCCTATTCTGCGGAGCGGCGGCCGG
dagBoptFw1	ATGGAATTCGAGCTCGGTACCCGGGGAAAGGAGGCCCTTCAGATGACCGTGCACAAGCGCGCATGC
dagBoptRv1	AGATCGCGGCGGTTCACCACCTGAAGGGCCTCCTTTCGGGGCGTTCGAA CTATTCTGCGGAGCGGCGGCCGG
dagoptAahgAFw1	CCGGCCGCCGCTCCGCAGAATAGTTCGAACGCCCCGAAAGGAGGCCCTTCAG GTGGTGAACCGCCGCGATCTGATC
dagoptAahgARv1	TGCATGCCTGCAGGTCGACTCTAGAGGATCCTACTGCTTCTTCACGCCCTTTGC
pECFw	TTTGCGCCGACATCATAACG
pECRV	TACTGCCGCCAGGCAAATTC

2.4 Cultivation

E. coli and *C. glutamicum* strains were routinely cultivated in the complex media LB or 2TY. Media was prepared according to the recipes given in Appendix A. Either LB or 2TY was used for liquid overnight cultivation, while plates were prepared using 2TY. When needed, the media was supplemented with 0.5 μ/mL of the appropriate antibiotics. Overnight incubation was performed at 37°C for *E. coli* and 30°C for *C. glutamicum* and *S. coelicolor*. The rotation was set to 225 rpm. For strains containing the pECXT99a plasmid, tetracycline was used, while for strains containing the pVWEx1 plasmid, kanamycin was used. In case of strains containing both plasmids, both antibiotics were added to the cultivation media.

2.5 Storage of bacterial strains

Glycerol stocks were prepared for storage of the bacterial strains constructed during this work. Freezing organisms in glycerol will stabilize the bacterial culture and prevent damage to the cell membrane, in addition to avoiding the formation of ice crystals that can dehydrate the bacteria[109]. For the preparation of glycerol stocks, colonies of the relevant bacterial strain were picked, inoculated in appropriate media, and incubated overnight. 670 μL of the overnight culture was mixed with 330 μL of 89% glycerol in 1 mL aliquots. All glycerol stocks were stored in the -80°C freezer of the model New Brunswick^{TM} Innovae C769 Ultra-Low temperature.

2.6 Sequencing

To validate the accuracy of DNA fragments assembled in the pECXT99a plasmid, they were sent for sequencing analysis. Plasmids isolated from successfully transformed *E. coli* DH5α cells were prepared for sequencing, following the prescribed guidelines for "LightRun Tubes" (Eurofins Genomics and GATC). In each sample tube, it was added 5 μL of DNA (50-100 ng/μL) to be sequenced along with 2.5 μL of sequencing primer and 2.5 μL MQ water. The sequencing process was conducted by Eurofins Genomics and GATC, utilizing the Sanger sequencing method. To evaluate the sequencing results, a multi-alignment of the DNA sequences against its template sequence was done in Clone Manager V9. In cases where specific mutations were identified in the inserted fragment, a BLASTp analysis of the translated protein sequence was carried out to identify if the mutation would impact the protein sequence. Furthermore, the integrity of promoter and ribosome binding site motifs was verified.

2.7 Molecular genetic techniques and strain construction

Standard molecular genetic techniques were carried out as described below. Molecular cloning uses a set of techniques to amplify and express DNA fragments. The fragment in question must be located, amplified, and purified before it can be ligated into the desired plasmid. In advance, the plasmid needs to be extracted, digested, and purified. Construction of the recombinant plasmid takes place inside the cloning host. After cloning, the recombinant plasmid can be transformed in an expression host and production can be evaluated. For the cloning, *E. coli* was used as the host. *C. glutamicum* was used as the expression host.

2.7.1 Isolation of genomic DNA

gDNA was isolated from *S. coelicolor* to have a genetic template for PCR amplification. For this purpose, the bacterium was cultivated in 5 mL liquid LB overnight, followed by centrifugation at 8000 rpm for 5 minutes. The NEB's monarch® Genomic DNA Purification was used to isolate the genomic DNA by following the Genomic DNA Purification Kit Protocol. All the solutions needed for the isolation were provided by the kit. To digest proteins and deactivate nucleases, the cell suspension was treated with Proteinase K before Ribonuclease was added to facilitate the degradation of RNA. Cell lysis buffer was added to lyse the cells, followed by binding the gDNA by introducing gDNA binding buffer. In a gDNA purification column, the

gDNA was washed with gDNA wash buffer and then eluted using 35-100 μL of gDNA elution buffer, which had been preheated to 50°C to enhance the yield of extracted gDNA.

2.7.2 Plasmid extraction

The *E. coli* strain containing the pECXT99a plasmid was cultivated overnight in 10 mL 2TY supplemented with 5 μL of tetracycline with a concentration of 5 mg/mL. The next day the culture was centrifuged at 8000 rpm for 5 minutes. Further, the ZR Plasmid miniprep™ from Zymo research was used for extraction of the plasmid DNA according to the Classic, Quick protocol. All the solutions needed for the extraction were provided by the kit. The sample was eluted with 30 μL MQ water. The concentration and purity of the sample containing the extracted plasmid was measured using the Microvolume UV-Vis Spectrophotometer NanoDrop™ One from ThermoFisher Scientific[110] where MQ water was used as blank.

2.7.3 Plasmid restriction

After extraction, the pECXT99a was linearized by the BamHI High Fidelity restriction enzyme as preparation for ligation with DNA fragments by Gibson assembly. A standard restriction mixture is shown in Table 2.5. Appropriate buffer and incubation temperature was found on a Performance Chart for NEB® Restriction Enzymes (NEB) to be the CutSmart™ Buffer and 37°C. The components of the mixture were mixed together, and the restriction enzyme was added lastly. The mixture was incubated at 37°C for 1.5 hour. Results after linearization was verified by gel electrophoresis.

Table 2.5: Reaction mixture for BamHI plasmid restriction of the pECXT99a plasmid.

Chemical component	Volume [μL]
CutSmart® Buffer	5
BamHI HiFi restriction enzyme	2
Plasmid DNA	10
MQ water	33
Final volume of the solution	50

2.7.4 Amplification of DNA fragments by Polymerase Chain Reaction (PCR)

Two different protocols were used, and both will be explained in the following section. All PCR programs were executed in a Mastercycler Nexus GX2 (Eppendorf). This method is based on the principle of repeated cycles of DNA denaturation, primer annealing, and DNA synthesis using a heat-stable DNA polymerase. This process exponentially amplifies a specific target DNA sequence, which enables the production of large quantities of DNA from a minimal starting amount[111]. The generated products were confirmed by gel electrophoresis after both cases of PCR procedures were performed.

High fidelity PCR

High-fidelity PCR is employed for applications that demand precise DNA amplification, such

as cloning, sequencing, and mutagenesis. In this study, the ColoneAmp^{TM} HiFi PCR premix Protocol-At-A-Glance from Takara[1] was followed. The recipe for the PCR tube solution and the thermal cycling conditions for this protocol is presented in Table 2.6. The CloneAmp^{TM} HiFi PCR Premix was premade. The duration of the extension step is dependent on the number of base pairs of the DNA sequence desired to amplify. Table 2.3 gives an overview of the origin of the genes, the sizes of the PCR products and annealing temperatures of the primers used in the HiFi PCR.

Table 2.6: Volume of the components in the reaction mix and the thermal cycle program for HiFi PCR according to the ColoneAmp^{TM} HiFi PCR premix Protocol-At-A-Glance from Takara[1] with a few adaptations in volume.

Reaction mixture component			**Volume [μL]**
CloneAmp^{TM} HiFi PCR Premix			12.5
Forward primer			0.5
Reverse primer			0.5
DNA template			1.0
MQ water			10.5
Total Volume			25
Step	**Temperature [°C]**	**Time [sec]**	**Number of cycels**
Denaturation	98	10	30-35
Annealing	55	15	30-35
Extention	72	5/kb	30-35
Soak	4	Indefinite	1

Colony PCR

Table 2.7: Volume of the components in the reaction mix and the thermal cycle program for colony PCR according to the GoTaq® PCR protocol from Promega[2].

Reaction mixture component			**Volume [μL]**
5x Green or colorless GoTaq® Reaction Buffer			5.0
Forward primer			0.5
Reverse primer			0.5
dNTPs			0.5
GoTaq®DNA Polymerase			0.2
MQ water			18.3
DNA template			-
Total Volume			25
Step	**Temperature [°C]**	**Time [min]**	**Number of cycles**
Initial denaturation	95	2	1
Denaturation	95	1	25-35
Annealing	55	1	25-35
Extention	72	1/kb	25-35
Final extention	72	5	1
Soak	4	Indefinite	1

Colony PCR offers the benefits of rapid and accurate detection and amplification of specific DNA or RNA fragments. In this study, the colony PCR was performed following the GoTaq® PCR protocol from Promega[2], ensuring reliable and efficient results.The procedure for the

PCR tube solution and the thermal cycling conditions for this protocol is presented in Table 2.7. The ingredients were mixed all together before being aliquoted in the PCR tubes. The duration of the extension step is dependent on the number of base pairs of the DNA sequence desired to amplify. The PCR tubes containing the reaction mix were inoculated with the DNA template by a sterile pipette tip.

2.7.5 Agarose gel electrophoresis

To evaluate and determine the length of DNA fragments, agarose gel electrophoresis was performed. Agarose gel electrophoresis is a technique for the separation of DNA or RNA fragments based on their size by using an electric field applied to an agarose gel matrix. Since DNA is negatively charged due to the phosphate backbone, the fragments migrate through the gel matrix towards the anode (positively charged electrode). Smaller DNA fragments experience less resistance and therefore move more quickly, resulting in their migration closer to the positive electrode. The electrophoresis was done as a checking point for PCR, DNA and plasmid extraction, plasmid restriction, Gibson assembly, cloning and transformations. The mobile and stationary phases consist of Tris-Acetate-EDTA (TAE) buffer and Agarose 0.8% with 20 μL GelRed and, respectively. 3 μL loading dye was mixed with 3 μL of the sample containing the DNA fragment to be evaluated by gel electrophoresis and loaded onto wells in the gel. To determine the size of the visual bands, the bands were compared to the standard DNA ladder Thermo Scientific^{TM} O'Generuler 1 kb DNA ladder, Ready-to-use-250-10,000 bp[112]. The resistance was set to 400 mA and the voltage 100 V. The gel electrophoresis was run for either 30 or 37 minutes depending on the size of the gel.

2.7.6 Purification of DNA fragments

To obtain pure DNA products, chemical components such as enzymes and buffers used in PCR and digestion of plasmids were removed by following a purification protocol. Both the PCR gene products and the restricted plasmids was purified following the QIAqick® PCR Purification Kit protocol from Qiagen. All solutions needed for the purification were provided by the kit. After purification, the concentration and purity of the sample was measured using the Microvolume UV-Vis Spectrophotometer NanoDrop^{TM} One from ThermoFisher Scientific[110]. MQ water was used as the blank for the NanoDrop and the samples were stored in the fridge until use.

2.7.7 Gibson assembly

To construct the expression plasmids the method of Gibson assembly was used. The DNA fragments of interest were assembled into linearized plasmids according to the protocol of Gibson et al.[113]. This protocol allows *in vitro* assembly of overlapping DNA regions which is created during PCR by DNA-specific primers. Precalculated volumes of plasmid and genes were added to 15 μL Gibson assembly Master-Mix containing the relevant enzymes, and the assembly is conducted in one isothermal reaction step at 50°C and 1 hour for optimal enzyme

activity. 5' exonuclease discloses the overlapping region to the complementary strand by hydrolyzing one DNA strand on each fragment. The complementary strands are annealed together by DNA ligase and gaps id filled by the action of DNA polymerase. The amount of plasmid and genes that need to be added to the Master-Mix depends on their respective concentration, as well as equimolar masses and length of the sequence in base pairs. The equimolar masses were calculated according to Equation 2.1. The isothermal cycle program was executed in a Mastercycler Nexus GX2 (Eppendorf).

$$\text{Insert quantity (ng)} = \text{Plasmid quantity (ng)} \cdot \frac{\text{Insert size (bp)}}{\text{Plasmid size (bp)}} \tag{2.1}$$

2.8 Construction of recombinant strains

In the field of molecular cloning, bacteria with high growth rates and efficient transformation capabilities are commonly employed as cloning hosts. *E. coli* DH5α, a genetically modified strain, is frequently utilized for this purpose. In this study, *E. coli* DH5α served as the general cloning host, and subsequently, the constructed plasmids were transformed into competent *C. glutamicum* cells through electroporation. Electroporation involves the application of an electrical pulse to transiently create pores in the cell membrane, enabling the uptake of exogenous DNA.

2.8.1 Preparation of competent *E. coli* cells

The preparation of chemically competent *E. coli* DH5α followed a protocol from Green and Rogers (2013), involving the use of media and buffers containing positive ions[114]. *E. coli* DH5α cells were cultured in 10 mL of Psi media overnight at 37°C and 225 rpm. From the pre culture, 1 mL was transferred to 100 mL of Psi media and grown until the optical density at 600 nm (OD_{600}) reached 0.40-0.43. The cells were then chilled on ice for 15 minutes and centrifuged at 8,000 rpm and 4°C for five minutes. After discarding the supernatant, the cell pellet was resuspended in 20 mL of Transformation buffer I (Tfb I) and incubated on ice for five minutes. Subsequently, the solution was centrifuged at 4,000 rpm and 4°C for five minutes. The resulting cell pellet was resuspended in 6 mL of Transformation buffer II (Tfb II) and divided into 100 μL aliquots. For long-term storage, the aliquots were flash-frozen using liquid nitrogen (N_2) and stored at -80°C.

2.8.2 Transformation of *E. coli* DH5α cells

Heat shock was done to enable the transformation of plasmids constructed by Gibson assembly in *E. coli* DH5α cells before further verification of the plasmids could be done. 10 μL of Gibson assembly reaction mix was added and mixed with competent *E. coli* DH5α cells that were thawed on ice. The cells were incubated on ice for 15 minutes before being heat shocked at 42°C for 1.5 minutes in an Eppendorf comfort thermomixer. After the heat shock, the cells were immediately transferred back to the ice to incubate for 1 minute. Further, 500 μL of LB

media was added to the cell cultures, before they were placed back in the thermomixer to incubate for 1 hour at 37°C and 450 rpm. After incubation, 100 μL of the culture was plated out on selective medium. The rest of the culture was centrifuged at 6000 rpm for 3 minutes before the supernatant was discarded and the pellet was plated out on selective medium. The plates were incubated overnight at 37°C. Single colonies from the plates were chosen for cloning verification via colony PCR and gel electrophoresis. The constructed plasmids were later sent to sequencing for confirmation before transformation in the expression host *C. glutamicum* took place.

2.8.3 Preparation of competent *C. glutamicum* cells

C. glutamicum competent cells were prepared to be used for strain construction as competent cells have a higher membrane permeability enabling the uptake of plasmids[114]. To make component cells, 5 mL of BHI was inoculated with wild type ATCC 13032 *C. glutamicum* cells and incubated overnight at 30°C. The morning after, 2 mL of the overnight culture was used to inoculate two 500 mL shake flasks containing 50 mL of BHIS solution. The cultures were further incubated at 30°C until an optical density (OD_{600}) of approximately 0.6 was reached. Then, 15 μL of ampicillin at a concentration of 5 mg/mL was added to the cultures, followed by further incubation at 30°C for 1-1.5 hours. After incubation, the cultures were centrifuged at 4000 rpm and 4°C for 7 minutes. The supernatant was then discarded, and the pellet was resuspended in 30 mL of EPB1 buffer. This centrifugation and resuspension process was repeated 3 times following the same procedure. Finally, the competent *C. glutamicum* cells were resuspended in 750 μL EPB2 buffer and 150 μL was aliquoted into 1.5 mL microcentrifuge tubes, which were stored at -80°C until further use.

2.8.4 Transformation of competent *C. glutamicum*

The constructed plasmids were transformed in competent *C. glutamicum* cells. One aliquot of the competent cells was thawed on ice before being mixed with 800 ng isolated plasmid DNA. The mix was transferred into a cold and dry electroporation cuvette before electroporation was executed in a single pulse at 2.5 kV, 25 Fv and 200Ω using GenePulser Xcell^{TM} (Bio-Rad). The suspension was then transferred to 46°C preheated 1 mL 2TY medium. The cultures were incubated at 46°C for 6 minutes, further one hour incubation at 30°C and 450 rpm in Eppendorf comfort thermomixer was followed. Lastly, the cells were plated out on selective medium and incubated for 1-2 days at 30°C.

2.9 Cultivation for experiments with the expression host

Experiments for evaluation of the growth of the constructed strains under different conditions were conducted to investigate three aspectes: the limiting concentration of agar in which CgEmpty is not able to grow; assessment of protein production and activity; the constructed strains' ability to degrade agar and utilize galactose as carbon source. For all experiments, minimal medium CGXII was used as flask media with different concentrations of glucose as

sole carbon source. To induce the cloned genes, the flask medium was supplemented with 1 mM IPTG. For overnight cultivation, each strain was cultivated in 500 mL shake flasks with 50 mL 2TY and 0.5 μL/mL of the appropriate antibiotics. The next day, OD_{600} measurements were taken to determine how much preculture was needed to inoculate three replicates of each strain with a starting OD_{600} of 1 approx. Before inoculation of flask media, the cells were washed in CGXII without carbon source in the following way. The measured amounts of cells were centrifugated at 4500 rpm for five minutes before the supernatant was removed and the cultures were resuspended and washed in 20 mL of CGXII. The cells were then centrifugated once more under the same conditions before the supernatant was removed and the inoculation of the flask media took place. The initial OD measurements were taken after inoculation before the flasks were placed on incubation. In all cases, growth experiments were performed in shake flasks in the New BrunswickTM Innovae 42® shaker incubator (Eppendorf) at 30°C and 150 rpm. The growth of the cells was tracked by preforming OD_{600} measurements. After ended growth experiment, all biomass calculations were done using the correlation: g/L biomass = $0.343 \cdot OD_{600}$ and growth rate calculation was done calculated in the exponential growth phase by construction an exponential fitted trend line[115]. Example calculations of this parameters is presented in Appendix B.

2.9.1 Toxicity test

CgEmpty strain was cultivated overnight and CGXII flask media with the following seven different concentrations of agar was prepared: 0.000 g/L, 0.025 g/L, 0.050 g/L, 0.075 g/L, 0.100 g/L, 0.200 g/L and 0.400 g/L. Three replicates of each of the strains was inoculated in the different agar concentrations aiming to reach a starting OD of 1. OD_{600} measurements was taken every second hour for the first ten hours of the experiments. The final OD_{600} samples were taken 24 and 26 hours. For the measurements, dilutions of the respective media were used as blank for the optical density measurements.

2.9.2 Protein activity assessment

The six constructed *C. glutamicum* strains containing the pECXT99a plasmid with inserted genes encoding for agarases in addition to a control strain with the empty plasmid (Table 2.2), were cultivated overnight. This was namely CgEmpty, CgB, CgA, CgAopt, CgAA, CgAAopt and CgAABopt. Three replicates of each strain inoculated different shake flasks containing CGXII flask media with 1% glucose. The cultures were then incubated at 30°C and 150 rpm for 26 hours with OD_{600} measurements taken every second hour for the first eight hours of the experiments. The final OD_{600} samples were taken at 24 and 26 hours. After 26 hours of growth in the flask media, the supernatants were collected after centrifuging the cultures in falcon tubes for 5 minutes at 8000 rpm to be used for further assessments.

2.9.3 Agar degradation test

To investigate the activity of the agarases produced by the constructed stains of *C. glutamicum*, an *in vivo* agar degradation assay was performed. Test tubes were filled with water containing

0.6% agar when the solution was warm. This was set to solidify with the test tubes tilted so that the agar would make an angled gel. 5 mL of the supernatants collected for protein assessments were added to the test tubes in order to assess the degradation of agar. The test tubes were placed in a Grant-bio PTR-35 Multi_Rotator at 30°C, which was set to reciprocal rotation with a 70° angle. The tubes were monitored for the first 10 minutes, but as no degradation was detected, they were checked every hour for three hours before being left to incubate overnight. After 24 hours, the supernatants were removed, and the visible degradation of the agar was visually assessed and photographed.

2.9.4 Bradford assay for the quantification of proteins

Bradford assay was used to evaluate the concertation of secreted proteins in the supernatants. A 1:10 dilution with Protein Assay Dye Reagent Concentrate (Bio-Rad) and MQ water was prepared and mixed with six different concentrations (0.0625;0.125;0.25;0.5;1;2 mg/ml) of Bovine Serum Albumin (BSA) in a 1:50 dilution to construct a standard curve for calculating the protein content of the collected supernatants. The cuvettes were incubated for 15 minutes in the dark at room temperature before optical density measurements (OD_{595}) of the known concentrations were performed. A linear trend line was fitted using the standard curve measurements as data points to calculate the protein concentrations in the supernatant extract, as demonstrated in Appendix B. Supernatants were mixed with the diluted Protein Assay Dye Reagent Concentrate in a 1:50 dilution followed by 15 minutes incubation on the dark. The optical density (OD_{595}) was measured using the Microvolume UV-Vis Spectrophotometer NanoDrop™ One from ThermoFisher Scientific [110]. The secreted protein concentration was calculated as shown in Appendix B.

3 Results

3.1 Evaluating *C. glutamicum* growth in the presence of agar

An agar toxicity assay was conducted to evaluate the growth of *C. glutamicum* on glucose-based minimal medium supplemented with different concentrations of agar. For this experiment, *C. glutamicum* transformed with plasmid pECXT99a, namely CgEmpty, was utilized. The objective was to ascertain the specific concentration of agar that would inhibit growth of the bacterium, providing a benchmark for future experiments. Figure 3.1 illustrates the growth curve of CgEmpty in CGXII flask media supplemented with 1% glucose, while employing seven different agar concentrations varying from 0.000 to 0.400 g/L.

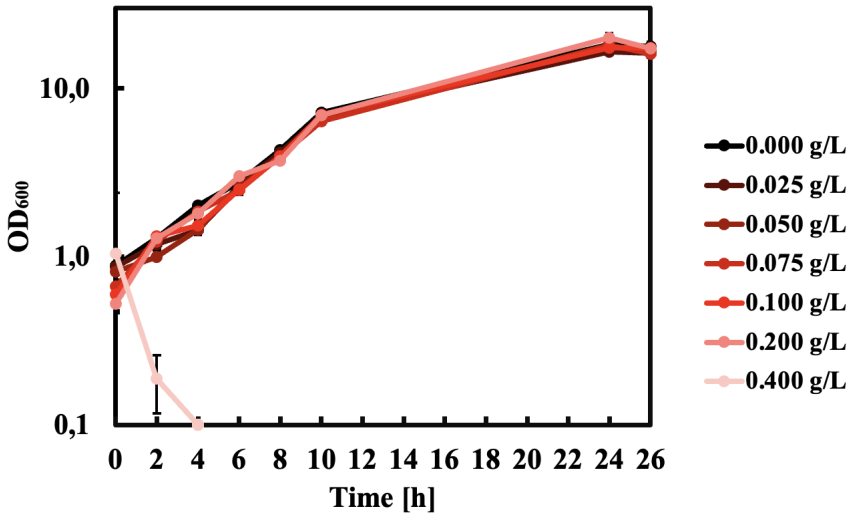


Figure 3.1: Growth of *C. glutamicum* (pECXT99a) in CGXII medium containing 1% glucose and varying concentrations of agar at 30°C. Values are presented as mean OD_{600} independent triplicates. Standard deviations are displayed as error bars.

Results of the calculated growth rates, final biomasses and biomass yields for the toxicity experiment are presented in Table 3.1. Example calculations of these parameters can be found in Appendix B.

Table 3.1: Growth rate, biomass and biomass yield calculated for growth of *C. glutamicum* (pECXT99a) in CGXII with 1% glucose and varying concentrations of agar. Final measurements for biomass calculations was taken after 26 h.

Agar concentration [g/L]	Growth rate [1/h]	Biomass [g/L]	Yield [g/g]
0.000	0.21 ± 0.00	6.07 ± 0.42	0.61 ± 0.04
0.025	0.21 ± 0.00	5.54 ± 0.12	0.55 ± 0.01
0.050	0.22 ± 0.00	5.73 ± 0.05	0.57 ± 0.01
0.075	0.22 ± 0.01	5.51 ± 0.29	0.55 ± 0.03
0.100	0.23 ± 0.02	5.94 ± 0.01	0.59 ± 0.00
0.200	0.24 ± 0.00	5.89 ± 0.00	0.59 ± 0.00
0.400	-	-	-

Based on the results obtained in this experiment, it is evident that CgEmpty strain is not able to grow when exposed to agar concentrations of 0.400 g/L and above in the growth medium. A notable observation, when considering standard deviation, it can be observed that the growth rates coincide for all lower agar concentrations, indicating that CgEmpty is able to thrive in media containing agar concentration of up to 0.200 g/L. The same overlapping trend is observed when examining the calculated biomasses, where all concentrations below 0.400 g/L exhibit overlapping within the standard deviations. At 0.400 g/L of agar, the cells did not show any growth following inoculation, and it was infeasible to detect any growth at four hours after inoculation. The media in the flask of 0.400 g/ agar was observed to have a remarkably high viscosity compared to the one at 0.200 g/L. As the cells did not grow at this concentration, this can indicate that the agar hindered even distribution of nutrients in the media. Out of the conditions tested in this experiment, 0.400 g/L was not a suitable concentration for *C. glutamicum* to grow. Therefore, it was decided to use 0.200 g/L for further experiments.

3.2 Establishment of agarases production in *C. glutamicum*

In order to establish production of agarases by *C. glutamicum*, the genes for the three agarases DagA, DagB and AhgA, namely *dagA, dagB* and *ahgA*, were assembled in the pECXT99a plasmid to create six different expression plasmids (Table 2.1). The tetracycline resistant pECXT99a plasmid was chosen as a shuttle plasmid as it can replicate in both *E. coli* and *C. glutamicum*. pECXT99a has an IPTG inducible promoter that ensures simultaneous expression of the cloned genes.

3.2.1 Amplification of agarase genes from *S. coelicolor* and *Z. galactanivorans*

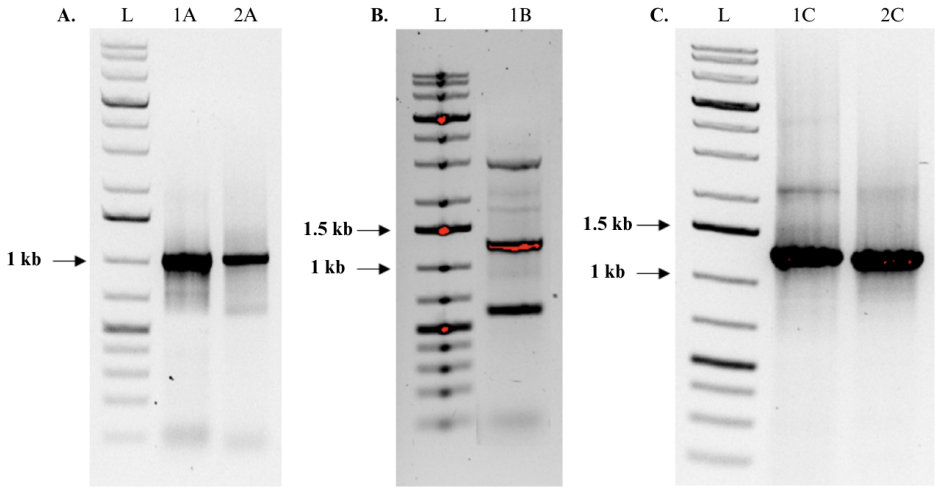


Figure 3.2: Agarose gel electrophoresis displaying PCR products obtained by performing HiFi PCR according to ColoneAmp™ HiFi PCR premix Protocol-At-A-Glance from Takara[111]: A. *dagA* (1001 bp) and *dagAopt* (1001 bp) in well 1A and 1B, respectively. B. *ahgA* (1306 bp) in well B1. C. *dagB1* (1272 bp) and *dagB2* (1252 bp) in well 1C and 2C, respectively. The wells denoted "L" contains the Thermo Scientific™ O'Generuler 1 kb DNA ladder[112].

The genes encoding for the three agarases were PCR amplified with primers that generated overlapping regions with other genes and the restricted pECXT99a plasmid (Table 2.4). The gene *dagA* (1001 bp) was amplified from genomic DNA while *dagAopt* (1001 bp) was amplified from codon optimized synthetic DNA. Amplified *dagA* and *dagAopt* are shown in Figure 3.2 A. in wells 1A and 2A, respectively. *ahgA* (1306 bp) is shown in Figure 3.2 B. in well 1B, while the two fragments of *dagB*, named *dagB1* (1272 bp) and *dagB2* (1252 bp), are displayed in Figure 3.2 C. in wells 1C and 2C. "L" is the symbol for the ladder. Despite the inability to achieve a single band for *ahgA*, due to the presence of a correctly sized concentrated band, further efforts were made to conduct colony PCRs. This yielded success which was subsequently validated through sequencing analysis.

3.2.2 Verifying the successfully constructed plasmids carrying agarases genes

Six plasmids (Table 2.1) were assembled by Gibson Assembly before being transformed to competent *E. coli* DH5α cells. Plasmid maps can be found in Appendix D. Successful transformation of the respective plasmids was verified by colony PCR. The pECXT99a-(*dagB*) plasmid was expected to show a 2524-sized band and the verified positives are shown in Figure 3.3 A. A band of 1001 bp was expected for both the pECXT99a-(*dagA*) and pECXT99a-(*dagAopt*) plasmids which can be seen in Figure 3.3 B. and C., respectively. The pECXT99a-(*dagA-ahgA-dagB*) was expected to show a band of the size of 4717 bp while bands measuring 2307 bp indicated the presence of both the pECXT99a-(*dagA-ahgA*) and pECXT99a-(*dagAopt-ahgA*) plasmids. These observations are illustrated in Figure 3.4 A., B., and C., respectively.

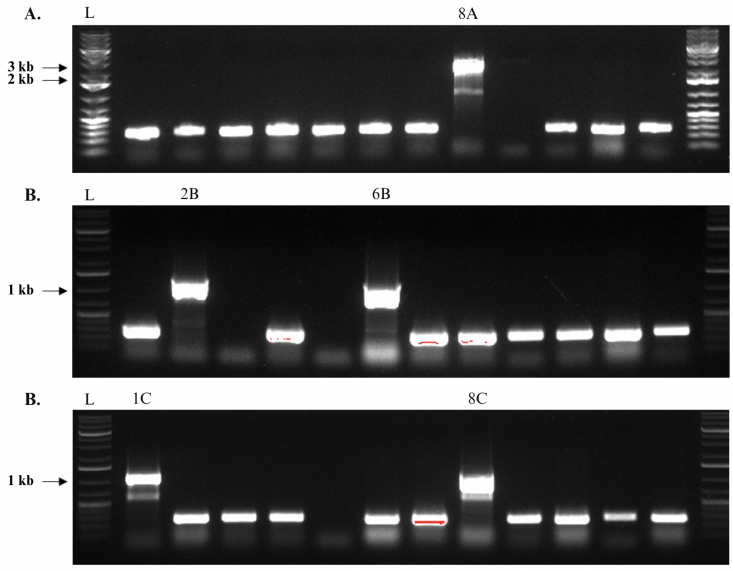


Figure 3.3: Agarose gel electrophoresis displaying colony PCR products representing positive clones of: A. *E. coli* (pECXT99a-*dagB*) of 1001 pb (well 8A), B. *E. coli* (pECXT99a-*dagA*) of 1001 bp (well 2B and 6B), C. *E. coli* (pECXT99a-*dagAopt*) of 1001 bp (well 1C and 6B). The wells denoted "L" contains the Thermo Scientific™ O'Generuler 1 kb DNA ladder[112].

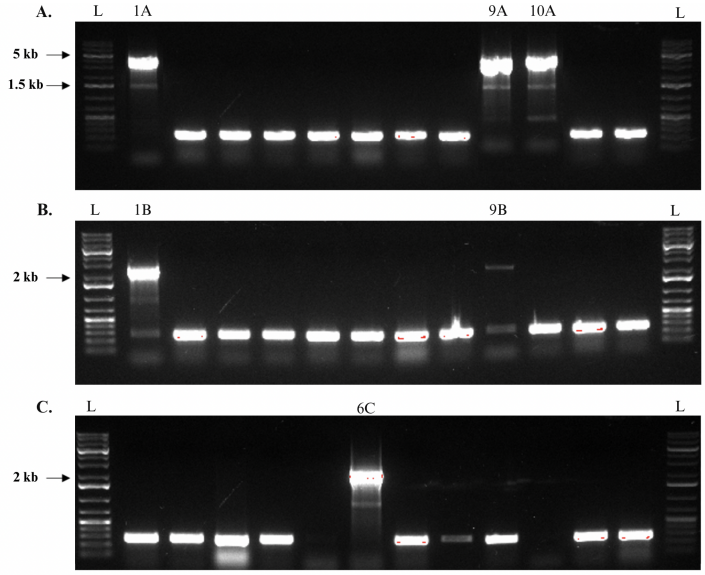


Figure 3.4: Agarose gel electrophoresis displaying colony PCR products representing positive clones of: A. *E. coli* (pECXT99a-*dagA-ahgA-dagB*) of 4717 pb (well 1A, 9A and 10A), B. *E. coli* (pECXT99a-*dagA-ahgA*) of 2307 bp (well 1B and 9B), C. *E. coli* (pECXT99a-*dagAopt-ahgA*) of 2307 bp (well 6C). The wells denoted "L" contains the Thermo Scientific™ O'Generuler 1 kb DNA ladder[112].

Sequencing analysis was employed to assess the sequences of the cloned agarase genes and to evaluate the presence of any deleterious mutations that could hinder gene expression or compromise protein functionality. After verification, a selected clone of each plasmid was transformed into competent *C. glutamicum* and *C. glutamicum* (pVWEx1-*galETKM-galP*) cells. The constructed bacterial strains were further used in experiments described in the following sections to study the expression of agarases for degradation of agar and for utilization of agar-derived galactose as carbon source in *C. glutamicum*.

3.3 *C. glutamicum* is able to produce and secrete agarases as well as digest agar under certain conditions

After the construction of *C. glutamicum* strains harboring plasmids expressing the cloned agarase genes was accomplished, a comprehensive analysis was performed to assess the growth characteristics of the strains, and the extracellular protein secretion patterns and enzymatic activities of the expressed and secreted proteins. This was performed by conducting growth experiments as outlined in subsection 2.9 on the seven strains, namely CgEmpty, CgA, CgAopt, CgB, CgAA, CgAAopt and CgAABopt.

3.3.1 Testing the growth performance of newly constructed strains

The strains were cultivated in CGXII medium supplemented with 1% glucose. After 26 hours of growth, the supernatants were collected for further experimentation. The growth curve of the seven strains is shown in Figure 3.5.

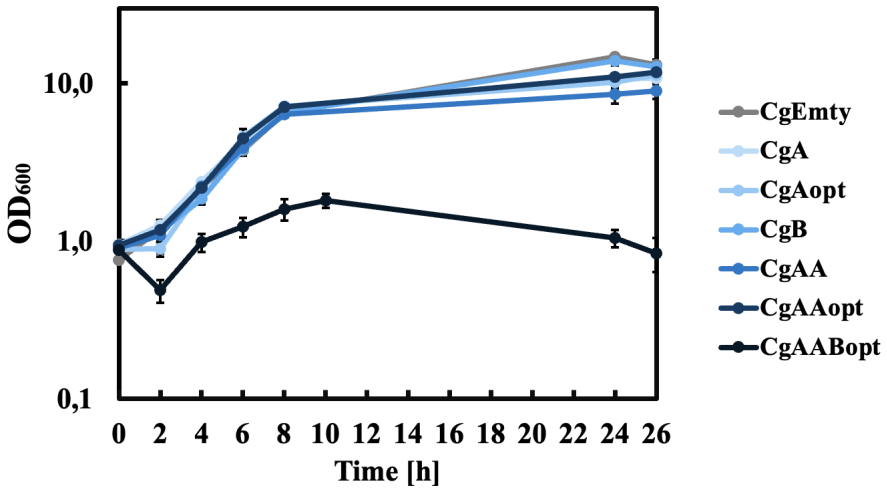


Figure 3.5: Growth of seven different *C. glutamicum* strains engineered for the production of agarases with genes from *S. coelicolor* and *Z. galactanivorans* at 30°C in CGXII medium supplemented with 1% glucose. Values are presented as mean OD_{600} of independent triplicates and standard deviations are displayed as error bars.

The calculated growth rate, final biomasses and biomass yields are presented in Table 3.2. The growth rate calculations were performed based on the exponential phase. An example of how these parameters were calculated is provided in the Appendix B.

Table 3.2: Growth rate, biomass and biomass yield calculated for seven different *C. glutamicum* strains harbouring genes encoding agarases from *S. coelicolor* and *Z. galactanivoras*. The parameters was calcuated after conducting an agar growth experiment in CGXII flask media suppelementet with 1% glucose. Final measurements for biomass calculations was taken after 26 h.

Name of strain	Growth rate [1/h]	Biomass [g/L]	Yield [g/g]
CgEmpty	0.28 ± 0.00	4.51 ± 0.02	0.41 ± 0.00
CgA	0.29 ± 0.02	3.70 ± 0.33	0.37 ± 0.03
CgAopt	0.35 ± 0.02	3.88 ± 0.24	0.39 ± 0.02
CgB	0.30 ± 0.01	4.39 ± 0.15	0.44 ± 0.01
CgAA	0.30 ± 0.05	3.08 ± 0.35	0.31 ± 0.03
CgAAopt	0.31 ± 0.03	4.04 ± 0.01	0.40 ± 0.00
CgAABopt	0.11 ± 0.01	0.36 ± 0.05	0.04 ± 0.00

With the exception of CgAABopt, the growth characteristics of the agarase expressing strains closely resembled those of the control, CgEmpty. The growth behavior of the stains harboring *dagA* or *dagAopt* alone, or in combination with *ahgA*, did not deviate significantly from CgEmpty. The same was the situation for CgB, carrying only *dagB*. However, combining all three genes in CgAABopt clearly impaired its growth, resulting in a reduced growth rate of 0.11 ± 0.01 h^{-1}, which is 2.5-fold lower than the growth rate of the negative control at 0.28 $\pm$ 0.00 h^{-1} (Table 3.2). CgAABopt displayed a shortened exponential phase and a noticeable decline in biomass after 26 hours of growth of 44%. In contrast, all other strains, exhibited higher growth rates compared to the control strain, albeit with a lower final biomass. Notably, CgB is the only strain showing overlapping biomass values with the control when considering

standard deviations. For the strains overexpressing optimized or non-optimized *dagA* alone or together with *ahgA*, there is a decrease in the biomass which can be an indication of a redirection of the carbon flux towards protein production, in this case, agarases.

3.3.2 Measuring secreted proteins by the engineered *C. glutamicum* strais

The Bradford assay method (Section 2.9.4) was employed to examine the secreted protein concentration in the collected supernatants of the strains CgEmpty, CgA, CgAopt, CgB, CgAA, CgAAopt, and CgAABopt. The findings of this analysis are presented in Figure 3.6, depicting the secretion of proteins in milligrams per milliliter relative to grams of cell dry weight [mg/ml · gCDW].

The Bradford assessment showed that the control CgEmpty, with a secreted protein level of 0.007 ± 0.001 mg/ml · gCDW protein secretion, exhibited a lower level of secreted protein in the supernatant compared to the engineered strains, except for CgAABopt. When accounting for standard deviations, CgAABopt displayed a similarly low quantity of secreted proteins in its supernatant with a level of 0.005 ± 0.001 mg/ml · gCDW. In contrast, all other strains demonstrated significantly higher concentrations of secreted proteins, with CgB exhibiting the lowest value among them at 0.023 ± 0.002 mg/ml · gCDW. According to the assessment, the strains CgA, CgAopt, CgAA and CgAAopt, show a 4.5-fold more secreted proteins than the control. This was also the strains that had a decrease in biomass.

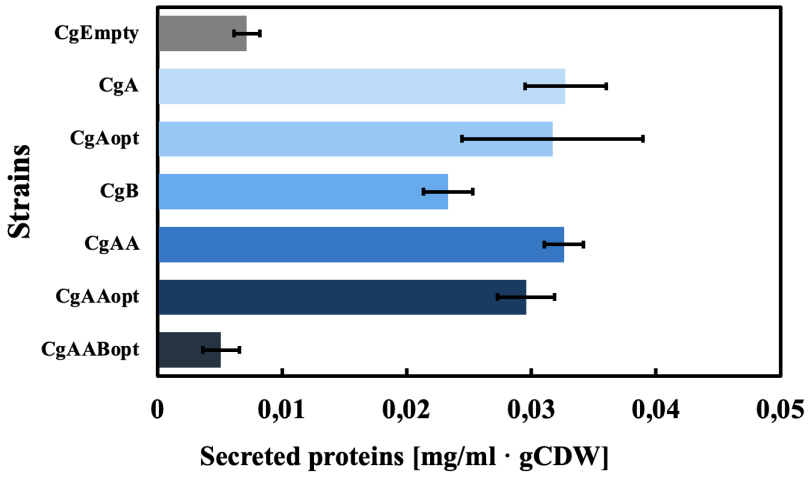


Figure 3.6: Measurement of secreted proteins of *C. glutamicum* strains engineered for agarase production by Bradford assay. Supernatant was collected after 24 hours of growth in CGXII supplemented with 1% glucose. Values are presented as mean OD_{600} independent triplicates and standard deviations are displayed as error bars.

3.3.3 Agar viscosity test for detecting *in vivo* extracellular agar-degrading activity

An *in vivo* viscosity test was executed to evaluate agar degradation activity. After 24 hours of growth at 30°C in CGXII media supplemented with 1% glucose, three replicates of each strain were examined by adding their respective supernatants to test tubes containing 0.6% agar that had been solidified in an angle of approximately 30°. If agar depolymerization was insufficient the solidified agar remained in the constructed shape; conversely, upon agar depolymerization, the gel would start to decompose, and a viscous solution would be found in the bottom of the tube. The findings are presented in Figure 3.7 with the control strain placed to the left, and Table 3.3 provides an assessment of the degradation for each test tube.

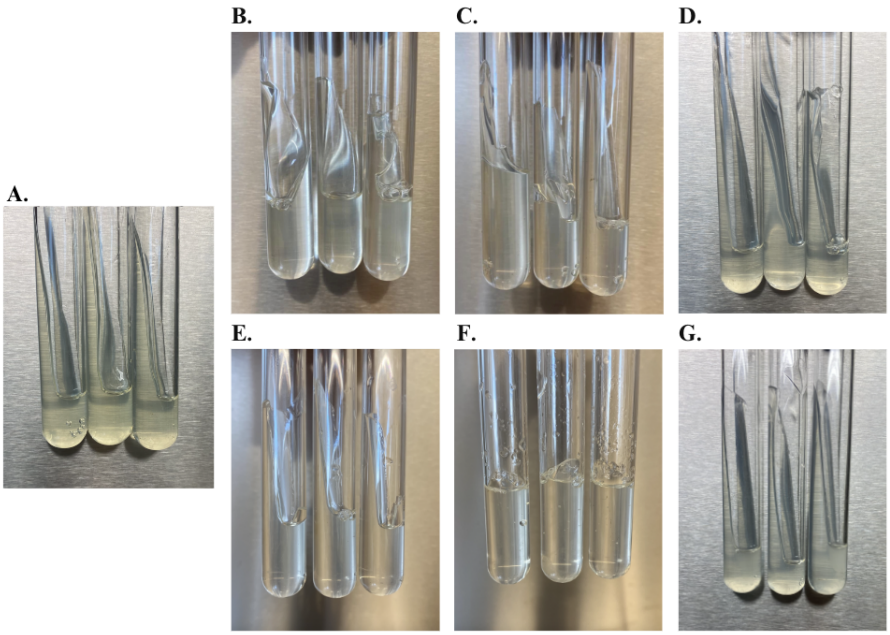


Figure 3.7: Extracellular degradation of agar by *C. glutamicum* containing the pECXT99a plasmid with different combinations of the agarases DagA and DagB from *S. coelicolor*, and AhgA from *Z. galactanivorans*. Supernatants of the strains was collected 26 h after inoculation: **A.** Control strain with empty pECXT99a plasmid, **B.** DagB, **C.** DagA, **D.** DagAopt, **E.** DagA and AhgA, **F.** DagAopt and AhgA, **G.** DagAopt, DagB and AhgA.

Table 3.3: Results of the agar viscosity test for detecting *in vivo* extracellular agar degrading activity where (-) means no degradation observed, (+) indicates some observed degradation and (++) signifies enough degradation observed for the agar to lose the jellified shape.

Strain	Replicate		
	1	2	3
Control	-	-	-
C. glutamicum (pECXT99a-*dagA*)	-	-	+
C. glutamicum (pECXT99a-*dagAopt*)	-	+	+
C. glutamicum (pECXT99a-*dagB*)	-	-	-
C. glutamicum (pECXT99a-*dagA-ahgA*)	+	+	+
C. glutamicum (pECXT99a-*dagAopt-ahgA*)	++	++	++
C. glutamicum (pECXT99a-*dagAopt-ahgA-dagB*)	-	-	-

CgAAopt exhibited the highest level of successful agar degradation, with high level of degradation in all three test tubes after 24 hours. Among the strains containing optimized or non-optimized *dagA* alone, or in combination with *ahgA*, all displayed at least one replicate with notable levels of degradation. It is evident that the optimized version of the gene *dagA* demonstrates enhanced efficiency compared to the non-optimized version, as it exhibits greater visible degradation capacity in both cases. Conversely, supernatants collected from strains harboring *dagB* showed no signs of degradation and closely resembled the control test tube. Even the combination of *dagB* with *dagAopt-ahgA* failed to exhibit any degradation. This observation may suggest potential issues with the *dagB*-gene, such as possible interference with the secretion system due to misfolding or improper folding. On account of showing the most efficient agar degradation, the combination same combination of genes as inn CgAAopt was used in further experiment for galactose utilization. Even though CgAABopt did not show any signs of degradation, this gene combination was also included in further experiments.

3.4 Establishment of galactose consumption in *C. glutamicum* as degradation product from agar

The strain *C. glutamicum* (pVWEx1-*galETKM-galP*) was engineered to enable galactose utilization by *C. glutamicum*. This strain, constructed by Frøystad M. (2023), was used as a platform strain in this work[105]. Consequently, the plasmids (pECXT99a-*dagAopt-ahgA*) and (pECXT99a-*dagAopt-ahgaA-dagB*) in addition to the empty pECXT99a plasmid were transformed into this strain yielding the strains *C. glutamicum* (pVWEx1-*galETKM-galP*)(pECXT-99a-*dagAopt-ahgA*), *C. glutamicum* (pVWEx1-*galETKM-galP*) (pECXT99a-*dagAopt-ahgaA-dagB*) and *C. glutamicum* (pVWEx1-*galETKM-galP*)(pECXT99a), namely CgGalAAopt, CgGalAABopt and CgGalEmpty, respectively. To assess the newly constructed strains for agar utilization, the growth performance was monitored, and the concentration of secreted proteins was measured.

To analyze the growth behavior of CgGalEmpty, CgGalAAopt and CgGalAABopt, they were cultivated in CGXII flask media supplemented with 0.2% agar as primary carbon source and varying concentrations of glucose as supportive carbon source. The glucose concentrations used were 0%, 0.2% and 1%, and the growth curve for each experiment is shown in Figure 3.8 A., B., and C., respectively.

In the case of growth on agar without glucose, the absence of observable growth suggests an inability of the constructed strains to utilize agar as sole carbon source. The slight increase in optical density observed might be attributed to evaporation rather than metabolic activity. When cultured CGXII media supplemented with 0.2% glucose, all three strains display a similar growth behavior. However, their growth is primarily owing to glucose consumption rather than agar utilization, as the strains CgGalAAopt and CgGalAABopt exhibit behavior akin to the control strain, CgGalEmpty. Notably, during the exponential growth, CgGalAAopt demonstrates a potentially higher growth rate. Due to logistical and temporal constraints, further measurements were not conducted in the following hours of the exponential phase.

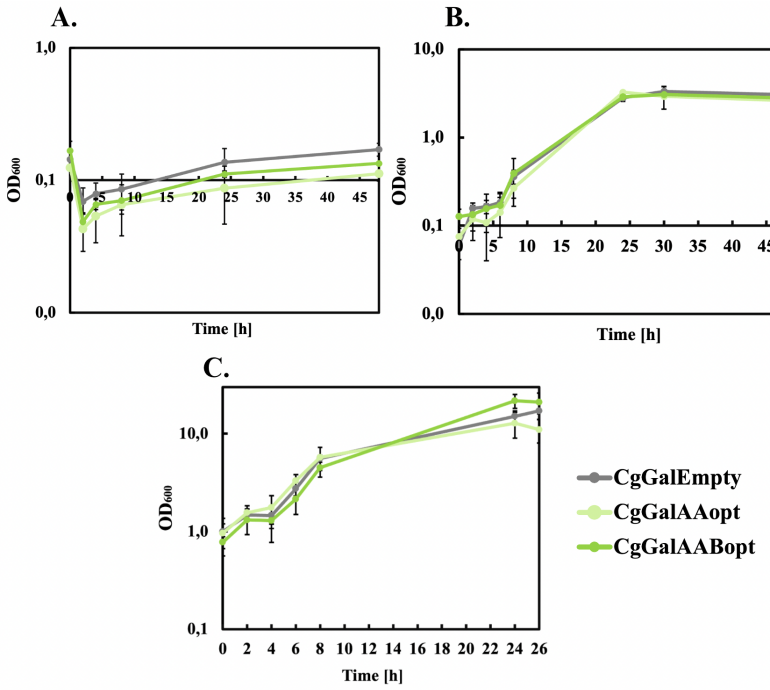


Figure 3.8: Growth curves of CgGalEmpty, CgGalAAopt and CgGalAABopt in CGXII media supplemented with 0.2% agar and different amount of glucose for evaluation of galactose utilization in *C. glutamicum*: A. 0% glucose B. 0.2% glucose C. 1% glucose.

Nevertheless, based on the predicting data point prior to reaching the stationary phase, it appears that CgGalAAopt may exhibit a higher growth rate. Under condition with 1% glucose supplemented to the flask media, the growth performance show similarities to the 0.2% glucose conditions. The three strains exhibit comparable growth behavior, making it challenging to conclusively determine whether galactose is being utilized or not. Likewise, CgGalAAopt show a tendency to have a higher growth rate, yet lack of optical density measurements precludes drawing definitive conclusions. In the absence of glucose supplementation, the strains show overlapping biomass values of approximately 0.5 g/L, taking into consideration the calculated standard deviations. This is a 2-fold lower than biomass values obtained under 0.2% glucose supplementation with values of 1.04 $\pm$ 0.06 g/L, 0.90 $\pm$ 0.46 g/L and 0.96 $\pm$ 0.48 g/L for CgGalEmpty, CgGalAAopt and CgGalAABopt, respectively. Furthermore, growth in the absence of glucose is more than a 10-fold lower in biomass compared to growth under the presence of 1% glucose, with biomass values of 5.17 $\pm$ 0.70 g/L, 4.30 $\pm$ 1.30 g/L and 7.44 $\pm$ 1.22 g/L, presented in the same order as previously stated. Under the latter conditions, the constructed strains exhibit a remarkably high standard deviation. Based on these assessments, it remains inconclusive whether successful construction of *C. glutamicum* strains that can utilize agar as carbon source has been achieved.

After cultivation of the strains CgGalEmpty, CgGalAAopt and CgGalAABopt in CGXII media containing 0.2% glucose, supernatants were collected for measurement of protein concentration by Bradford assay. Figure 3.9 depicts the results presented as secretion of proteins in

milligrams per milliliter relative to grams of cell dry weight [mg/ml · gCDW].

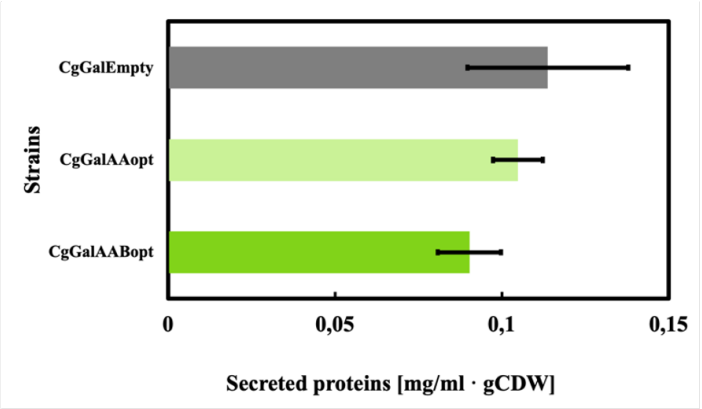


Figure 3.9: Measurement of secreted proteins of *C. glutamicum* strains engineered for agarase production by Bradford assay. Supernatant was collected after 24 hours of growth in CGXII supplemented with 1% glucose. Values are presented as mean OD_{600} independent triplicates and standard deviations are displayed as error bars.

Taking into consideration the standard deviations, the quantity of secreted proteins is similar in all three cases, with values of 0.090 ± 0.009 mg/ml · gCDW, 0.105 ± 0.007 mg/ml · gCDW and 0.114 ± 0.024 mg/ml · gCDW for CgGalEmpty, CgGalAAopt and CgGalAABopt, respectively. Hence, based on this assessment, it was not observed any indication of agarases being secreted.

The carbon source that was available in the culture conditions tested was not enough to observe significant differences between secreted protein concentrations. Nevertheless, the digestion of agar was visually confirmed. After a cultivation period of 48 hours in CGXII media supplemented with 0.2% glucose, culture samples of 1 mL of each replicate were transferred to an Eppendorf tube and centrifuged for 5 minutes at 4.500 rpm. Figure 3.10 illustrates the isolated cultures in the following order: A. the control condition, B. CgGalAAopt, and C. CgGalAABopt.

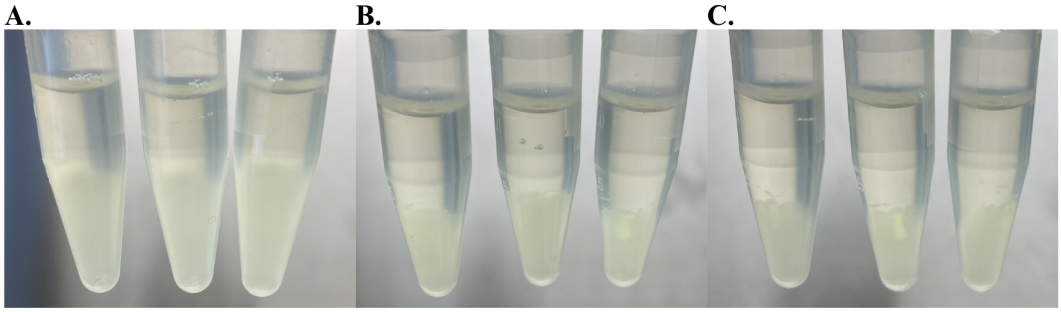


Figure 3.10: Collected supernatants after cultivation of *C. glutamicum* strains engineered to degrade and utilize agar as sole carbon source in CGXII supplemented 0.2% agar and 0.2% glucose. The supernatants were centrifuged at 4.500 rpm for 5 minutes. A. CgGalEmpty B. CgGalAAopt C. CgGalAABopt.

A higher volume of pellet and higher turbidity was observed in the culture obtained from the control replicates. In this case, the pellet consists of agar and cell mass. The volume of the pellet is observed to be approximately half the size of the strains constructed for agarase degradation and consumption. Due to the differences, one can conclude that degradation of agar took place, but the utilization of agar as carbon source could not be demonstrated in this work. Furthermore, this test supports the fact that the strain constructed to overexpress *dagB* seems to work despite previously collected data. Nevertheless, as the volume extracted from the cultures was equal and and the biomass values overlap in all three cases, one can conclude that the volume difference of the supernatants in Figure 3.10 is due to agar degradation.

4 Discussion

4.1 *C. glutamicum* as host for production of agarases

In the field of contemporary industrial biotechnology, glucose-based feedstocks stand as the predominant choice for production of value-added compounds[7]. However, the utilization of sugars in bacterial fermentations requires further assessment as exploitation of alternative carbon sources has the promising potential to provide a broader production diversity, minimize costs, and improve sustainability[6;7]. Furthermore, the use of sugars derived from terrestrial crops for biotechnology processes such as glucose, fructose or sucrose directly competes with the usage of resources for the production of human and animal nutrition, resulting in increased prices and lower availability of those resources[7;8]. Consequently, it is necessary to identify and employ non-food compounds as alternative fermentation substrates, thereby addressing the objectives of sustainability, economic efficiency, and production yields and hence, complying with several Sustainable Development Goals defined by the United Nations[116].

Seaweeds, as the third generation of feedstocks, represent a promising alternative to land-derived sugars as renewable substrates for microbial-based production owing to its abundance, high growth rate, low lignin content and environmentally friendly nature[117;14;39]. The multifarious macroalgae contain diverse polysaccharides that possess novel chemical and bioactive properties to be exploited. The most commercialized ones include alginate and laminarin, from brown seaweed, and carrageenan and agar, found in red seaweed[25]. The latter is an easily accessible polysaccharide that holds promising industrial applications in the food and pharmaceutical industry[36], besides being used for biotechnological purposes due to its gelling properties[37]. The chemical structure of agar is mainly built up of 1,3-linked β-D-galactose (G) and 1,4-linked 3,6-anhydro-α-L-galactose (ALG). Therefore, the complete degradation of agar results in the formation of D-galactose and 3,6-anhydro-L-galactose that can be utilized by specialized microorganisms[51;53]. For instance, organisms such as *Lactococcus lactis* subsp. *cremoris, Zymomonas mobilis* and *Escherichia coli* have the ability to metabolize galactose[7;40;103], and the genus *Saccharophagus* and *Streptomyces* accommodate an array of microorganisms such as *Saccharophagus degradans* and *Streptomyces coelicolor* that can degrade agar[40;118]. To develop sustainable production lines of value-added chemicals from seaweed-based feedstocks, metabolic engineering can be applied to commonly used industrial workhorses. This will prevent the need to develop new knowledge, equipment and methodology for non-commonly used bacteria harboring native abilities for agar utilization, in addition, to secure effectivity in the form of yield and cost.

The microbial host of this project, *C. glutamicum* is typically used in large-scale production of amino acids, especially L-lysine and L-glutamate, whose market surpasses 6 million tons per year[119]. With the applications of novel genome editing techniques and the advancement in system and synthetic biology tools, the repertoire of *C. glutamicum* has expanded significantly from food and feed to include the chemical industry, cosmetics, and healthcare[119]. However, despite its versatility, *C. glutamicum*, lacks the inherent capability of utilizing seaweed-based carbohydrates which includes agar. Nevertheless, it was chosen as the expression host for this

project as it has proven to be a robust workhorse that easily allows genetic engineering necessitating to enable this property. *C. glutamicum* has been extensively metabolically engineered in numerous studies for several purposes as for instance to establish the expansion of its product portfolio towards bio-based industrial production and to enable non-traditional substrates as alternative carbon sources[120;115;7;88]. For instance, *C. glutamicum* has been engineered to utilize the plant-based carbohydrate starch as sole carbon source to produce L-lysine by heterologous expression of the α-amylase gene from *Streptococcus bovis*[121]. *C. glutamicum* has also been modified for growth on lignocellulosic pentoses such as xylose and arabinose by the implementation of *xylA* and *araBAD* genes from *Xanthomonas campestris* and *E. coli*, respectively[7]. Additionally, metabolically engineered *C. glutamicum* strains have successfully degraded cellulosic compounds such as cellulose and cellobiose by insertion of the *cpbA* gene from *Clostridium cellulovorans*[7]. Glucose, galactose, xylose, and arabinose are sugar monomers can be found in various seaweed species[122]. Previous research has also successfully introduced both lactose- and galactose-metabolizing genes from lactic acid bacteria into a L-lysine-overproducing *C. glutamicum* strain for growth on galactose[95]. Figure 4.1 provides a simplified illustration of relevant pathways implemented in *C. glutamicum* for the utilization of non-native carbon sources. Remarkably, studies by Jeon et al. (2021) have successfully engineered *C. glutamicum* strains for the production of neoagarobiose, a rare anti-melanogenesis reagent and moisturize reagent form agar, by expression of two β-agarases from *S. coelicolor* and *Gilvimarinus chinensis*[123]. Considering these examples, *C. glutamicum* has shown a potential to become a competitive expression host for polysaccharide-specific enzymes for degradation and usage of monosaccharides as an alternative carbon source.

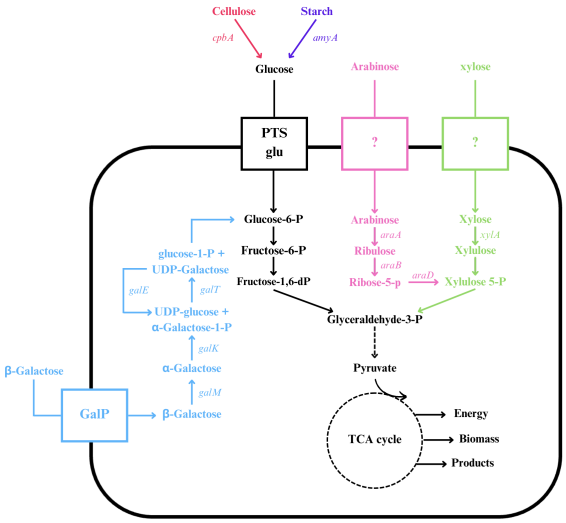


Figure 4.1: Simplified illustration of relevant pathways implemented in *C. glutamicum* for the utilization of non-native carbon sources. Xylose pathway (green): *xylA*, xylose isomerase. Arabinose pathway (pink): *araA*, arabinose isomerase; *araB*, ribulokinase; *araD* ribulose 5-phosphate 4-epimerase. Galactose pathway (blue): *galM*, aldose-1-epimerase; *galK* galactokinase; *galT*, UDP-glucose-1-P-uridylyltransferase; and *galE*, UDP-galactose-4-epimerase. Cellulose pathway (red): *cpbA*, scaffolding protein. Starch pathway (purple): *amyA*, α-amylase[7].

4.2 Establishing production of agarases in *C. glutamicum*

Seaweed fosters a diverse and abundant microbial community, encompassing archaea, bacteria, fungi, microalgae, protozoa, and viruses. This intricate symbiotic society is characterized by mutualistic interactions, where microorganisms and seaweed depend on each other for their respective growth, development, and maturation[124]. Consequently, these microbes have adapted to grow on seaweed and can utilize its structural and storage carbohydrates as carbon sources[124]. These unique adaptations possess potential to be exploited for biotechnological applications. For instance, *Saccharina japonica*, a species of brown seaweed, was used as a substrate for *Monascus purpureus* for the production of the cholesterol-lowering drug, lovastatin[125]. The same seaweed species has also been used to manufacture value-added chemicals such as cadaverine and C30 terpenoids by the use of the methylotrophic bacteria *Bacillus methanolicus*[126]. In another example, the red seaweed species *Gracilaria* has been fermented by *Lactobacillus acidophilus* and *Lactobacillus plantarum* for the production of lactic acid[127]. Additionally, *C. glutamicum* was engineered to enable access to mannitol as sole carbon source by using the mannitol-specific phosphotransferase system from *Bacillus subtilis*. Next, hydrolysate from the brown seaweed from *Laminaria hyperborea* containing mainly mannitol and glucose was used to grow the new *C. glutamicum* strain in flask and bioreactor fermentations while overproducing riboflavin[115].

The majority of microorganisms able to hydrolyze and metabolize agar as carbon and energy sources have been identified to live on different red seaweeds in seawater and marine sediments[40]. The production of agarases have been documented in a diverse array of microbes and is typically linked to degradation of the related polymer carrageenan, another major constituent of red seaweed. For instance, κ-Carragenases have emerged from β-agarases and so they belong to the same glycoside hydrolase family[128]. Hence, several microorganisms that degrade agar are additionally capable of degrading carrageenan, such as *Pseudoalteromonas* and *Zobellia*[129]. This highlights the natural occurrence of carbohydrate degradation in nature. Genetic engineering of industrial organisms has exploited this property in various fields. For instance, *E. coli* has in several studies been engineered for recombinant agarase production[130;131]. Cloning of different genes encoding β-agarases from *Pseudomonas* sp. has successfully been established in *E. coli* several times[130;131]. Production of extracellular κ-carrageenase by genes cloned from *Zobellia* sp. has also been accomplished in this bacterium[128].

In this work, the focus has been on utilizing the three agarases AhgA, DagA and DagB encoded by *ahgA*, *dagA* and *dagB*, respectively, where the former comes from the marine bacterium *Zobellia galactanivorans* and the two latter, from the soil bacterium *S. coelicolor*. According to literature, these three enzymes are β-agarases that recognize the (G-ALG) repeating units and degrade agar by hydrolyzing the β-(1,4) glycosidic bonds[40]. *ahgA* encodes for an exolytic 3,6 anhydro-α-L-galactosidase that releases 3,6-anhydro-L-galactose from the non-reducing end of neoagaro-oligosaccharides[68;40]. *dagA* encodes an endo-type agarase that catalyzes the degradation of agar into the two neoagaro-oligosaccharides neoagarotetraose and neoagaro-

hexaose[40;76]. *dagB* encodes a putative hydrolase that hydrolyses agar into the heterodimer neoagarobiose[76].

The regulation mechanisms underlying agar degradation in both *S. coelicolor* and *Z. galactanivorans* are complex and intricately controlled, as indicated by limited available information and emerging research findings[40;68]. Detailed biochemical investigations have been initiated to unravel the intricate enzymatic systems involved in agar degradation. Utilizing genomics and proteomics analyses, putative agarolytic pathways have been proposed. For instance, Chi et al. (2012) conducted a systematic review of agar degradation in *S. coelicolor*, *S. degradans*, and *Z. galactanivorans*, shedding light on the subject[40;132]. Figure 4.2 A. and B. illustrates the agar degradation pathways in *Z. galactanivorans* and *S. coelicolor*, respectively. The regulatory systems in both organisms share a common feature where extracellular agarases initiate the breakdown of agar prior to its internalization for further degradation. In both cases, the ultimate degradation products, D-galactose and anhydro-L-galactose, enter the central metabolism, although the latter undergoes additional enzymatic transformations before being fully metabolized[132]. The main difference between agar degradation in the two bacteria lies in the mode of importation of agar units. While *Z. galactanivorans* imports monomeric units into the cytoplasm, *S. coelicolor*, on the other hand, imports dimers.

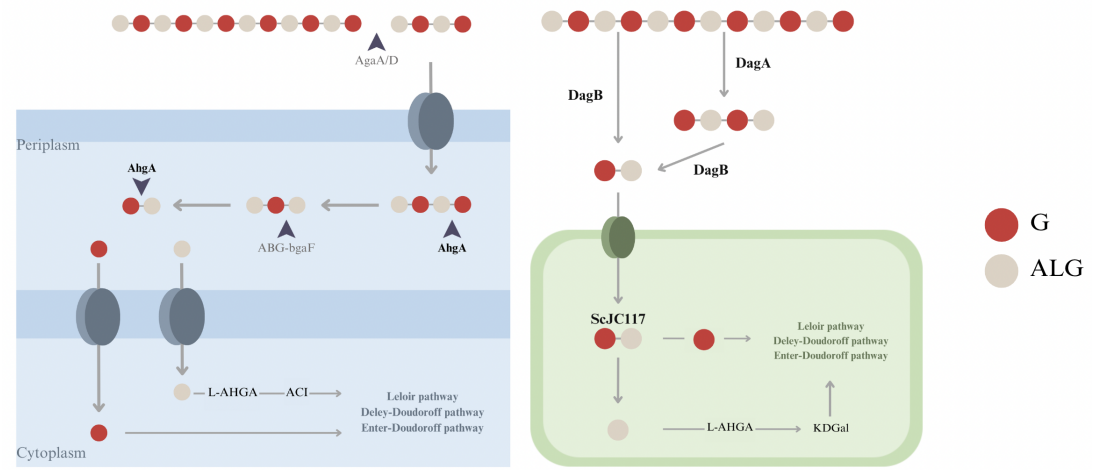


Figure 4.2: The native metabolic pathways for degradation of agar in agar-decomposing microorganisms *Z. galactanivorans* (left) and *S. coelicolor* (right). G: D-galactose; ALG: anhydro-L-galactose; L-AHGA: L-3,6-anhydrogalactonate; ACI: 3,6-anhydrogalactonate cycloisomerase; AhgA: exolytic 3,6 anhydro-A-L-galactosidase; AgaA/D:ext-β-agarases; DagB: putative hydrolase; DagA: extracellular agarase; ScJC117: α-neoagarobiose hydrolase; KDGal: 2-keto-3-deoxy-galactonate[132].

4.3 Protein transport systems involved in agarases secretion

The selection of AhgA, DagA, and DagB as the focus of this study was based on their classification as secreted enzymes, as documented in the literature, distinguishing them from other agarases that exhibit activity exclusively within the periplasm or cytoplasmic compartments[40]. The Sec and Tat pathways represent the primary bacterial secretion systems em-

ployed for protein transport across the cytoplasmic membrane. The Sec and Tat pathways involve a single secretion step but differ in protein transport mechanisms, with the Sec pathway folding proteins subsequent to secretion and the Tat pathway folding proteins prior to secretion[133]. Signal peptides are essential for the secretion of extracellular proteins, and while the signal peptides of Sec- and Tat-dependent proteins share similar characteristics, they also possess distinct specificities[134]. *C. glutamicum* exhibits low levels of endogenous extracellular proteins and lacks detectable extracellular hydrolytic enzyme activity which makes it highly advantageous as a host cell for the secretory production of heterologous proteins, essential enzymes, and pharmaceutical proteins[134]. For instance, research by Kikuchi et al. (2009) demonstrated the efficiency of *C. glutamicum* to be superior in Tat pathway-dependent secretion of green fluorescent protein compared to *B. subtilis* and *Staphylococcus carnosus*, suggesting its potential as a valuable host for heterologous protein production[135].

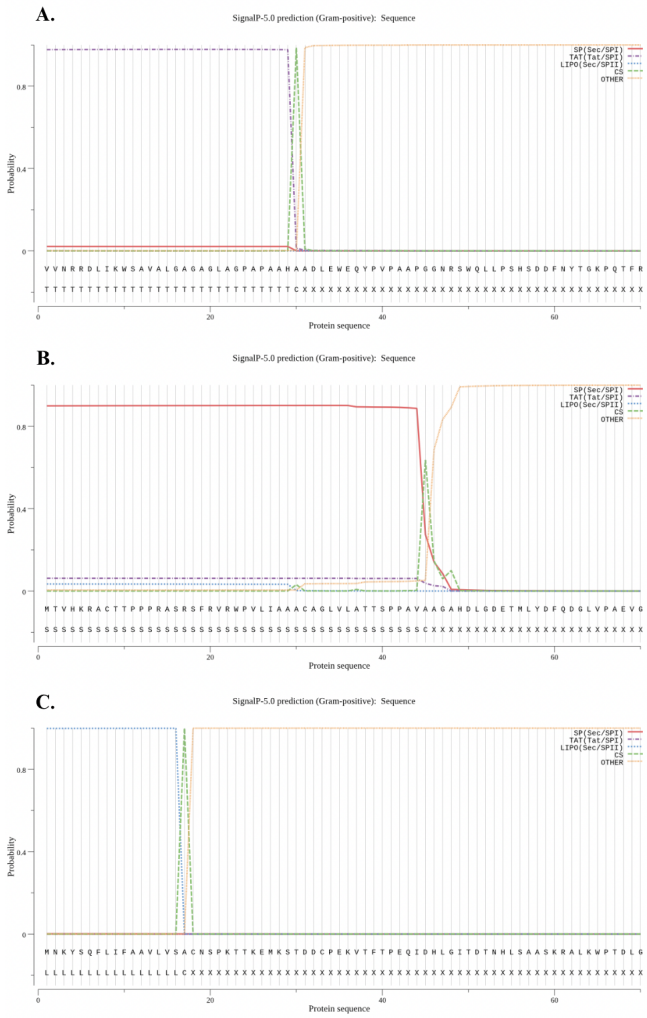


Figure 4.3: Predictions of signal peptides of three agarases using the free tool SignalP 5.0 prediction made by DTU, Denmark[136]. DagA, DagB from *S. coelicolor* and AhgA from *Z. galactanivorans* is shown in A., B., and C., respectively

In this study, the secretion pathway dependency of the three agarases (DagA, DagB, and AhgA) cloned in *C. glutamicum* was assessed using the SignalP 5.0 prediction tool developed by DTU, Denmark[136]. The results of the prediction are depicted in Figure 4.3 A, B, and C for DagA, DagB and AhgA, respectively, illustrating the secretion protein type indicated by the color of the line corresponding to the first peak. It was observed that both DagB and AhgA are dependent on the Sec pathway for secretion, whereas DagA utilizes the Tat pathway. Consequently, the folding of DagB and AhgA occurs post-secretion, while DagA undergoes folding prior to secretion. Although both DagB and AhgA rely on the Sec pathway, AhgA is classified as a lipoprotein characterized by the presence of a lipid moiety attached to a cysteine residue at the N-terminal of the protein, whereas DagB is a secretory protein destined for extracellular secretion[137]. Previous studies have proved that DagA is secreted by the Tat system, whose secretion was enhanced by overexpressing the tat subunits genes A and C[123]. The identification of signal peptides confirms the secretion of all three relevant enzymes.

Comparative studies expressing the same protein in both *E. coli* and *C. glutamicum* have demonstrated higher yields of secreted proteins from *E. coli*[138]. However, employing *C. glutamicum* as an expression host may offer advantages in terms of protein stability, due to its low protease activity[138]. Hence, *C. glutamicum* presents a favorable choice as a host for the production and secretion of agarases, as its secreted proteins are not susceptible to extracellular protease activity resulting in enhanced production yields as the occurrence of proteolytic degradation of secreted proteins is infrequent[123]. In contrast, *B. subtilis*, another commonly used host organism, is known to secrete eight proteases, whereof the majority of its proteolytic activity is attributed to subtilisin and neutral protease[139]. These findings further emphasize the robustness of *C. glutamicum* and provide additional justification for selecting this bacterium as the expression host in this particular study.

4.4 Assessment of microbial robustness

For economic reasons, chemical treatment by thermic acid or base hydrolysis stands as the dominant method to hydrolyze seaweed before enzymatic degradation[53;140]. However, enzymatic processes are regarded as more sustainable, more time effective and proven to produce lesser toxic by-products[53]. Hence, enzymatic pretreatment is preferred compared to other methods as it preserved the bioactive properties of the oligosaccharide produced[53]. Both G and ALG have the potential to be converted into high-value chemicals as well as binging used for biorefining and biofuel production[40]. For instance, galactose can be converted to a low-calorie sweetener, D-tagatose, by L-arabinose isomerase or to isopropyl β-D-1-thiogalactopyranoside[40]. Importantly, seaweed hydrolysate produced by acidic treatment contains a larger number of growth inhibitors, such as phenolic compounds, aliphatic acids (e.g., acetate and lactate), and furans (such as furfural and 5-hydroxymethylfurfural (5HMF)), where the latter is known as the main inhibitor of red seaweed[140]. Detoxification is necessary before fermentation when employing bacteria that lack native detoxification pathways.

Among the aforementioned inhibitors, furfural strongly inhibits bacterial growth. However, tudies by Sakai et al. (2007) and Tsuge et al. (2014) have demonstrated that *C. glutamicum* inherent the native ability to detoxify furfural under both aerobic and anaerobic conditions[141;142]. This is a tolerance that can be enhanced through recombinant methods, such as long-term adaptive evolution, by continuous exposure to the inhibitors[143]. Moreover, recombinant strains of *C. glutamicum* have been tested as a cell factory for the production of molecules with possible toxic properties that in certain concentrations inhibit bacterial growth. Additionally *C. glutamicum* exhibits the native ability to metabolize various compounds such as acetate and lactate, even in the presence of glucose[144]. When establishing new microbial processes, it is crucial to assess whether the chosen host will be suitable or not for the desired process. Conducting a toxicity assessment on *C. glutamicum* is a common method used to evaluate its compatibility as a host. Research conducted by Pérez-García et al. (2016) has established *C. glutamicum* strains for the production of L-pipecolic acid and the response of the recombinant strains was determined by performing a toxicity assessment analyzing their growth behavior after extracellular supplementation of pipecolic acid. The result of the assessment was an inhibitory constant of (K_i) of approximately 3.5 M[145]. A study by Jorge et al. (2016) successfully constructed *C. glutamicum* strains with a new metabolic route capable of producing the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) by glucose fermentation. In a toxicity assessment, the research proved that *C. glutamicum* was a suitable host for GABA production by analyzing the growth of the constructed strains in minimal medium containing different concentrations of GABA[146]. The K_i was found to be approximately 1.1 M.

In order to evaluate the suitability of *C. glutamicum* as an expression host for agar degradation, a toxicity test was performed following standard practices to assess the growth behavior of this bacterium under novel conditions. This assessment aimed to determine if *C. glutamicum* could sustain sufficient growth when supplementing the media with agar. The toxicity test followed a methodology similar to the aforementioned toxicity assessments, involving the exposure of *C. glutamicum* carrying the empty pECXT99a plasmid to seven different concentrations of agar. Of the tested conditions in this work, it was observed that a concentration of 0.400 g/L agar was not suitable for growth. One possible explanation for the limited growth is the observed high viscosity of the flask media which may have impeded oxygen access and hindered equal nutrient distribution, ultimately leading to cell death. However, research by Nishimura et al. (2007) and Michel A. et al. (2015) have proven *C. glutamicum* to be facultative anaerobe, metabolizing glucose, fructose, sucrose, and ribose through the glycolysis or the pentose phosphate pathway via mixed acid fermentation[147;148]. In the absence of oxygen, *C. glutamicum* does not reach its maximum growth potential due to the energy consumption associated with adapting to alternative electron acceptors and regulating gene expression[147]. Consequently, the lack of oxygen would result in reduced growth rate and biomass formation, but it would not completely inhibit cell growth. Therefore, the limited growth at 0.400 g/L agar is most likely attributed to poor nutrient distribution as liquid media supplemented with agar reduces exposure of cell surface to nutrients as has been observed before in experiments

with organisms such as *Bacillus cereus*[149].

4.5 *C. glutamicum* for degradation of agar and utilization of agar-derived galactose as carbon source

To date, limited research has been conducted on recombinant agar degradation and utilization. Nonetheless, successful heterologous production of exo- and endo-type β-agarases has been demonstrated in various host systems, including *E. coli*[150;151;152], *B. subtilis*[153] *Streptomyces lividans*[53;79] and *C. glutamicum*[123]. For instance, β-agarase gene *agaXa* cloned from *Catenovulum* has been expressed in *E. coli* and tested for endolytic agarose degradation yielding neoagarohexaose, neoagarooctaose, neoagarodecaose, and neoagarododecaose[154]. In another study, the expression of β-agarase derived from *Agarivorans* sp. was achieved in *B. subtilis*, enabling cost-effective and efficient large-scale production of the enzyme with a significantly higher yield compared to its overexpression in *E. coli*[153]. However, the production yields in most of the studies fell below the desired levels, and often necessitated purification of the enzymes, thereby impeding the development of an economically feasible bioprocess[123]. In the research of Jeon et al. (2021), *C. glutamicum* demonstrated successful conversion of agar into neoagarobiose when expressing two β-agarases, namely the endo-type DagA from *S. coelicolor* and the exo-type EXB3 from *G. chinensis*[123]. The study confirmed 54% and 24.5% successful secretion of DagA and EXB3, respectively.

Within this thesis, the growth assessment of *C. glutamicum* strains harboring plasmids overexpressing the cloned agarase genes revealed that the strains expressing one or two agarases exhibited similar growth patterns to the control strain. However, these strains exhibited a 4.5-fold increase in secreted protein concentration, as determined by the Bradford assessment. Interestingly, these strains also displayed the lowest biomass values, suggesting that the carbon flux likely was sent towards protein production in addition to the central metabolism. Conversely, the strain overexpressing all three agarases, specifically optimized *dagA* and *ahgA* in conjunction with *dagB*, displayed poor growth and no additional protein activity compared to the control. These observations suggest a potential bottleneck in protein secretion as a plausible explanation. Both DagB and AhgA utilize the Sec secretion system, which may be susceptible to blockage due to heavy traffic. Furthermore, the substantial size of DagB could potentially impede its secretion process especially if problems occur during protein folding. Similar secretion problems have previously been identified in earlier studies. A study conducted by Chen et al. (2015) has identified possible bottlenecks in the Sec-secretions system of *B. subtilis* when overexpressing genes from *Bacillus licheniformis* and *Geobacillus stearothermophilus* production of α-amylases[155]. They found instability regarding protein folding to be the most important bottleneck but that balanced expression of Sec-pathway components such as *prsA* and partial *dnaK* operon enhanced production. Jurischka et al. (2020) discovered that secretion bottlenecks in *C. glutamicum* can vary depending on the specific target proteins involved. Factors such as protein folding properties, dependence on folding factors, and interactions with the cell envelope components can contribute to this variability. Additionally, the transport mechanism across the mycolic acid outer membrane layer remains unknown but

may restrict the secretion of heterologous proteins into the culture supernatant[156]. The findings provide support for the hypothesis that problems associated with protein secretion may contribute to the low efficiency of agar degradation. However, further evaluation of this matter can be assessed via SDS-page of the supernatants and internal cell contents from the agarases producers.

As of now, the available literature regarding the recombinant utilization of agar as a carbon source is limited. Nevertheless, it is known that galactose can be metabolized by ethanol-fermenting bacteria and yeasts, such as *E. coli, Z. mobilis*, and *Saccharomyces cerevisiae*[40]. Furthermore, studies have been conducted to uncover novel approaches for the use of alternative carbohydrates derived from various seaweeds for efficient utilization in industrial processes in addition to potential substrate exploitation. For instance, Doi et al. (2017) conducted research demonstrating the successful extracellular degradation of alginate through the expression of *alyB* from *Vibrio algivorus* in *E. coli*. The recombinant strains, in conjunction with six additional genes (*alyD, oalA, oalB, oalC, dehR*, and *toaA*), exhibited the ability to utilize degraded soluble alginate as sole carbon source[157]. In a separate study, Wargacki et al. (2012) engineered a microbial platform within *E. coli* that could concurrently degrade, uptake, and metabolize alginate. This was achieved by incorporating genes from *Vibrio splendidus*, which encoded enzymes responsible for alginate transport and metabolism, along with an engineered system for extracellular alginate depolymerization[158].

Overexpression of the *galETKM* operon in this study along with the agarases showed low efficiency of degradation and utilization of agar-derived galactose as carbon source considering quantitative methods. As previous studies by Frøystad M. (2023)[105] have proven successful galactose utilization by expression of this operon, the hypothesis of the agarase secretion bottleneck is strengthened. On the other hand, degradation of agar was visually confirmed, therefore it is essential to identify the degradation products for instance by using thin layer chromatography methods[159].

4.6 Outlook

Gaining a complete understanding of the agar-degrading system in microorganisms is imperative for the effective utilization of red algal biomass. However, there are still missing links in the pathway, posing a challenge to elucidate its regulatory network[40]. To address the gaps and advance research in this field, it is essential to employ comprehensive molecular investigations into agarase enzymes synthesis, folding, secretion, and enzymatic activity. By elucidating potential limitations and confirming key bottlenecks, the efficiency of recombinant bacterial degradation of agar and utilization of agar-derived galactose as carbon source can be improved.

One approach to investigating the location and secretion status of agarases is the utilization of analytical techniques such as SDS-page (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), which enables the separation of low-molecular-mass proteins[160]. For instance, in the study by Jeon et al. (2021), SDS-page confirmed that the majority of DagA produced

was present in the cytoplasm, indicating successful production but highlighting the need for secretion optimization[123]. In addition, the application of mass spectrometry (MS), a widely used analytical technique in protein research, can provide valuable insights into the identification and characterization of peptides, proteins, and enzymes[161]. Complementary techniques such as HPLC (high-performance liquid chromatography) and TLC (thin-layer chromatography) can also be utilized to study the degradation products or intermediates produced during recombinant degradation of agar[162]. For instance, TLC has been utilized to analyse alginate degradation products in a study by Dobruchowska J. et al. (2022) revealing two alginate lyases' ability to depolymerize alginate[163].

Additionally, it is essential to comprehensively evaluate other relevant parameters that may influence the efficiency of the agar degradation process. These parameters include optimizing growth conditions, the temperature to achieve optimal protein activity and exploring the potential synergistic effects of combining different agarases. While this work utilized a temperature of 30°C, previous studies have demonstrated that *Z. galactanivorans* exhibits optimal growth and enzymatic activity at relatively low temperatures, typically ranging from 10°C to 20°C[72]. Conversely, the purified DagA enzyme exhibited maximum agarase activity at a temperature of 40°C and pH 7.0[53].

Furthermore, studies by Seo et al. (2014) revealed that DagB, despite its exo-lytic activity, exhibits a slow hydrolysis rate when breaking down agarose. To enhance the efficiency of neoagarobiose, they anticipate that a combination of DagB agarase and AgaG1 agarase would be highly effective as AgaG1 agarase is known for fast release of neoagarotetraose, the final substrate for the DagB agarase[151]. Moreover, within the chromosomal DNA of *S. coelicolor*, there are 15 open reading frames (ORFs) located between the *dagA* and *dagB* genes. It is anticipated that a majority of these ORFs play a role in the breakdown of agarose[164]. Exploring the possible synergetic effects of incorporating additional genes or different agarases combinations could potentially enhance the efficiency of the process. Given that agar is a complex polysaccharide with sulfate groups, different combinations of enzymes and rational engineered enzymes can offer improved catalytic activities and enhance the overall efficiency of agar degradation.

Lastly, this work does not explore previously reported technologies for improving the secretion of agarases in *C. glutamicum*. For instance, Jeon et al. (2021) demonstrated removal of two endogenous protein genes (cg2052 and cg1514) to enhance the purity of secreted DagA, along with coexpression of two Tat pathway components (TatA and TatC) to improve secretion efficiency[123]. This is supported by research by Kikuchi et al. (2009) that highlights the role of overexpressing TatABC in enhancing Tat-dependent protein secretion in *C. glutamicum*[135]. Notably, in secretory production, the purity and production titer of target proteins may be improved by reducing the secretion of competitive proteins[123]. Additionally, identifying the optimal signal peptide for a target protein can contribute to enhanced secretion efficiency. This can be accomplished by screening a wide range of signal peptides either through signal peptide variation using extensive libraries or through the optimization of an existing signal peptide

using strategies such as site-directed or random mutagenesis[165]. Specifically, in this context, native signal peptides from *C. glutamicum* can be explored as potential replacements for those derived from *S. coelicolor* and *Z. galactinovoras*.

Considering the results of this thesis, engineered *C. glutamicum* showed slow degradation of agar under certain conditions which could not support *C. glutamicum* agar-based growth. Also, protein secretion was impaired when producing the enzyme DagB in combination with AhgA. Therefore, further research within this line should focus on the enhancement of both catalytic efficiency and agarases secretion.

5 Conclusion

In this work, *Corynebacterium glutamicum* strains producing and secreting different agarases from *Z. galactanivorans* and *S. coelicolor* were successfully constructed. In particular, the combination of the agarases AhgA and DagA showed the best performance in terms of agar degradation. Additionally, production and secretion of agarases was coupled with galactose utilization by an engineered *C. glutamicum* strain harboring the *galETKM* operon and the *galP* gene enabling galactose uptake and consumption. This was done to establish agar degradation and utilization of agar-derived galactose as carbon source in the bacterium to explore a sustainable alternative to sugar-based feedstocks.

The qualitative results suggest low efficiency of agar degradation and galactose utilization. Nevertheless, agar degradation by the newly constructed strains was visually confirmed after 24 hours. Hence, this work lays the initial cornerstone of agar utilization as carbon source by *C. glutamicum* for biotechnology processes and, in consequence, the utilization of red seaweed biomass as promising microbial feedstock.

References

[1] Cloneamp™ hifi pcr premix protocol-at-a-glance, (2012). URL https://www.takarabio.com/documents/User%20Manual/CloneAmp%20HiFi%20PCR%20Premix%20Protocol/CloneAmp%20HiFi%20PCR%20Premix%20Protocol-At-A-Glance_092612.pdf. Accessed: 11.04.23.

[2] Gotaq® dna polymerase (m300) protocol, (2018). URL https://no.promega.com/resources/protocols/product-information-sheets/g/gotaq-dna-polymerase-m300-protocol/. Accessed: 11.04.23.

[3] Mesbah N. M. Industrial biotechnology based on enzymes from extreme environments. *Frontiers in Bioengineering and Biotechnology*, 10, (2022).

[4] Moldes A., Vecino X., Rodríguez-López L., Rincón-Fontán M., and Cruz J. M. *New and Future Developments in Microbial Biotechnology and Bioengineering*, pages 163–185. Elsevier, 1 edition, (2020).

[5] Keasling J. D. Manufacturing molecules through metabolic engineering. *Science*, 330 (6009):1355–1358, (2010).

[6] Koutinas A. A., Vlysidis A., Pleissner D., Kopsahelis N., Garcia I. L., Kookos I. K., Papanikolaou S., Kwanb T. H., and Ki Lin C. S. Valorization of industrial waste and by-product streams *via* fermentation for the production of chemicals and biopolymers. *Chemical Society Reviews*, 8:2375–2878, (2014).

[7] Wendisch V. F., Brito L. F., Lopez M. G., Hennig G., Pfeifenschneider J., Sgobba E., and Veldmann K. H. The flexible feedstock concept in industrial biotechnology: Metabolic engineering of *Escherichia coli*, *Corynebacterium glutamicum*, *Pseudomonas*, *Bacillus* and yeast strains for access to alternative carbon sources. *Journal of Biotechnology*, 234(20):139–157, (2016).

[8] Sgobba E., Blöbaum L., and Wendisch V. F. Production of food and feed additives from non-food-competing feedstocks: Valorizing n-acetylmuramic acid for amino acid and carotenoid fermentation with *Corynebacterium glutamicum*. *Frontiers in microbiology*, 9:2046, (2018).

[9] Salim I., González-García S., Feijoo G., and Moreira M. T. Assessing the environmental sustainability of glucose from wheat as a fermentation feedstock. *Journal of Environmental Management*, 257:323–332, (2019).

[10] Song L., Yang D., Liu R., Liu S., Dai L., and Dai X. Microbial production of lactic acid from food waste: Latest advances, limits, and perspectives. *Bioresource technology*, 345:126052, (2022).

[11] Prathiba S., Kumar P. S., and Vo D. V. N. Recent advancements in microbial fuel cells:

A review on its electron transfer mechanisms, microbial community, types of substrates and design for bio-electrochemical treatment. *Chemosphere*, 286(3):131856, (2022).

[12] Pandey A., Ricke S. C., Gnansounou E., Larroche C., and Dussap C. G. *Biofuels - Alternative Feedstocks and Conversion Processes*, chapter 5, pages 101–122. Academic Press, 1 edition, (2011).

[13] Ward O. P. and Singh A. Bioethanol technology: Developments and perspectives. *Advances in Applied Microbiology*, 51:53–80, (2002).

[14] Tabassum M. R., Xia A., and Murphy J. D. Potential of seaweed as a feedstock for renewable gaseous fuel production in ireland. *Renewable and Sustainable Energy Reviews*, 68:136–146, (2017).

[15] Subhadra B. and Edwards M. An integrated renewable energy park approach for algal biofuel production in united states. *Energy Policys*, 38(9):4897–4902, (2010).

[16] Jung K. A., Lim S. R., Kim Y., and Park J. M. Potentials of macroalgae as feedstocks for biorefinery. *Bioresource Technology*, 135:182–190, (2013).

[17] Wei N., Quarterman J., and Jin Y. S. Marine macroalgae: an untapped resource for producing fuels and chemicals. *Energy Policys*, 31(2):70–77, (2013).

[18] Duarte C. M., Losada I. J., Hendriks I. E., Mazarrasa I., and Marbà N. The role of coastal plant communities for climate change mitigation and adaptation. *Nature Climate Change*, 3:961–968, (2013).

[19] Jönsson M., Allahgholi L., Sardari R. R. R., Hreggviðsson G. O., and Karlsson E. N. Extraction and modification of macroalgal polysaccharides for current and next-generation applications. *Molecules*, 25(4):930, (2020).

[20] Tiwari B. K. and Troy D. J. *Seaweed Sustainability*, chapter 1, pages 1–6. Academic Press, 1 edition, (2015).

[21] Lahaye M. and Jegou D. Chemical and physical-chemical characteristics of dietary fibres from ulva lactuca (l.) thuret and enteromorpha compressa (l.) grev. *Journal of Applied Phycology*, 5:195–200, (1993).

[22] Ito K. and Hori K. Seaweed: Chemical composition and potential food uses. *Food Reviews International*, 5:101–144, (1989).

[23] El-Din S. M. M. and El-Ahwany A. M. D. Bioactivity and phytochemical constituents of marine red seaweeds (j*ania rubens, Corallina mediterranea* and *Pterocladia capillacea*). *Journal of Taibah University for Sciencea*, 10(4):471–484, (2016).

[24] Chu W. L. Biotechnological applications of microalgae. *International Medical University*, 6(1):24–37, (2012).

[25] Venkatesan J., Lowe B., Anil S., Manivasagan P., Al Kheraif A. A., Kang K. H., and Kim S. K. Seaweed polysaccharides and their potential biomedical applications. *Starch*, 67(5-6):381–390, (2015).

[26] Leonel Pereira. *Alginates: Recent Uses of This Natural Polymer*. IntechOpen, 1 edition, (2020).

[27] Rashedy S. H., Abd El Hafez M. S. M., Dar M. A., Cotas J., and Pereira L. Evaluation and characterization of alginate extracted from brown seaweed collected in the red sea. *Applied Sciences*, 11(14):6290, (2021).

[28] Moroney N. C., O'Grady M. N., O'Doherty J. V., and Kerry J. P. Effect of a brown seaweed (*Laminaria digitata*) extract containing laminarin and fucoidan on the quality and shelf-life of fresh and cooked minced pork patties. *Meat Science*, 94(3):304–311, (2013).

[29] Costa L. S., Fidelis G. P., Cordeiro S. L., Oliveira R. M., Sabry D. A., Câmara R. B. G., Nobre L. T. D. B., Costa M. S. S. P., Almeida-Lima J., Farias E. H. C., Leite E. L., and Rocha H. A. O. Biological activities of sulfated polysaccharides from tropical seaweeds. *Biomedicine and Pharmacotherapy*, 64(1):21–28, (2010).

[30] Kim E. J., Fathoni A., Jeong G. T, Jeong H. D., Nam T. J., Kong I. S., and Kim J. K. *Microbacterium oxydans*, a novel alginate- and laminarin-degrading bacterium for the reutilization of brown-seaweed waste. *Journal of Environmental Management*, 130 (30):153–159, (2013).

[31] Kadam S. U., Tiwari B. K., and O'Donnell C. P. Extraction, structure and biofunctional activities of laminarin from brown algae. *International Journal of Science and Technology*, 50(1):24–31, (2015).

[32] De Ruiter G. A. and Rudolph B. Carrageenan biotechnology. *Trends in Food Science and Technology*, 8(12):389–395, (1997).

[33] Necas J. and Bartosikova L. Carrageenan: a review. *Veterinární medicína*, 58(4):187–205, (2013).

[34] Therkelsen G. H. *Industrial Gums*, chapter 7. Academic Press, 3 edition, (1993).

[35] Hii S. L., Lim J. Y., Ong W. T., and Wong C. L. Agar from malaysian red seaweed as potential material for synthesis of bioplastic film. *Journal of Engineering Science and Technology*, 7:1–15, (2016).

[36] Jayakody M. M., Vanniarachchy M. P. G., and Wijesekara I. Seaweed derived alginate, agar, and carrageenan based edible coatings and films for the food industry: a review. *Journal of Food Measurement and Characterization*, 22:1195–1227, (2022).

[37] Stephen A. M., Phillips G. O., and Williams P. A. *Meat Products Handbook*. Taylor and Francis Group, 2 edition, (2006).

[38] Cosenza V. A., Navarro D. A., Ponce N. M. A., and Stortz C. A. Seaweed polysaccharides: Structure and applications. *Industrial Applications of Renewable Biomass Products*, (2017).

[39] Álvarez Viñas M., Flórez-Fernández N., Torres M. D., and Domínguez H. Successful approaches for a red seaweed biorefinery. *Marine Drugs*, 17(620):1–18, (2019).

[40] Chi W. J., Chang Y. K., and Hong S. K. Agar degradation by microorganisms and agar-degrading enzymes. *Applied Microbiology and Biotechnology*, 94:917–930, (2012).

[41] Yoon H. S., Nelson W., Lindstrom S. C., Boo S. M., Pueschel C., Qiu H., and Bhattacharya D. *Rhodophyta*, chapter 4, pages 89–133. Springer International Publishing, 2 edition, (2017).

[42] Yoon J. J., Kim Y. J., Kim S. H., Ryu H. J., Choi J. Y., Kim G. S., and Shin M. K. Production of polysaccharides and corresponding sugars from red seaweed. *Advanced Materials Research*, 93-94:463–466, (2010).

[43] Nagarajan D., Chen C. Y., Ariyadasa T. U., Lee D. J., and Chang J. S. Macroalgal biomass as a potential resource for lactic acid fermentation. *Chemosphere*, 309(2): 136694, (2022).

[44] Kovaleski G., Kholany M., Dias L. M. S., Correia S. F. H., Ferreira R. A. S., Coutinho J. A. P., and Ventura S. P. M. Extraction and purification of phycobiliproteins from algae and their applications. *Green and Sustainable Chemistry*, 10, (2022).

[45] Liu C. and Zou D. Effects of elevated co2 on the photosynthesis and nitrate reductase activity of pyropia haitanensis (bangiales, rhodophyta) grown at different nutrient levels. *Chinese Journal of Oceanology and Limnology*, 33(2):419–429, (2014).

[46] Carpena M., Garcia-Perez P., Garcia-Oliveira P., Chamorro F., Otero P., Lourenço-Lopes C., Cao H., Simal-Gandara J., and Prieto M. A. Biological properties and potential of compounds extracted from red seaweeds. *Phytochem Rev.*, 1:1–32, (2022).

[47] Kim H. M., Wi S. G., Jung S., Song Y., and Bae H. J. Efficient approach for bioethanol production from red seaweed *Gelidium amansii*. *Bioresource Technology*, 175:128–134, (2015).

[48] A. Peñuela, D. Robledo, N. Bourgougnon, G. Bedoux, E. Hernández-Núñez, and Y. Freile-Pelegrín. Environmentally friendly valorization of solieria filiformis (gigartinales, rhodophyta) from imta using a biorefinery concept. *Marine Drugs*, 16(12):487, (2018).

[49] Liu Z., Gao T., Yang Y., Meng F., Zhan F., Jiang Q., and Sun X. Anti-cancer activity of porphyran and carrageenan from red seaweeds. *Molecules*, 24(23):4286, (2019).

[50] Wang J. and Yin Y. Fermentative hydrogen production using pretreated microalgal biomass as feedstock. *Microbial Cell Factories*, 17(22), (2018).

[51] Varshosaz J., Zaki M. R., Minaiyan M., and Banoozadeh J. Preparation, optimization, and screening of the effect of processing variables on agar nanospheres loaded with bupropion hcl by a d-optimal design. *BioMed Research International*, pages 1–13, (2015).

[52] Bera K., Sarwa K. K., and Mazumder B. Metformin hcl loaded mucoadhesive agar microspheres for sustained release. *Asian Journal of Pharmaceutics*, 7(2):75–82, (2013).

[53] Temuujin U., Chi W. J., Lee S. Y., and Chang Y. K. Hong S. K. Overexpression and biochemical characterization of daga from *Streptomyces coelicolor* a3(2): an endo-type beta-agarase producing neoagarotetraose and neoagarohexaose. *Applied Microbiology and Biotechnology*, 92:749–759, (2011).

[54] Park S. H., Lee C. R., and Hong S. K. Implications of agar and agarase in industrial applications of sustainable marine biomass. *Applied Microbiology and Biotechnology*, 104:2815–2832, (2020).

[55] Sambhwani K., Kazi M. A., and Mantri V. A. *Gracilaria*: An emerging source of agar feedstock—with special reference to industrially important species. *Sustainable Global Resources of Seaweeds*, 2:55–76, (2022).

[56] Adams J. M. M., Toop T. A., Donnison I. S., and Gallagher J. A. Seasonal variation in *Laminaria digitata* and its impact on biochemical conversion routes to biofuels. *Bioresource Technology*, 102(21):9976–9984, (2011).

[57] Wang J., Jiang X., Mou H., and Guan H. Anti-oxidation of agar oligosaccharides produced by agarase from a marine bacterium. *Journal of Applied Phycology*, 16:333–340, (2004).

[58] Yun E. J., Shin M. H., Yoon J. J., Kim Y. J., Choi I. G., and Kim K. H. Production of 3,6-anhydro-l-galactose from agarose by agarolytic enzymes of *Saccharophagus degradans* 2-40. *Process Biochemistry*, 46(1):88–93, (2011).

[59] Fu X. T. and Kim S. M. Agarase: Review of major sources, categories, purification method, enzyme characteristics and applications. *Marine Drugs*, 8(1):200–218, (2010).

[60] Suzuki H., Sawai Y., Suzuki T., and Kawai K. Purification and characterization of an extracellular alpha-neoagarooligosaccharide hydrolase from *Bacillus* sp. mk03. *Journal of Bioscience and Bioengineering*, 93(5):456–463, (2002).

[61] Morrice L. M., McLean M. W., Williamson F. B., and Long W. F. beta-agarases i and ii from *Pseudomonas atlantica*. purifications and some properties. *European Journal of Biochemistry*, 135(3):553–558, (1983).

[62] Hu Z and. Lin B. -K., Xu Y., Zhong M. Q., and Liu G. M. Production and purification of agarase from a marine agarolytic bacterium *Agarivorans* sp. hz105. *Journal of Applied Microbiology*, 106(1):181–190, (2009).

[63] Sugano Y., Terada I., Arita M., Noma M., and Matsumoto T. Purification and characterization of a new agarase from a marine bacterium, vibrio sp. strain jt0107. *Applied and Environmental Microbiology*, 59(5):1549–1554, (1993).

[64] Vera J., Alvarez R., Murano E., Slebe J. C., and Leon O. Identification of a marine agarolytic *Pseudoalteromonas Isolate* and characterization of its extracellular agarase. *Applied and Environmental Microbiology*, 64(11):4378–4383, (1998).

[65] van der Meulen H. J. and Harder W. Production and characterization of the agarase of *Cytophaga flevensis*. *Antonie van Leeuwenhoek*, 41:431–447, (1975).

[66] Ohta Y., Hatada Y., Miyazaki M., Nogi Y., Ito S., and Horikoshi K. Purification and characterization of a novel alpha-agarase from a *Thalassomonas* sp. *Current Microbiology*, 50:212–216, (2005).

[67] Lakshmikanth M., Manohar S., and Lalitha J. Purification and characterization of beta-agarase from agar-liquefying soil bacterium, *Acinetobacter* sp., ag lsl-1. *Process Biochemistry*, 44(9):999–1003, (2009).

[68] Thomas F., Bordron P., Eveillard D., and Michel G. Gene expression analysis of *Zobellia galactanivorans* during the degradation of algal polysaccharides reveals both substrate-specific and shared transcriptome-wide responses. *Frontiers in Microbiology*, 8, (2017).

[69] Correc G., Hehemann J. H., Czjzek M., and Helbert W. Structural analysis of the degradation products of porphyran digested by *Zobellia galactanivorans* beta-porphyranase a. *Carbohydrate Polymers*, 83(1):277–283, (2011).

[70] Hehemann J. H., Correc G., Thomas F., Bernard T., Barbeyron T., Jam M., Helbert W., Michel G., and Czjek M. Biochemical and structural characterization of the complex agarolytic enzyme system from the marine bacterium *Zobellia galactanivorans*. *Protein Structure and Folding*, 287(36):30571–30584, (2012).

[71] Allouch J., Jam M., Helbert W., Kloareg B., Henrissat B., and Czjzek M. The three-dimensional structures of two beta-agarases. *Journal of biological chemistry*, 287(47): 47171–47180, (2003).

[72] Rebuffet E., Groisillier A., Thompson A., Jeudy A., Barbeyron T., Czjzek M., and Michel G. Discovery and structural characterization of a novel glycosidase family of marine origin. *Environmental microbiology*, 13(5):1253–1270, (2011).

[73] The UniProt Consortium. Uniprot: the universal protein knowledgebase in 2023. *Nucleic Acids Research*, 51(1):523–531, (2023).

[74] Bentley S. D., Chater K. F., Cerdeño-Tárraga A. M., Challis G. L., Thomson N. R., James K. D., Harris D. E., Quail M. A., Kieser H., Harper D., Bateman A., Brown S., Chandra G., Chen C. W., Collins M., Cronin A., Fraser A., Goble A., Hidalgo J.,

Hornsby T., Howarth A., Huang C. H., Kieser T., Larke L., Murphy L., Oliver K., O'Neil S., Rabbinowitsch E., Rajandream M. A., Rutherford K., Rutter S., Seeger K., Saunders D., Sharp S., Squares R., Squares S., Taylor K., Warren T., Wietzorrek A., Woodward J., Barrell B. G., Parkhill J., and Hopwood D. A. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* a3(2). *Nature*, 9(417):141–147, (2002).

[75] Vockenhuber M. P. and Suess B. Streptomyces coelicolor srna scr5239 inhibits agarase expression by direct base pairing to the daga coding region. *Microbiology (Reading)*, 158(2):424–435, (2012).

[76] Romero-Rodríguez A., Rocha D., Ruiz-Villafan B., Tierrafría V., Rodríguez-Sanoja R., Segura-González D., and Sánchez S. Transcriptomic analysis of a classical model of carbon catabolite regulation in *Streptomyces coelicolor*. *BMC Microbiology*, 16(77), (2016).

[77] Angell S., Lewis C. G., Buttner M. J., and Bibb M. J. Glucose repression in *Streptomyces coelicolor* a3(2): a likely regulatory role for glucose kinase. *Molecular and General Genetics*, 244:135–143, (1994).

[78] Alkotaini B., Han N. S., and Kim B. S. Fusion of agarase and neoagarobiose hydrolase for mono-sugar production from agar. *Applied Microbiology and Biotechnology*, 101: 1573–1580, (2017).

[79] Temuujin U., Chi W. J., and Chang Y. K. Hong S. K. Identification and biochemical characterization of sco3487 from *Streptomyces coelicolor* a3(2), an exo- and endo-type beta-agarase-producing neoagarobiose. *Applied Microbiology and Biotechnology*, 92: 749–759, (2011).

[80] Jang M. K., Lee S. W., Lee D. G., Kim N. Y., Yu K. H., Jang H. J., Kim S., Kim A., and Lee S. H. Enhancement of the thermostability of a recombinant beta-agarase, agab, from *Zobellia galactanivorans* by random mutagenesis. *Biotechnology Letters*, 32:943–949, (2010).

[81] Luti K. J. K. and Mavituna F. *Streptomyces coelicolor* increases the production of undecylprodigiosin when interacted with *Bacillus subtilis*. *Biotechnology Letters*, 33: 113–118, (2011).

[82] Mesbah N. M. Industrial biotechnology based on enzymes from extreme environments. *Frontiers in Bioengineering and Biotechnology*, 10, (2022).

[83] Kumar R. R and Prasad S. Metabolic engineering of bacteria. *Indian Journal of Microbiology*, 51(3):403–409, (2011).

[84] Becker J., Rohles C. M., and Wittmann C. Metabolically engineered *Corynebacterium glutamicum* for bio-based production of chemicals, fuels, materials, and healthcare products. *Metabolic engineering*, 50:122–141, (2018).

[85] Yukawa H. and Inui M. *Corynebacterium glutamicum: Biology and Biotechnology*. Springer-Verlag Berlin Heidelberg, 1 edition, (2013).

[86] Kalinowski J. et al. The complete *Corynebacterium glutamicum* atcc 13032 genome sequence and its impact on the production of l-aspartate-derived amino acids and vitamins. *Journal of Biotechnology*, 104(1-3):5–25, (2003).

[87] Kirchner O. and Tauch A. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. *Journal of Biotechnology*, 104(1-3):287–299, (2003).

[88] Heider S. A. E. and Wendisch V. F. Engineering microbial cell factories: Metabolic engineering of *Corynebacterium glutamicum* with a focus on non-natural products. *Biotechnology Journal*, 10(8):1170–1184, (2013).

[89] Messelink J. J. B., Meyer F., Bramkamp M., and Broedersz C. P. Single-cell growth inference of corynebacterium glutamicum reveals asymptotically linear growth. *eLife*, 10, (2021).

[90] Henke N. A., Peters-Wendisch P., and Wendisch V. F. Carotenoid production by *Corynebacterium*: The workhorse of industrial amino acid production as host for production of a broad spectrum of c40 and c50 carotenoids. *Carotenoids*, 1:159–173, (2017).

[91] Pérez-García F., Zierta C., Risseb J. M., and Wendisch V. F. Improved fermentative production of the compatible solute ectoine by *Corynebacterium glutamicum* from glucose and alternative carbon sources. *Journal of Biotechnology*, 258:59–68, (2017).

[92] Käß F., Hariskos I., Michel A., Brandt H-J., Spann R., Junne S., Wiechert W., Neubauer P., and Oldiges M. Assessment of robustness against dissolved oxygen/substrate oscillations for *C. glutamicum* dm1933 in two-compartment bioreactor. *Bioprocess and Biosystems Engineering*, 37:1151–1162, (2014).

[93] Ikeda M. Sugar transport systems in *Corynebacterium glutamicum*: features and applications to strain development. *Applied Microbiology and Biotechnology*, 96: 1191–1200, (2012).

[94] Zahoor A., Lindner S. N., and Wendisch V. F. Metabolic engineering of *Corynebacterium glutamicum* aimed at alternative carbon sources and new products. *Computational and Structural Biotechnology Journal*, 3(4), (2012).

[95] Barrett E., Stanton C., Zelder O., Fitzgerald G., and Ross R. P. Heterologous expression of lactose- and galactose-utilizing pathways from lactic acid bacteria in *Corynebacterium glutamicum* for production of lysine in whey. *Applied and Environmental Microbiology*, 70(5):2861–2866, (2004).

[96] Görke B. and Stülke J. Carbon catabolite repression in bacteria: Many ways to make the most out of nutrients. *Bioprocess and Biosystems Engineering*, 6(8):613–624, (2008).

[97] Lindner S. N., Seibold G. M., Henrich A., Krämer R., and Wendisch V. F. Phosphotransferase system-independent glucose utilization in *Corynebacterium glutamicum* by inositol permeases and glucokinases. *Applied and environmental microbiology*, 77(11): 3571–81, (2011).

[98] Coelho A. I., Berry G. T., and Rubio-Gozalbo M. E. Galactose metabolism and health. *Current Opinion in Clinical Nutrition and Metabolic Care*, 18(4):422–427, (2015).

[99] Ra H. C., Kim Y. J., Lee S. Y., Jeong G. T., and Kim S. K. Effects of galactose adaptation in yeast for ethanol fermentation from red seaweed, *Gracilaria verrucosa*. *Bioprocess and Biosystems Engineering*, 38:1715–1722, (2015).

[100] Blombach B. and Seibold G. M. Carbohydrate metabolism in *Corynebacterium glutamicum* and applications for the metabolic engineering of l-lysine production strains. *Applied Microbiology and Biotechnology*, 86(5):1313–1322, (2010).

[101] Brabetz W., Liebl W., and Schleifer K. H. Studies on the utilization of lactose by *Corynebacterium glutamicum*, bearing the lactose operon of *Escherichia coli*. *Archives of Microbiology*, 155(6):607–612, (1991).

[102] Weickert M. J. and Adhya S. The galactose regulon of *Escherichia coli*. *Molecular Microbiology*, 10(2):245–251, (1993).

[103] Grossiord B. P. et al. Characterization, expression, and mutation of the *Lactococcus lactis* galpmkte genes, involved in galactose utilization via the leloir pathway. *J Bacteriol*, 185(3):870–878, (2003).

[104] Riley M. et al. *Escherichia coli* k-12: a cooperatively developed annotation snapshot - 2005. *Nucleic Acids Res*, 34(1):1–9, (2006).

[105] Frøystad M. From seaweed to sustainable microbial feedstocks. *Department of biotechnology and food science*, (2023).

[106] Kirchner O. and Tauch A. Tools for genetic engineering in the amino acid-producing bacterium corynebacterium glutamicum. *Journal of biotechnology*, 104(1-3):287–299, (2003).

[107] Abe S., Takayama K-I., and Kinoshita S. Taxonomical studies on glutamic acid-producing bacteria. *The Journal of General and Applied Microbiology*, 13(3):279–301, (1967).

[108] Hanahan D. Studies on transformation of escherichia coli with plasmids. *Journal of molecular biology*, 166(4):557–580, (1983).

[109] Bacterial culturing from glycerol stocks, (2021). URL

https://www.escolifesciences.com/news/bacterial-culturing-from-glycerol-stocks. Accessed: 01.04.23.

[110] Nanodrop™ one/onec microvolume uv-vis spectrophotometer, (2021). URL https://www.thermofisher.com/order/catalog/product/ND-ONE-W. Accessed: 17.04.23.

[111] van Pelt-Verkuil E., van Belkum A., and Hays J. P. *Principles and Technical Aspects of PCR Amplification*. Springer Science and Business Media,, (2008).

[112] Thermo scientific™ o'generuler 1 kb dna ladder, ready-to-use- 250-10,000 bp, (2021). URL https://www.fishersci.no/shop/products/fermentas-o-generuler-ready-to-use-1kb-dna-ladder/11551615. Accessed: 05.05.23.

[113] Gibson D. G., Young L., Chuang R-Y., Venter J. C., Hutchison C. A. 3rd, and Smith H. O. Enzymatic assembly of dna molecules up to several hundred kilobases. *Nature methods*, 6:343–345, (2009).

[114] Green R. and Rogers E. J. Transformation of chemically competent e. coli. *Methods in enzymology*, 529:329–36, (2033).

[115] Pérez-García F., Klein V. J., de Brito L. F., and Brautaset T. From brown seaweed to a sustainable microbial feedstock for the production of riboflavin. *Frontiers in bioengineering and biotechnology*, 12(10), (2022).

[116] Take action for the sustainable development goals, (2023). URL https://www.un.org/sustainabledevelopment/sustainable-development-goals/. Accessed: 23.05.23.

[117] Bart J. C. J., Gucciardi E., and Cavallaro S. Renewable lubricants. *Science and Technology*, pages 1–9, (2013).

[118] Ekborg N. A., Gonzalez J. M., Howard M. B., Taylor L. E., Hutcheson S. W., and Weiner R. M. *Saccharophagus degradans* gen. nov., sp. nov., a versatile marine degrader of complex polysaccharides. *International journal of systematic and evolutionary microbiology*, 55(4):1–6, (2005).

[119] Becker J., Rohles C. M., and Wittmann C. Metabolically engineered *Corynebacterium glutamicum* for bio-based production of chemicals, fuels, materials, and healthcare products. *Metabolic engineering*, 50:122–141, (2018).

[120] Pérez-García F., Zierta C., Risseb J. M., and Wendisch V. F. Improved fermentative production of the compatible solute ectoine by corynebacterium glutamicum from glucose and alternative carbon sources. *Journal of Biotechnology*, 258:59–68, (2017).

[121] Tateno T., Fukuda H., and Kondo A. Direct production of l-lysine from raw corn starch by *Corynebacterium glutamicum* secreting *Streptococcus bovis* alpha-amylase

using cspb promoter and signal sequence. *Applied Microbiology and Biotechnology*, 77:533–541, (2007).

[122] Olsson J., Toth G. B., and Albers E. Biochemical composition of red, green and brown seaweeds on the swedish west coas. *Journal of Applied Phycology*, 32:3305–3317, (2020).

[123] Jeon E. J., Choi J. W., Cho M. S., and Jeong K. J. Enhanced production of neoagarobiose from agar with *Corynebacterium glutamicum* producing exo-type and endo-type beta-agarases. *Microbial biotechnology*, 14(5):2164–2175, (2021).

[124] Ren C. G., Liu Z. Y., Wang X. L., and Qin S. The seaweed holobiont: from microecology to biotechnological applications. *Microb Biotechnology*, 15(3):738–754, (2022).

[125] Suraiya S., Kim J. H., Tak J. Y., Siddique M. P., Young C. J., Kim J. K., and Kong I. S. Influences of fermentation parameters on lovastatin production by *Monascus purpureus* using *Saccharina Japonica* as solid fermented substrate. *Lebensmittel-Wissenschaft und-Technologie*, 92:1–9, (2018).

[126] Hakvåg S., Nærdal I., Heggeset T. M. B., Kristiansen K. A., Aasen I. M., and Brautaset T. Production of value-added chemicals by bacillus methanolicus strains cultivated on mannitol and extracts of seaweed saccharina latissima at 50 degree c. *Frontiers in microbiology*, 9(11):680, (2020).

[127] Monteiro P., Lomartire S., Cotas J., Pacheco D., Marques J. C., Pereira L., and Gonçalves A. M. M. Seaweeds as a fermentation substrate: A challenge for the food processing industry. *Biotechnological Food-Oriented Processes*, 9(11):1953, (2021).

[128] Ghanbarzadeh M., Golmoradizadeh A., and Homaei A. Carrageenans and carrageenases: versatile polysaccharides and promising marine enzymes. *Phytochemistry Reviews*, 17:535–571, (2018).

[129] Liu Z., Li G., Mo Z., and Mou H. Molecular cloning, characterization, and heterologous expression of a new kappa-carrageenase gene from marine bacterium *Zobellia* sp. zm-2. *Applied Microbiology and Biotechnology*, 97:10057–10067, (2013).

[130] Kang N. Y., Choi Y. L., Cho Y. S., Kim B. K., Jeon B. S., Cha J. Y., Kim C. H., and Lee Y. C. Cloning, expression and characterization of a beta-agarase gene from a marine bacterium, *Pseudomonas* sp. sk38. *Biotechnology Letters*, 25:1165–1170, (2003).

[131] Ha J. C., Kim G. T., Kim S. K., Oh T. K., Yu J. H., and Kong I. S. beta-agarase from *Pseudomonas* sp. w7: purification of the recombinant enzyme from *Escherichia coli* and the effects of salt on its activity. *Biotechnology and applied biochemistry*, 26(1): 1–6, (1997).

[132] Jiang C., Liu Z., Cheng D., and Mao X. Agarose degradation for utilization: Enzymes,

pathways, metabolic engineering methods and products. *Biotechnology Advances*, 45: 107641, (2020).

[133] Green E. R. and Mecsas J. Bacterial secretion systems – an overview. *Microbiol Spectr.*, 4(1):10, (2016).

[134] Liu X., Zhang W., Zhao Z., Dai X., Yang Y., and Bai Z. Protein secretion in *Corynebacterium glutamicum*. *Crit Rev Biotechnol*, 37(4):541–551, (2017).

[135] Kikuchi Y., Itaya H., Date M., Matsui K., and Wu L. F. Tatabc overexpression improves *Corynebacterium glutamicum* tat-dependent protein secretion. *Appl Environ Microbiol.*, 75(3):603–607, (2009).

[136] Signalp - 5.0, (2023). URL `https://services.healthtech.dtu.dk/services/SignalP-5.0/`. Accessed: 01.06.23.

[137] Juncker A. S., Willenbrock H., von Heijne G., Brunak S., Nielsen H., and Krogh A. Prediction of lipoprotein signal peptides in gram-negative bacteria. *Protein Science*, 12 (8):1652–1662, (2003).

[138] Lee M. J. and Kim P. Recombinant protein expression system in *Corynebacterium glutamicum* and its application. *Front Microbiol.*, 9:2523, (2019).

[139] Maurizi M. R. Proteases and protein degradation in *Escherichia coli*. *Experientia*, 48 (2):178–201, (1992).

[140] Nguyen T. H., Sunwoo I. Y., Jeong G. T., and Kim S. K. Detoxification of hydrolysates of the red seaweed *Gelidium amansii* for improved bioethanol production. *Applied Biochemistry and Biotechnology*, 188:977–990, (2019).

[141] Sakai S., Tsuchida Y., Nakamoto H., Okino S., Ichihashi O., Kawaguchi H., Watanabe T., Inui M., and Yukawa H. Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested *Corynebacterium glutamicum* r. *Applied and environmental microbiology*, 3(7):2349–2353, (2007).

[142] Tsuge Y., Hori Y., Kudou M., Ishii J., Hasunuma T., and Kondo A. Detoxification of furfural in *Corynebacterium glutamicum* under aerobic and anaerobic conditions. *Applied Microbiology and Biotechnology*, 98:8675–8683, (2014).

[143] Wang X., Khushk I., Xiao Y., Gao Q., and Bao J. Tolerance improvement of *Corynebacterium glutamicum* on lignocellulose derived inhibitors by adaptive evolution. *Applied microbiology and biotechnology*, 102(1):377–388, (2018).

[144] Engels V. and Wendisch V. F. The deor-type regulator sugr represses expression of ptsg in *Corynebacterium glutamicum*. *Journal of Bachteriology*, 189(8):2955–2966, (2007).

[145] Pérez-García F., Peters-Wendisch P., and Wendisch V. F. Engineering corynebacterium

glutamicum for fast production of l-lysine and l-pipecolic acid. *Applied microbiology and biotechnology*, 100(18):8075–8090, (2016).

[146] Jorge J. M. P., Leggewie C., and Wendisch V. F. A new metabolic route for the production of gamma-aminobutyric acid by *Corynebacterium glutamicum* from glucose. *Amino Acids*, 48:2519–2531, (2016).

[147] Michel A., Koch-Koerfges A., Krumbach K., Brocker M., and Bott M. Anaerobic growth of *Corynebacterium glutamicum* via mixed-acid fermentation. *Applied and Environmental Microbiology*, 81(21), (2015).

[148] Nishimura T., Vertès A. A., Shinoda Y., Inui M., and Yukawa H. Anaerobic growth of *Corynebacterium glutamicum* using nitrate as a terminal electron acceptor. *Applied Microbiology and Biotechnology*, 75:889–897, (2007).

[149] Stecchini M. L., Del Torre M., Donda S., Maltini E., and Pacor S. Influence of agar content on the growth parameters of *Bacillus cereus*. *International Journal of Food Microbiology*, 64(1-2):81–88, (2001).

[150] Ko Y. J., Park E., Song J., Yang t. H., Lee H. J., Kim K. H., and Choi I. G. Functional cell surface display and controlled secretion of diverse agarolytic enzymes by *Escherichia coli* with a novel ligation-independent cloning vector based on the autotransporter yfal. *Applied and Environmental Microbiology*, 78(9):4–11, (2012).

[151] Seo Y. B., Lu Y., Chi W. J., Park H. R., Jeong K. J., Hong S. K., and Chang Y. K. Heterologous expression of a newly screened beta-agarase from *Alteromonas* sp. gnum1 in *Escherichia coli* and its application for agarose degradatio. *Process Biochemistry*, 49 (3):430–436, (2014).

[152] Yoon S. Y., Lee H. M., Kong J. N., and Kong K. H. Secretory expression and enzymatic characterization of recombinant *Agarivorans albus* beta-agarase in *Escherichia coli*. *Preparative Biochemistry and Biotechnology*, 47(10):1037–1042, (2017).

[153] Lee D. G., Jang M. K., Lee O. H., Kim N. Y., Ju S. A., and Lee S. H. Over-production of a glycoside hydrolase family 50 beta-agarase from *Agarivorans* sp. ja-1 in *Bacillus subtilis* and the whitening effect of its product. *Biotechnology Letters*, 30:911–918, (2008).

[154] Xie W., Lin B., Zhou Z., Lu G., Lun J., Xia C., Li S., and Hu Z. Characterization of a novel beta-agarase from an agar-degrading bacterium *Catenovulum* sp. x3. *Applied Microbiology and Biotechnology*, 97:4907–4915, (2013).

[155] Chen J., Fu G., Gai Y., Zheng P., Zhang D., and Wen J. Combinatorial sec pathway analysis for improved heterologous protein secretion in *Bacillus subtilis*: identification of bottlenecks by systematic gene overexpression. *Microbial Cell Factories*, 14(92), (2015).

[156] Jurischka S., Bida A., Dohmen-Olma D., Kleine B., Potzkei J., Binder S., Schaumann G., Bakkes P. J., and Freudl R. A secretion biosensor for monitoring sec-dependent protein export in *Corynebacterium glutamicum*. *Microbial Cell Factories*, 19:11, (2020).

[157] Juncker A. S., Willenbrock H., von Heijne G., Brunak S., Nielsen H., and Krogh A. Identification of enzymes responsible for extracellular alginate depolymerization and alginate metabolism in *Vibrio algivorus*. *Applies Microbiology and Biotechnology*, 101 (4):1581–1592, (2017).

[158] Wargacki A. J., Leonard E., Win M. N., Regitsky D. D., Santos C. N. S., Kim P. B., Cooper S. R., Raisner R. M., Herman A., Sivitz A. B., Lakshmanaswamy A., Kashiyama Y., Baker D., and Yoshikuni Y. An engineered microbial platform for direct biofuel production from brown macroalgae. *Science*, 335(6066):308–313, (2012).

[159] Poole C. F. Thin-layer chromatography: challenges and opportunities. *Journal of Chromatography A*, 1000(1-2):963–984, (2003).

[160] Biochemical characterization and mutational studies of a novel 3-methlyadenine dna glycosylase ii from the hyperthermophilic thermococcus gammatolerans. *DNA Repair*, 97, (2021).

[161] Domon B. and Aebersold R. Mass spectrometry and protein analysis. *Science*, 312 (5771):212–217, (2006).

[162] Dołowy M. and Pyka A. Application of tlc, hplc and gc methods to the study of amino acid and peptide enantiomers: a review. *Biomedical Chromatography*, 28(1):84–101, (2014).

[163] Dobruchowska J. M., Bjornsdottir B., Fridjonsson O. H., Altenbuchner J., Watzlawick H., Gerwig G. J., Dijkhuizen1 L., Kamerling J. P., and Hreggvidsson G. O. Enzymatic depolymerization of alginate by two novel thermostable alginate lyases from *Rhodothermus marinus*. *Front. Plant Sci.*, 13, (2022).

[164] Tsevelkhoroloo M., Dhakshnamoorthy V., Hong Y. S., Lee C. R., and Hong S. K. Bifunctional and monofunctional alpha-neoagarooligosaccharide hydrolases from *Streptomyces coelicolor* a3(2). *Applied Microbiology and Biotechnology*, 107:3997–4008, (2023).

[165] Freudl R. Signal peptides for recombinant protein secretion in bacterial expression systems. *Microbial Cell Factories*, 17(52), (2018).

[166] Grimsley G. R. and Pace C. N. Spectrophotometric determination of protein concentration. *Current protocols in protein science*, (2003).

A Recipes for media and solutions

This section contains recipes for the media and chemicals used in this study.

A.1 Complex media

LB

LB media was prepared by adding the components in Table A.1 to water while stirring. The antibiotic tetracycline was added when cultivating cells harboring the pECXT99a plasmid. Both kanamycin and tetracycline was added when cultivating cells carrying both the pECXT99a and pVWEX1 plasmids. After preparation, the mixtures were autoclaved at 121°C for 15 minutes.

Table A.1: Components in the LB medium.

Chemical component	Concentration [g/L]	Measure	Unit
Yeast extract	10.0	5.0	g
Tryptone	16.0	8.0	g
NaCl	5.0	2.5	g
MQ water	-	500	mL
Final volume of the solution		500	mL

2TY

2YT is a standard microbial growth medium used for cultivation of *E. coli* and *C. glutamicum*. It media was prepared by adding the components in Table A.2 to water while stirring. The antibiotic tetracycline was added when cultivating cells harboring the pECXT99a plasmid. Both kanamycin and tetracycline was added when cultivating cells carrying both the pECXT99a and pVWEX1 plasmids. After preparation, the mixtures were autoclaved at 121°C for 15 minutes. 2TY plates were prepared by adding 15 g/L agar to the solution under preparation.

Table A.2: Components in the 2TY medium.

Chemical component	Concentration [g/L]	Measure	Unit
Yeast extract	10.0	10.0	g
Tryptone	16.0	16.0	g
NaCl	5.0	5.0	g
MQ water	-	500	mL
Final volume of the solution		500	mL

BHI

Table A.3 gives an overview of the chemical components used in the preparation of the BHI solution. After preparation, the mixtures were autoclaved at 121°C for 15 minutes. BHI was used when cultivating *C. glutamicum* to prepare competent cells.

Table A.3: Components in the BHI medium.

Chemical component	Concentration [g/L]	Measure	Unit
BHI	37.00	14.8	g
MQ water	-	400	mL
Final volume of the solution		400	mL

Sorbitol

The sorbitol solution was prepared following the recipe of Table A.4. After preparation, the mixtures were autoclaved at 121°C for 15 minutes. Sorbitol was used in the preparation of BHIS which was further used for cultivation during the making of competent cells.

Table A.4: Components in the Sorbitol solution.

Chemical component	Concentration [g/L]	Measure	Unit
D-Sorbitol	455	45.5	g
MQ water	-	100	mL
Final volume of the solution		100	mL

BHIS

BHIS was used whenever competent *C. glutamicum* or *C. glutamicum* (pVWEx1-*galETKM-galP*) cells was prepared. It was prepared by mixing BHI and sorbitol media in a ratio of 1:5.

A.2 Selective media

CGXII salt solution

Table A.5 provides an overview of the chemicals needed to produced the salt solution CGXII. The pH of the solution was corrected to 7 with potassium hydroxide before the final volume was adjusted. After preparation, the mixtures were autoclaved at 121°C for 15 minutes. The CGXII salt solution was used when growth experiments was preformed.

Table A.5: The chemicals of the CGXII salt solution.

Chemical component	Concentration	Unit	Measure	Unit
$(NH_4)_2SO_4$	10	g/L	10	g
KH_2PO_4	1	g/L	1	g
K_2HPO_4	1	g/L	1	g
UREA	5	g/L	5	g
MOPS	42	g/L	42	g
Ca-stock 1000X	1	mL/L	1	mL
Mg-stock 1000X	1	mL/L	1	mL
MQ water	-	-	795	mL
KOH 5 M - pH correction to 7			Needed amount	
Final volume of the solution			795	mL

Glucose 40%

The stock solution of glucose 40% was prepared using the recipe of table Table A.6. Glucose 40% was further diluted and used in preparation of flask media when preforing growth experiments.

Table A.6: Glucose 40% preparation as glucose stock solution.

Chemical component	Concentration [g/L]	Measure	Unit
Glucose	0.4	200	g
MQ water	-	500	mL
Final volume of the solution		500	mL

Mg-stock 1000X

The Mg-stock stock 1000X solution was prepared after the Table A.7. After preparation the solution was sterilized by filtration.

Table A.7: Components in the Mg-stock 1000X solution.

Chemical component	Concentration [g/L]	Measure	Unit
$MgSO_4$ x $7H_2O$	250	12.5	g
MQ water	-	10	mL
Final volume of the solution		50	mL

Ca-stock 1000X

The Ca-stock stock 1000X solution was prepared after the Table A.8. After preparation the solution was sterilized by filtration.

Table A.8: Components in the Ca-stock 1000X solution.

Chemical component	Concentration [g/L]	Measure	Unit
$CaCl_2$ x $2H_2O$	13.25	0.6625	g
MQ water	-	10	mL
Final volume of the solution		50	mL

Biotin

The biotin solution was prepared according to the Table A.9. After preparation, the solution was sterilized by filtration before being kept on storage at -20°C.

Table A.9: Components in the biotin solution.

Chemical component	Concentration	Measure	Unit
Biotin	0.2 g/L	0.002	g
NaOH	1 M	10	mL
Final volume of the solution		10	mL

Protocatechuic acid (PCA)

The PCA solution was prepared according to the Table A.10. After preparation, the solution was sterilized by filtration before being kept on storage at -20°C.

Table A.10: Components in the PCA solution.

Chemical component	Concentration	Measure	Unit
3,4-dihydroxybenzoic acid	30 g/L	0.3	g
NaOH	1 M	10	mL
Final volume of the solution		10	mL

Isopropyl β-D-1-thiogalactopyranoside (IPTG)

The IPTG solution was prepared according to the Table A.11. After preparation, the solution was sterilized by filtration before being kept on storage at -20°C.

Table A.11: Components in the PCA solution.

Chemical component	Concentration	Measure	Unit
IPTG	238.31 g/L	2.38	g
MQ water	-	10	mL
Final volume of the solution		10	mL

Trace element solution (TES)

The TES solution was prepared according to the Table A.12. pH was adjusted to 1 with 0.5 M Hcl. After preparation, the solution was sterilized by filtration before being kept on storage at 4°C.

Table A.12: Components in the TES solution.

Chemical component	Concentration [g/L]	Measure	Unit
$FeSO_4$ x $7H_2O$	16.40	1.640	g
$MnSO_4$ x H_2O	10.00	1.000	g
$ZnSO_4$ x $7H_2O$	1.00	0.100	g
$CuSO_4$ x $5H_2O$	0.31	0.031	g
$NiCl_2$ x $6H_2O$	0.02	0.002	g
Final volume of the solution		100	mL

Flask media

Flask media was prepared according to the Table A.13 and used for cultivation of cells when conducting growth experiments.

Table A.13: An overview of the ingredients of the components in the minimal media used in growth experiments.

Chemical component	Measure	Unit
CGXII	20	mL
Glucose 1%	625	μL
Biotin	25	μL
PCA	25	μL
IPTG 0.5M	25	μL
Trace element solution	25	μL
Kanamycin	25	μL
Sterile water	25	mL
Final volume of the solution	25	mL

A.3 Antibiotics and EBP buffers

Tetracycline stock solution

To prepare the tetracycline stock solution the antibiotics was diluted in ethanol (Table A.14). The stock solution was sterilized utilizing filtration and stored at -20°C.

Table A.14: Chemicals used in preparation of tetracycline stock solution.

Chemical component	Concentration	Measure	Unit
Tetracycline	10.0 g/L	0.1	g
Ethanol	70%	10	mL
Final volume of the solution		10	mL

Kanamycin stock solution

To prepare the kanamycin stock solution the antibiotics was diluted in ethanol (Table A.15). The stock solution was sterilized utilizing filtration and stored at -20°C.

Table A.15: Chemicals used in preparation of kanamycin stock solution.

Chemical component	Concentration	Measure	Unit
Kanamycin	50.0 g/L	0.5	g
Ethanol	70%	10	mL
Final volume of the solution		10	mL

EPB buffers

The EPB buffers 1 and 2 was utilized in the making of competent *C. glutamicum* or *C. glutamicum* (pVWEx1-*galETKM-galP*) cells. Table A.16 shows the recipe for preparing the two buffers. NaOH 1 M was utilized to adjust the pH of the solution to 7.2 before the the mixtures were autoclaved at 121°C for 15 minutes for sterilization.

Table A.16: Components of the EPB1 and EPB2 buffers.

Chemical component	Measure		Unit
	EPB1	**EPB1**	
HEPES	4.04	0.06	g
Glycerin 89%	36.6	6.85	mL
MQ water	800	50	mL
NaOH 0.1 M - pH correction to 7.2	Needed amount	Needed amount	
Final volume of the solution	800	50	mL

B Calculations

B.1 Example calculation of protein concentration using Bradford assay

After preforming growth experiment on the strains constructed for agar degradation, the concentration of proteins in the collected supernatant was calculated by Bradford assay. To prepare a standard curve, six different concentrations with bovine serum albumin (BSA) was used to construct a standard curve. BSA was diluted to six different concentrations and the measured OD_{595} is given in Table B.1.

Table B.1: BSA concentrations (mg/mL) and measured absorbance at 595 nm used for constructing a standard curve.

BSA concentration [mg/ml]	Absorbance (595 nm)
2.0000	1.126
1.0000	0.727
0.5000	0.518
0.2500	0.303
0.1250	0.157
0.0625	0.042

The standard curve was generated by plotting the BSA concentration against their measured absorbance at 595 nm as can be seen in Figure B.1. A linear trend line was fitted to the data points and used for further calculations of the protein concentrations of the collected supernatants. To find the best fitted trend line (R^2 = 0.9795), the data points of the two highest concentrations was excluded.

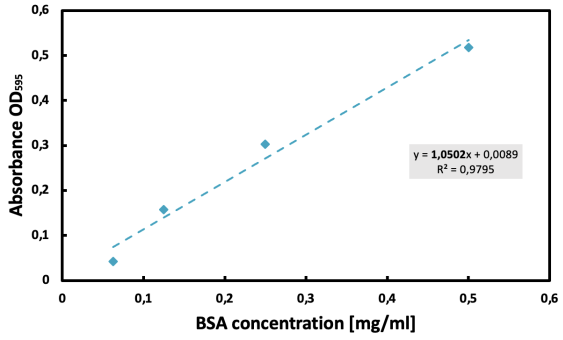


Figure B.1: Standard curve (R^2 = 0.9795) of 4 different BSA concentrations and their respective absorbance measured at 595 nm. Constructed for calculation of the protein concentration in the supernatants of agar degrading strains using Bradford assay. Obtained linear equation: y = 1.0502x + 0.0089

To calculate the protein concentrations, Beer-Lamberts law was assumed to be valid stating that the concentration of the solution is proportional to the measured absorbance within the linear area[166]. An example on how the concentrations of the secreted protein (C) was calculated after dilution 1:10 is given in Equation B.1.

$$\text{Concentration of secreted protein} = \frac{\text{OD}_{595} - \text{b}}{\text{a}} \cdot \text{dilution factor}$$

$$\text{C} = \frac{0.021 - 0.0089}{1.0502} \cdot 10$$

$$\text{C} = 0.125 \text{ mg/mL} \tag{B.1}$$

B.2 Example calculation of growth rate and biomass

When preforming growth experiments, the growth rate was calculated when the bacteria was in the exponential phase. To illustrate this, Figure B.2 A shows the growth rate of *C. glutamicum* (pECXT99a-*dagAopt-ahgA*) in CGXII media with 1% glucose where the exponential phase lasts from 2 to 8 hours after inoculation. Figure B.2 B displays the data points for the exponential growth phase with an linear fitted trend line (R^2=0.9955). The growth rate was extracted from the slope of the obtained equation y = 0.5755e$^{0.3244}$ which in this case indicates that the growth rate is 0.32 h^{-1}.

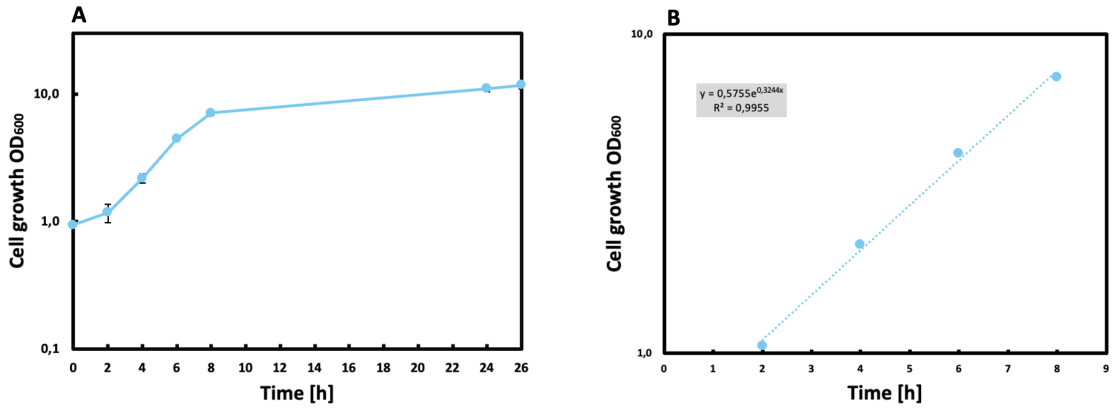


Figure B.2: **A**: Growth curve OD_{600} of *C. glutamicum* (pECXT99a-*dagAopt-ahgA*) in CGXII meda with 1 % glucose. **B**: Exponential fitted trend line (R^2 = 0.9955) for the exponential phase for calculations of growth rate using the linear equation y = 0.5755e$^{0.3244}$.

The biomass yield Y_{XS} was calculated based on the highest OD_{600} measurements. This value is multiplied by a factor of 0.343 as shown in Equation B.2. This is the biomass result for growth of *C. glutamicum* (pECXT99a-*dagAopt-ahgA*) with the highest observed OD_{600} at 11.767.

$$\text{Biomass yield} = \text{Final biomass} \cdot 0.343$$

$$Y_{XS} = 11.767 \cdot 0.343 = 4.04 \tag{B.2}$$

Calculation of the standard deviation σ was done according to equation Equation B.3

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})}{n-1}} \tag{B.3}$$

n represents the total number of samples. x_i is the value og sample i, while $\bar{x}$ is the average value of all samples taken. The latter was calculated using Equation B.4.

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \tag{B.4}$$

C Production of agarases in *C. glutamicum*

C.1 pECXT99a isolation and restriction

Figure C.1 shows a circular and linearized pECXT99a (7509 bp), displayed in well 1 and 2, respectively. Successful linearization is confirmed by a single band after BamHI-digestion of the circular plasmid.

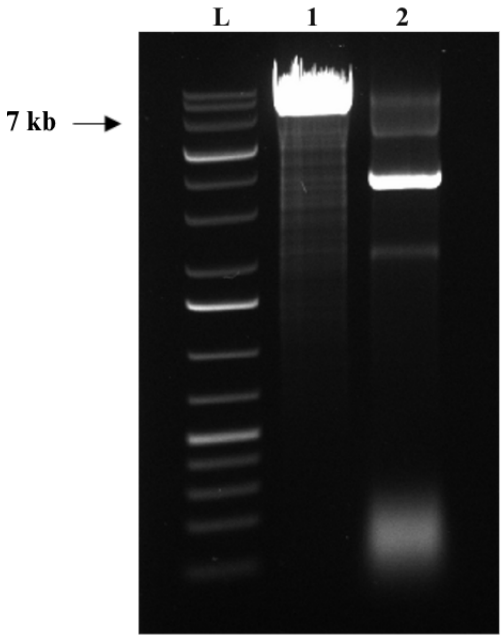


Figure C.1: Agarose gel electrophoresis displaying circular (well 1) and BamHI digested (well 2) pECXT99a plasmid of 7509 bp. The wells denoted "L" contains the Thermo Scientific™ O'Generuler 1 kb DNA ladder[112].

D Plasmid maps

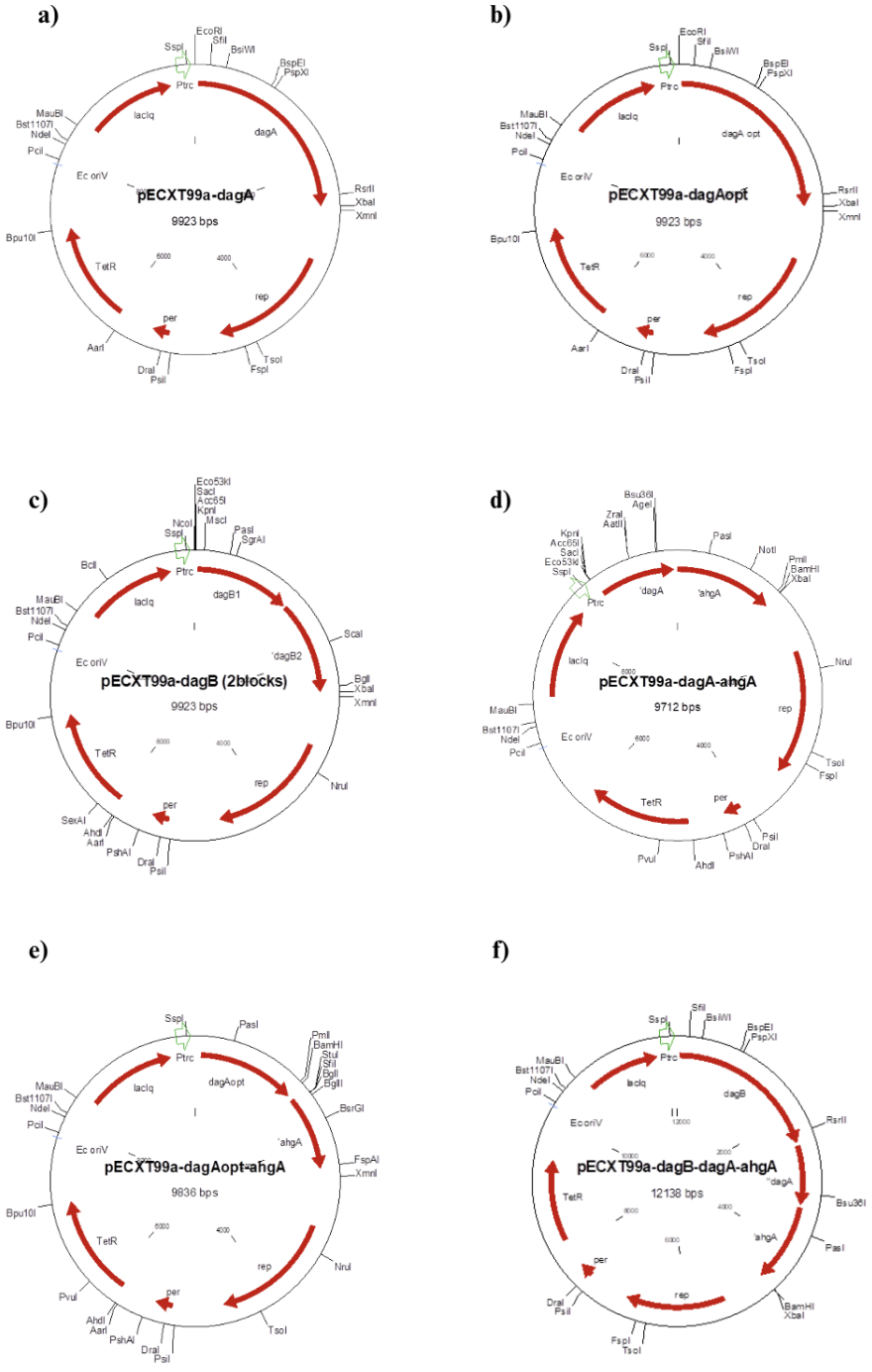
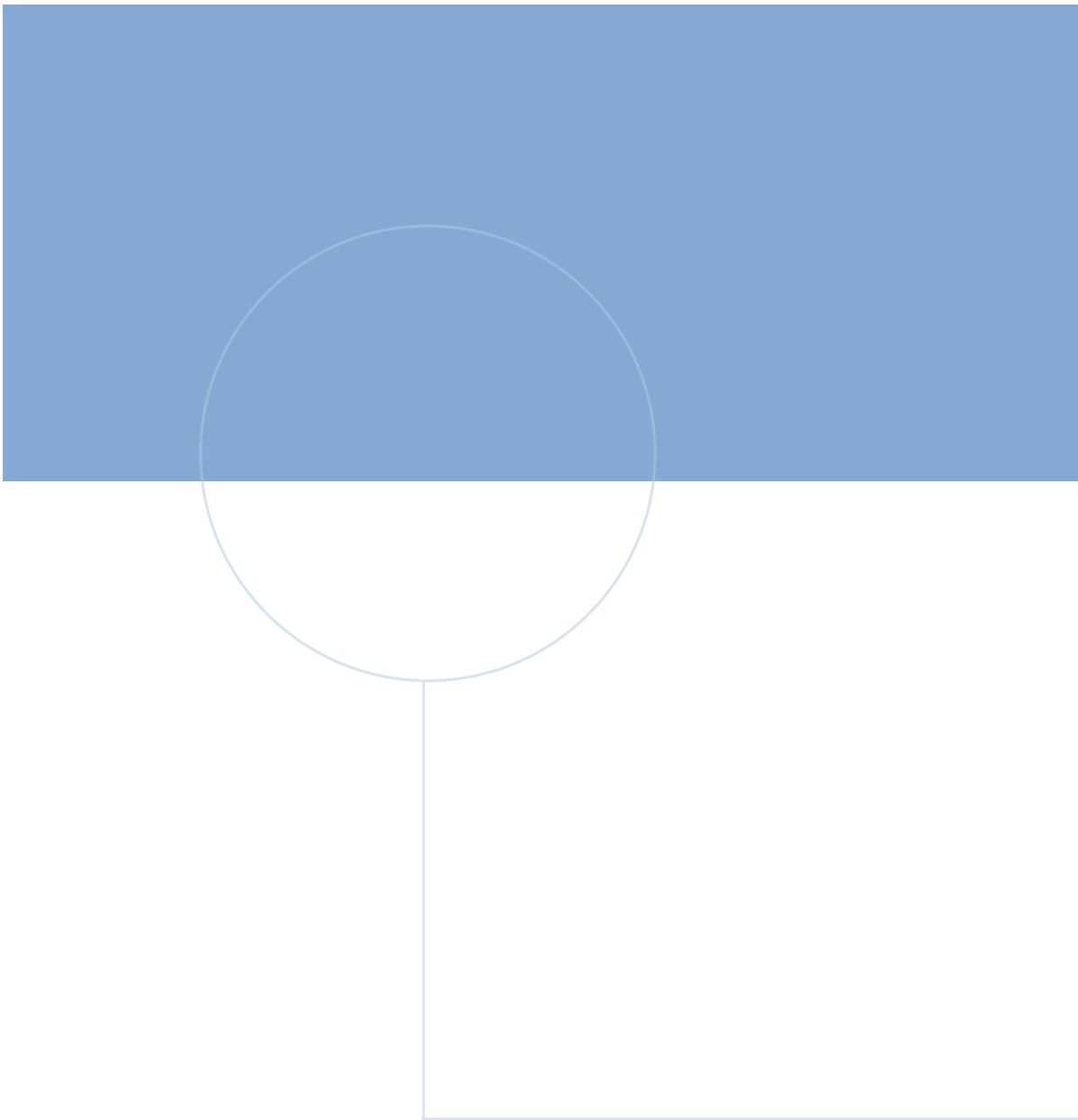


Figure D.1: Plasmid maps for agarase production: **a)** pECXT99a-(*dagA*), **b)** pECXT99a-(*dagAopt*), **c)** pECXT99a-(*dagB*), **d)** pECXT99a-(*dagA-ahgA*), **e)** pECXT99a-(*dagAopt-ahgA*) and **f)** pECXT99a-(*dagB-dagA-ahgA*)



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