Cyanobacteria as a novel green route for maximizing the utilization of insect meals as feed ingredient for fish in Aquaculture

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Resumo

A crescente procura por produtos do mar exige uma pressão contínua sobre a Aquacultura. Para garantir dietas sustentáveis, os insetos representam uma fonte promissora de ingredientes para alimentação. No entanto, o seu potencial é limitado pela presença de quitina, um polissacarídeo estrutural do exoesqueleto dos insetos. A quitina é indigestível, apresenta uma estrutura semelhante à das fibras e atua como um componente antinutricional que impede os peixes, como o robalo-europeu, de absorver nutrientes, interferindo particularmente na utilização de proteínas e lípidos.

O objetivo deste trabalho foi avaliar o potencial da cianobactéria modelo *Synechocystis* sp. PCC 6803 (S6803) como plataforma biológica para produzir quitinases, com a finalidade de a incorporar em dietas para robalo-europeu contendo farinha de inseto (FI), aumentando a digestibilidade da quitina no trato intestinal dos peixes e, consequentemente, melhorando a bioacessibilidade das proteínas e lípidos nela retidos. Para tal, foram utilizadas estirpes de S6803 geneticamente modificadas que expressam uma proteína heteróloga, a quitinase, capaz de hidrolisar o polímero de quitina. Ambas as estirpes, bem como uma estirpe selvagem de S6803, foram incorporadas a 1.15% em dietas para robalo-europeu com níveis elevados de FI (30% de inclusão, *Hermetia illucens*), sendo utilizada a mesma dieta à base de FI como controlo. Cada dieta foi administrada a grupos triplicados de peixes (peso inicial: 27 g) durante 10 dias.

Com estas dietas, foi realizado um ensaio de digestibilidade para determinar os coeficientes de digestibilidade aparente (CDA) da matéria-seca, proteína, quitina e energia das dietas, assim como as atividades das enzimas digestivas amílase, lípase e protéases totais alcalinas no intestino. Foi igualmente avaliado o efeito da biomassa das cianobactérias no estado oxidativo dos peixes através da determinação da atividade de enzimas chave do stress oxidativo no fígado.

Foi testada previamente a atividade quitinolítica dos diferentes tratamentos para avaliar a sua atividade após liofilização, assim como o crescimento das culturas ao longo do tempo e ao longo dos diferentes volumes (120 mL, 0,6 L, 8 L). Assim, esses resultados mostraram que a atividade quitinolítica mantém-se depois da liofilização, uma perspetiva positiva para o futuro da produção de S6803 numa escala maior. Contudo, o volume onde se obteve a produção mais rápida foi o 0,6 L, um volume pequeno quando o objetivo é produzir em grande escala. Assim, é necessário otimizar o volume de produção para alcançar um crescimento mais rápido. Os resultados não revelaram diferenças significativas entre os tratamentos experimentais e a dieta controlo para

nenhum dos parâmetros de digestibilidade ou das atividades enzimáticas avaliadas, bem como no estado oxidativo dos peixes.

Estes resultados indicam que a inclusão de S6803, nas condições testadas, não resultou em melhorias na digestibilidade da quitina e dos restantes nutrientes da dieta ou no aumento da capacidade antioxidante dos peixes. Investigações futuras devem focar-se no aumento da expressão enzimática, na otimização da entrega e na estabilidade do gene-alvo. Este trabalho contribui para o esforço mais amplo de desenvolver alimentos de aquacultura sustentáveis e melhorados por biotecnologia, particularmente no contexto de dietas que incluem ingredientes derivados de insetos.

Palavras-chave: Cianobactérias; Quitinase; Farinha de Inseto; Nutrição; Aquacultura Sustentável

Abstract

The increasing demand for seafood products places constant pressure on Aquaculture. To ensure sustainable diets, insects represent a promising source of feed ingredients. However, their potential is limited by the presence of chitin, a structural polysaccharide essential to the insect exoskeleton. Chitin is highly indigestible, has a fibre-like structure, and acts as an antinutritional component that prevents fish, such as European seabass (ESB), from absorbing nutrients, particularly interfering with the utilization of proteins and lipids.

The aim of this work was to evaluate the potential of the model cyanobacterium *Synechocystis* sp. PCC 6803 (S6803) as a biological platform to produce chitinases, with the purpose of incorporating this cyanobacterium into European seabass diets containing insect meal (IM), thereby increasing chitin digestibility in the fish intestinal tract and, consequently, improving the bioaccessibility of the proteins and lipids trapped within it. For this purpose, genetically modified strains of S6803 expressing a heterologous protein - chitinase, an enzyme capable of hydrolyzing the chitin polymer were used. Both engineered strains, as well as a wild-type (WT) strain of S6803, were incorporated at 1.15% into diets for European seabass with high levels of IM inclusion (30% *Hermetia illucens*), and the same IM-based diet was used as a control. Each diet was administered to triplicate groups of fish (initial weight: 27 g) for 10 days.

Using these diets, a digestibility trial was carried out to determine the apparent digestibility coefficients (ADCs) of dry matter, protein, chitin and energy, as well as the activities of the digestive enzymes' amylase, lipase and total alkaline proteases in the intestine. The effect of the cyanobacterial biomass on the oxidative status of the fish was also evaluated by determining the activity of key oxidative stress enzymes in the liver.

The chitinolytic activity of the different treatments was previously tested to assess their activity after lyophilization, as well as the growth of the cultures over time and across different volumes (120 mL, 0.6 L, 8 L). These results showed that chitinolytic activity is maintained after lyophilization, a positive outlook for the future of large-scale S6803 production. However, the fastest production was obtained in the 0.6 L volume, which is small when the goal is large-scale production. Therefore, optimization of the most suitable production volume for faster growth must be improved.

The results did not reveal significant differences between the experimental treatments and the control diet for any of the digestibility parameters or the enzymatic activities assessed, nor in the oxidative status of the fish.

These results indicate that the inclusion of S6803, under the conditions tested, did not lead to improvements in chitin digestibility or in the digestibility of the remaining dietary nutrients, nor to an increase in the antioxidant capacity of the fish. Future research should focus on increasing enzyme expression, optimising delivery, and improving the stability of the target gene. This work contributes to the broader effort to develop sustainable, biotechnology-enhanced aquaculture feeds, particularly in the context of diets that include insect-derived ingredients.

Keywords: Cyanobacteria; Chitinase; Insect Meal; Nutrition; Sustainable Aquaculture

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List of Abbreviations

4MU – 4-methylumbelliferone

ADC – Apparent digestibility coefficients

AOAC – Association of Official Analytical Chemists

BG11 – Blue green medium

BHT – Butylated hydroxytoluene

BOGA – Aquatic Organism Bioterium

BSA – Bovine albumin serum

CAT – Catalase

CIIMAR – Interdisciplinary Center for Marine and Environmental Research

CL – Crude lipid

CP – Crude protein

CTR – Control diet

DHA – Docosahexaenoic acid

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediaminetetraacetic acid

EPA – Eicosapentaenoic acid

ESB – European Seabass

FAO – The Food and Agriculture Organization

FM – Fish meal

FO – Fish oil

GlcNac – N-acetylglucosamine

GR – Glutathione reductase

GPx – Glutathione peroxidase

HCl – Hydrochloric acid

IM – Insect Meal

LMM – Linear mixed model

N – Nitrogen

NADPH and NADP+ – Nicotinamide Adenine Dinucleotide Phosphate (Reduced and Oxidized)

NaOH – Sodium hydroxide

Np*chiA* – *Nostoc punctiforme* PCC 73102 chitinase encoding gene A

NpChiA - *Nostoc punctiforme* PCC 73102 chitinase

OD_{730} – Optical density at 730 nm

PCR – Polymerase Chain Reaction

PF – Plant-based feedstuffs

PUFAs – Polyunsaturated fatty acids

ROS – Reactive oxygen species

RT – Room Temperature

S6803 – *Synechocystis* sp. PCC 6803

SD – Standard deviation

SL9 – genetically modified *Synechocystis* sp. PCC 6803 strain harboring the plasmid pSEVA251 with the promoter $P_{trc.x.lacO}$ driving the expression of the Np*chiA* gene

SR1 – *Synechocystis* sp. PCC 6803 harboring the plasmid pSEVA251 with the promoter P_{rbcL} driving the expression of the Np*chiA* gene

TBA – Thiobarbituric acid

TBARS – Thiobarbituric acid-reacting substances

TBS – Tris-buffered saline solution

TCA - Trichloroaceitic acid

UV – Ultraviolet

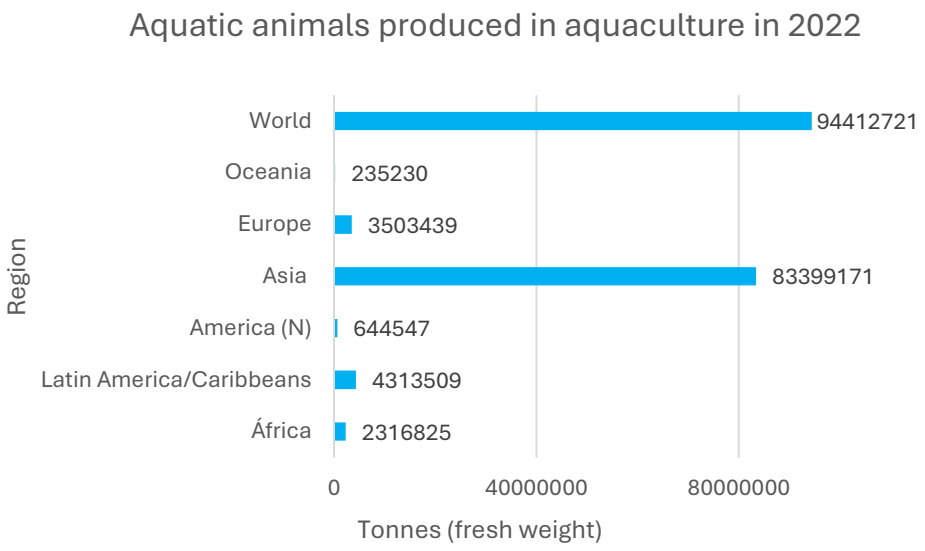
WT – wild-type

1. Introduction

1.1. Global aquaculture production

Aquaculture is a key global sector that supports nutrition and food security, helping to address hunger and malnutrition, especially in coastal communities. Global aquaculture output kept rising throughout 2020, 2021, and 2022. Nonetheless, growth varied across regions, countries, and territories, reflecting significant differences in production scale, distribution, farming practices, efficiency, and management approaches. World aquaculture production reached a record 130.9 million tonnes in 2022, an increase of 8.1 million tonnes compared to the 122.8 million tonnes recorded in 2020. This included around 94.4 million tonnes of aquatic animals (live-weight equivalent) and 36.5 million tonnes of algae (wet weight) (Figure 1A and B) (FAO, 2024). Furthermore, for the first time in history, in 2022, aquaculture surpassed capture fisheries (91 million tonnes) in aquatic animal production, reaching 94.4 million tonnes (Figure 2). Of the 94.4 million tonnes, Asian countries produced 87.9% of the total aquaculture production of aquatic animals (China, India, and Indonesia), followed by countries in Latin America and the Caribbeans (Chile and Ecuador) (7.3%), Europe (Norway, Spain, France, Greece, and Italy) (3.5%), Africa (0.8%), North America (0.4%) and Oceania (0.2%) (FAO, 2024). Overall, this reflects a shift in dietary habits by populations, which leads to a growing demand for aquatic foods driven by the increasing world population.

(A)



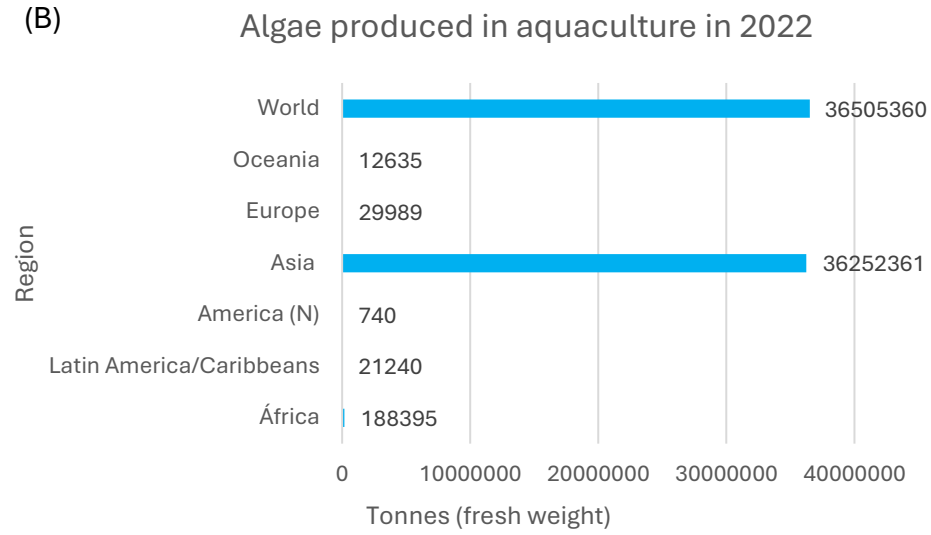


Figure 1. Total aquaculture production of aquatic animals (A) and algae (B) in 2022, by region (tonnes). Data taken from FAO (2024).

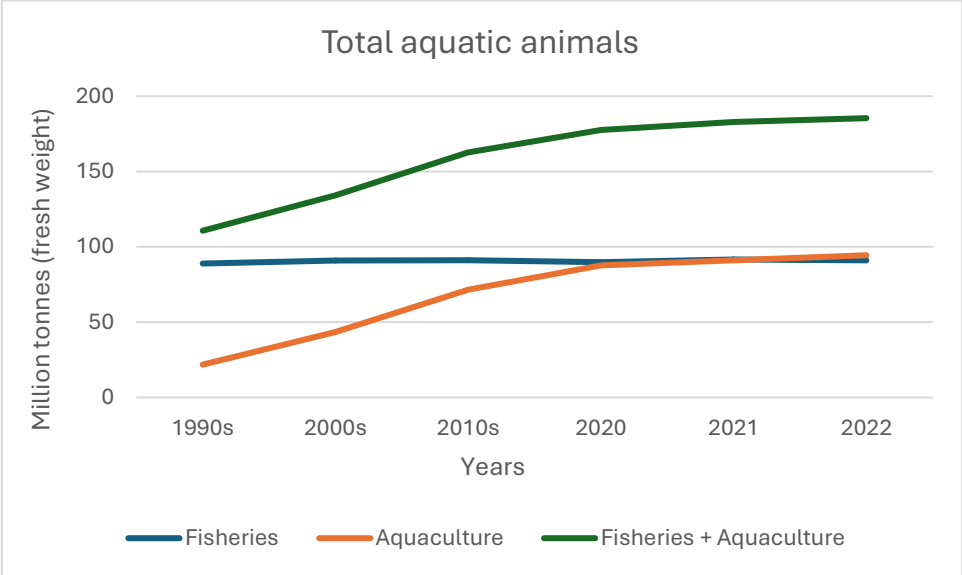


Figure 2. Aquaculture and fisheries trends over the years: total aquatic animals in fisheries and aquaculture (million tonnes). Data taken from FAO (2024).

This can be explained following the stabilization of fisheries since the 90's (Figure 2), while aquaculture has been steadily growing during the same period. In fact, aquaculture is one of the fastest-expanding industries in the world, with growth rates exceeding 10% each year (Olmos Soto, 2017). However, it is mostly Asian countries that utilize aquaculture production to its full potential, contributing up to 87.9% of the total (Figure 3), with Latin America and Europe following behind.

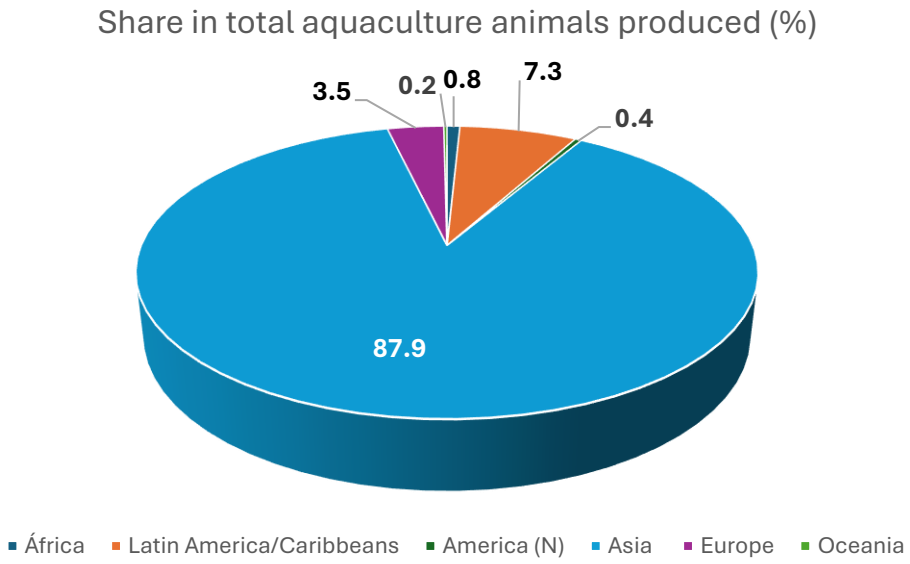


Figure 3. Share in world total aquaculture animals produced by region (percentage). Data taken from FAO (2024).

Moreover, only ten countries (China, Indonesia, India, Vietnam, Bangladesh, Norway, Egypt, Chile, Myanmar, and Ecuador) account for around 88% of total aquaculture production (FAO, 2024), indicating substantial room for growth in other regions. As for the Mediterranean and Black Sea countries, aquaculture production reached 2.97 million tonnes in 2023 (FAO, 2025). In the case of Portugal it represents only 20.872 tonnes of aquaculture production in 2023, an increase of 10,9% when compared to the previous year (INE, 2025). Ultimately, expanding aquaculture in these areas could enhance competitiveness within the sector, increase product availability, and help reduce prices.

1.2. European Seabass production in aquaculture

European Seabass (*Dicentrarchus labrax*) or ESB for short, is a carnivorous fish found in the Northeast Atlantic and Mediterranean Sea. It inhabits a variety of coastal environments, including estuaries, rivers and brackish water lagoons. As such, it can support high temperature and salinity variations (5-28ºC and 3-35‰, respectively). Their breeding season occurs during the winter (December to March) in the Mediterranean populations and up to June for the Atlantic populations. Their pelagic eggs are present in littoral areas with high salinity or estuaries. They prey on crustaceans, mollusks, and smaller fish. It has a silvery-grey body and highly valued delicate flesh, being highly valued by European consumers.

Although ESB can be farmed in seawater ponds, the bulk of production comes from sea cages and tanks in a flow-through system. During the late 1960s, France and Italy competed to develop reliable mass-production techniques for juvenile ESB, and, by the late 1970s, these techniques were developed well enough in most Mediterranean countries to provide hundreds of thousands of larvae. ESB was the first marine non-salmonid species to be commercially cultured in Europe and at present is the most important commercial fish widely cultured in Mediterranean areas (FAO, 2015). Greece, Turkey, Italy, Spain, Croatia, and Egypt are the biggest ESB producers. In Portugal it was the third most produced marine fish in 2023, with 1703 tonnes produced, a growth of 15% when compared to the previous year. Seabass production was followed by seabream (2798 tonnes) and turbot (3209 tonnes) (INE, 2025).

ESB is particularly valuable because formulated diets are available that can be used from first feeding under experimental conditions. These diets do not compromise growth when compared with live feeds, making ESB an excellent model species for nutrition studies (Carter, 2015).

1.3. Importance of fish meal and fish oil in fish nutrition

Fish meal (FM) and fish oil (FO) are highly nutritious ingredients and key components of aquaculture feeds, supplying essential amino acids and lipids that fish cannot synthesize and must obtain from the diet (FAO, 2024; Olmos Soto, 2017). Among these lipids are polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), long-chain omega-3 fatty acids that provide consumers with numerous health benefits, including improved cardiac function, reduced blood pressure, enhanced cognitive performance, and reduced symptoms of depression. They also play a role in preventing or modulating metabolic and inflammatory diseases, such as type 2 diabetes and cancer (Calder, 2015).

FM and FO are derived from small pelagic fish like anchovy, herring, sardines, mackerel, which are primarily harvested and processed for feed production (FAO, 2024). Their high-quality protein and fatty acid contents drive to a high demand contributing to the depletion of these fish stocks. Given that these stocks are finite and that fish for aquaculture feed competes with other industries such as poultry, livestock and pig farming, the availability of FM and FO is likely to decline, leading to higher feed prices (Olmos Soto, 2017). Since feed costs represent the largest portion of aquaculture production expenses (50 to 70%), reducing them is essential (Olmos Soto, 2017);

however, alternative ingredients must still provide all species with the necessary nutrients and energy.

Plant-based feedstuffs (PF) have become the main substitutes for FM in aquafeeds (R. L. Olsen & Hasan, 2012), as they are generally more economical and more sustainable than FM. A study (Kaushik et al., 2004) showed the possibility of replacing more than 90% FM for plant origin ingredients in ESB without finding significant differences among diets in digestibility coefficients, voluntary feed intake, growth rates, feed efficiency and daily nitrogen gains. Still, it was observed an increase in fat deposition with increasing levels of fish meal replacement (Kaushik et al., 2004). Another study (Azeredo et al., 2017) covered fish immunity by replacing 70% of FM for plant protein sources. Their results showed that plant sources promoted inflammatory signals, such as increased plasma nitric oxide and lysozyme levels. Moreover, the expression of other immune parameters like immunoglobulins, monocytes percentage and gut interleukin 10 gene expression was inhibited (Azeredo et al., 2017). Recently, efforts to replace FM in aquafeeds have focused on the use of probiotic-fermented soybean meal in spotted seabass (*Lateolabrax maculatus*) (Zhang et al., 2025). Studies have shown that this ingredient can replace up to 40% of FM without compromising fish performance, with an optimal substitution level of around 21-28% based on weight gain. However, higher replacement levels (50-60%) have been associated with negative effects on growth, serum immunity, hepatic antioxidant capacity, and intestinal health (Zhang et al., 2025). These adverse effects are mainly associated with PF anti-nutritional factors, unbalanced essential amino acid profiles (lysine and methionine), and low palatability. In response, researchers are still searching for ways to efficiently incorporate plant-based ingredients into aquafeeds and develop cost-effective diets. However, PF also compete with other sectors of the food industry for both human and livestock consumption. Their global scarcity and rising prices further highlight the need for sustainable, environmentally friendly alternatives that do not compete directly with the human food supply.

1.4. Insects as feed ingredients

There is an increasing interest in finding novel and sustainable ingredients to replace dietary FM and PF. A sustainable alternative in Europe since its approval in 2017 under European Union Regulation 2017/893 is insect meal (IM). Some of the insect species approved by European regulations are *Hermetia illucens*, *Tenebrio molitor* and *Musca domestica.* IM from these species contain around 42-63.3 % crude protein (dry matter) (Alfiko et al., 2022), a well-balanced essential amino acid profile, high lipid content (10-

30%), being a good source of vitamins such as vitamin B12, and bioavailable minerals such as iron and zinc (Gasco et al., 2020). In contrast, IM generally contains lower levels of PUFAs (EPA and DHA), and higher proportions of omega-6 fatty acids compared to FM (Barroso et al., 2014; Hua et al., 2019). This limitation can, however, be mitigated by manipulating the rearing substrate, as the nutrient composition of IM is strongly influenced by the composition of their diet (Magalhães et al., 2017).

Recent studies indicate that insects have the potential to improve flesh quality, strengthen the immune system, and reduce disease susceptibility in farmed fish, promoting sustainable and productive aquaculture systems (Fantatto et al., 2024). Replacing 15% FM (at 25 and 50%) with an IM blend (*H. illucens* and *T. molitor*) has been shown to support growth performance and feed efficiency in ESB, stimulating proliferation of probiotic species in the gut microbiota with increased bacterial richness and diversity in fish fed IM (Kalemi et al., 2025). This study also showed no adverse effects either on feed intake, mortality rates or overall fish health.

Another study with ESB using a similar IM mixture and replacing 3, 25 and 50% of 15% FM showed that feed intake, feed efficiency and growth remained similar across groups, with reduced fecal phosphorus losses and longer anterior intestine villi on 50% substitution of IM group displaying an intestinal morphology modulation in the IM treatments (Costa et al., 2026). Additionally, muscle of fish fed with IM50% presented the highest oxygen radical absorbance capacity, suggesting higher antioxidant capacity.

Finally, in a study with *Hermetia illucens* pre-pupae meal (Magalhães et al., 2017) was formulated to include up to 19.5% of *H. illucens*, replacing up to 45% of FM in diets for European seabass. In this study no adverse effects were observed on growth performance, feed utilization and digestibility by the inclusion of 19.5% of *H. illucens* (Magalhães et al., 2017). Other study using defatted *Tenebrio molitor* larvae meal (Basto et al., 2021) to replace FM intended to determine its maximum inclusion level without compromising growth performance, general metabolism, and flesh quality traits in ESB. It was determined that diets with up to 80% of *T. molitor* substituting 36% of FM showed no detrimental effects on nutrient digestibility, growth performance and associated genetic pathways, as well as fillet nutritional value (Basto et al., 2021).

However, one of the main limitations of using IM in diets for carnivorous fish is the presence of chitin since it is thought to be an anti-nutritional component that prevents fish from absorbing nutrients, particularly interfering with the use of proteins and lipids (Nogales-Mérida et al., 2019; Alfiko et al., 2022).

1.5. Chitin and chitinases

Chitin is a polysaccharide that consists of long-chain polymers of N-acetylglucosamine (GlcNac) joined by β1→4 bonds (Rangel, 2023). Chitin is a structural polysaccharide, occurring mainly in Arthropoda (including insects and crustaceans) and in Fungi, and it is also present in some mollusks and certain annelid species (Younes & Rinaudo, 2015). It is characterized by its rigidity, elasticity and waterproofing (Merzendorfer & Zimoch, 2003; Arakane & Muthukrishnan, 2010), being the most abundant polymer in the marine environment (Tamadoni Jahromi & Barzkar, 2018) and, therefore, an important source of energy, carbon, and nitrogen in the aquatic environment (Younes & Rinaudo, 2015). In the case of Black Soldier Fly (*Hermetia Illucens)* chitin content ranged between 8% and 24% and differed between different lifecycle stages (larvae, prepupae, pupae, flies, shedding and cocoons). Sheddings, prepupae and cocoons were the stages more rich in chitin when compared to the larvae stage (Soetemans et al., 2020).

Carnivorous fish like ESB cannot properly digest chitin, despite having the necessary enzymes (Henry et al., 2015). Its degradation into smaller molecules depends on the activity of chitinases, chitobiases, and lysozyme (Fines & Holt, 2010; Henry et al., 2015).

However, the dense chitin-protein matrix restricts the action of digestive enzymes, thus reducing the access of chitinases, proteases, and lipases, lowering the digestibility of chitin, proteins and lipids (Henry et al., 2015). Therefore, approaches that facilitate the breakdown of chitin into smaller, more digestible components are required.

To overcome the digestive challenges associated with chitin in IM, the compound can be removed through biological or physico-chemical methods before the ingredient is incorporated into fish feeds. However, each approach presents specific limitations. Biological treatments generally exhibit slow hydrolysis rates, require long processing times, and handle only small quantities of raw material (Mérida et al., 2019; Karlsen & Skov, 2022). In contrast, physico-chemical methods tend to be expensive, environmentally unfriendly, and may modify the nutritional profile of the ingredient or generate inhibitory compounds, potentially creating new antinutritional factors. Moreover, the addition of exogenous enzymes to diets containing IM demonstrated to be ineffective and even counterproductive, as it lowered nutrient digestibility probably due to microbiota disruption (Gasco et al., 2016).

Chitinolytic enzymes, known as chitinases, are produced by a wide range of organisms, including bacteria, fungi, insects, plants, and animals, for purposes including nutrition, morphogenesis, and defense (Adrangi & Faramarzi, 2013). Chitinases are responsible for the hydrolysis of glycosidic linkages within chitin chains (β1→4, β1→3 and β1→6), having a beta-glucosidase activity. These enzymes break chitin into lesser subunits, until they become monomers, simpler molecules more easily absorbed and digested (Rangel, 2023).

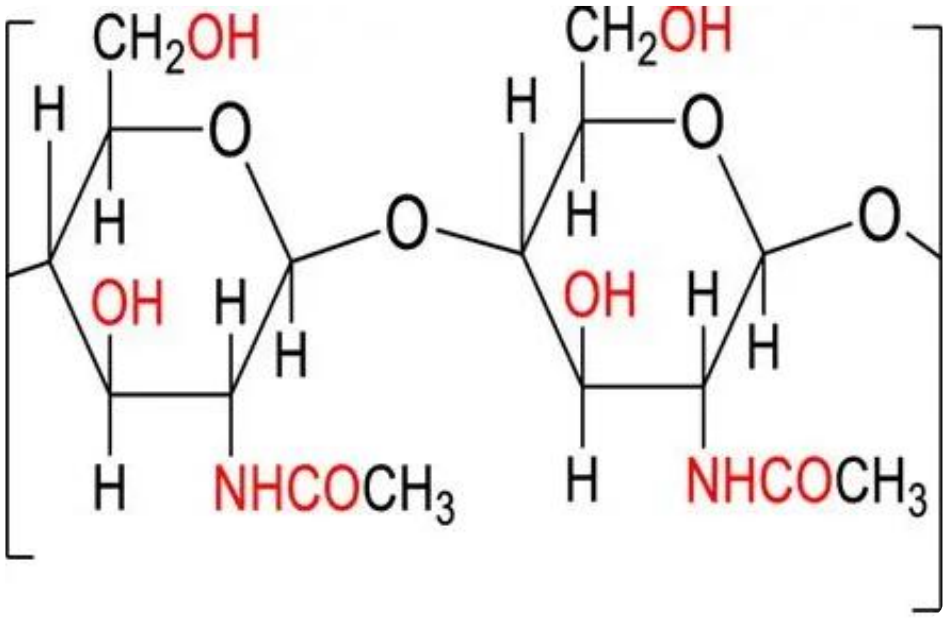


Figure 4: Chitin structure; DOI:10.1007/s10924-021-02154-x

Most microorganisms able to catabolize chitin are heterotrophic bacteria and archaea. Accordingly, Rangel et al. (2024) isolated two chitinolytic probiotics closely related to *Bacillus licheniformis* (Fish Isolates FI645 and FI658) from the ESB gut. Dietary supplementation of an experimental diet with 30% defatted *H. illucens* larvae meal with FI645 at a concentration of 2×10^9 colony forming units (CFU) kg^{-1} enhanced chitin digestibility and improved ESB feed and protein efficiency ratios (Rangel et al., 2024). Additionally, FI645 also increased ESB cumulative survival rate from 52.5% to 77.5% compared to those fed with the control diet when challenged with *Vibrio anguillarum* (Rangel et al., 2024).

Recently, it was characterized the presence of chitinase coding genes and chitin-degrading activity in autotrophic microorganisms, namely marine cyanobacteria (Capovilla et al., 2023), opening for potential studies on their use as a novel method for helping fish in chitin degradation.

1.6. Cyanobacteria as a vehicle for chitinase expression

Cyanobacteria constitute a unique phylogenetic group of organisms belonging to the domain Bacteria, characterized by their capacity for oxygenic photosynthesis, using water as an electron source and carbon dioxide as a carbon source (Garcia-Pichel et al., 2020). They fix atmospheric carbon dioxide and, in some cases, dinitrogen (N_2), thereby enhancing nutrient availability in nutrient-poor environments (Seth et al., 2021). This makes them essential primary producers, playing a crucial role in global carbon and nitrogen cycles. They exhibit great biological adaptability, and can be found in diverse ecosystems, both terrestrial, marine and freshwater (Bouyahya et al., 2024) and can exist either as free-living organisms or in symbiotic associations with various hosts, such as fungi, plants, and protists (Garcia-Pichel et al., 2020). The ubiquity of this group has exposed them to different biotic and abiotic stressors, stimulating the development of considerable genetic complexity in this group which, in turn, results in the production of secondary metabolites with high biotechnological value (Mazard et al., 2016). They have complex morphologies, with variable shapes and sizes, and members of this group may be unicellular, colonial or filamentous (Flores & Herrero, 2010). They further demonstrate a high capacity for cellular specialization, capable of developing specialized structures with specific functions such as heterocysts (for atmospheric nitrogen fixation), akinetes (which confer resistance), or hormogonia (motile filaments, facilitating dispersal) (Flores & Herrero, 2010).

As mentioned above, its ability to use solar energy and atmospheric carbon dioxide as energy and carbon sources, respectively, represent a valuable benefit under laboratory conditions, which value their rapid growth, their relatively simple genetics and the availability of well-characterized molecular and genetic tools in microbial research. Because of that several cyanobacterial species have been established as model organisms in this field of research (Lindblad et al., 2012; Sun et al., 2018). One such case is *Synechocystis* sp. PCC 6803, being the first photosynthetic organism, whose genome has been fully sequenced, placing it in a special position as a key model for investigating photosynthesis, stress responses, and metabolic regulation (Kaneko et al., 1996). Its innate ability to take up external DNA, combined with its highly efficient homologous recombination, allows for accurate genetic modification, a key requirement for applications in synthetic biology and metabolic engineering (Herrero et al., 2016; Sun et al., 2018). Several genetic manipulation tools are known, which facilitate the genomic alteration of this strain, allowing it to be used for genetic modification to obtain value-added bioproducts (Patel et al., 2023).

In recent years, the incorporation of cyanobacteria and microalgae into feed or supplements has gained increased attention (Nagappan et al., 2021), as cyanobacteria helps to promote the health and survival of aquatic animals, when used as supplements. Microalgal and cyanobacterial biomass is rich in key biochemical components, including lipids, proteins, carbohydrates, and a variety of pigments (Apandi et al., 2018). In their natural environment, microalgae, but also cyanobacteria constitute a primary food source for fish, which supports their use as promising ingredients in aquafeed formulation. They supply nearly all essential nutrients such as PUFAs, amino acids, vitamins, and minerals required for fish nutrition (Benemann, 1992; Knuckey et al., 2005; Carneiro et al., 2020). Owing to this well-balanced nutritional profile, photoautotrophic microorganisms are increasingly recognized as valuable feed additives or supplements, with the potential to substitute FM and other conventional components in aquaculture and livestock diets (Ansari et al., 2021). Microalgae have been used as feed additives at a large scale for fish and prawn larvae, crustaceans, and molluscan (Belay et al., 1996; Borowitzka, 1997) and microalgae such as *Chlorella* sp. and *Scenedesmus* sp. are most used in aquaculture feed due to their excellent nutritional value and suitability (Khatoon et al., 2010). In the case of cyanobacteria, *Spirulina (Arthrospira platensis)* is considered a prospective protein source in aquaculture. However, full substitution of FM with *Spirulina* on fish feeds presented some implications for system productivity and end-product quality, depending on animal production systems. Fish productivity and product quality were negatively affected by *Spirulina* (Altmann & Rosenau, 2022). The authors concluded that high substitution levels of *Spirulina* often lead to a reduction in growth performance and increase in feed conversion ratio in carnivorous fish. Still, *Spirulina* fish feed could completely replace FM on omnivorous fish (Altmann & Rosenau, 2022). Another work concluded that *Spirulina* meal could be used within a range from 22% to 25% as FM substitute without compromising growth and feed utilization in various fish species (Li et al., 2022). Also, regarding supplementation, the optimal level observed for *Spirulina* ranged from 1.46% to 2.26%. These findings (Li et al., 2022) show that cyanobacteria such as *Spirulina* have a chance to become an alternative to FM or even serving as a more sustainable alternative in the FM industry, if some small challenges regarding protein quality and production can be overcome.

On another note, but with biotechnology at the forefront, microbial photosynthetic organisms such as microalgae and cyanobacteria are increasingly being recognized as a promising system for producing recombinant antigens designed for vaccines targeted at fish and other aquatic organisms (Yang et al., 2023; Chen et al., 2025), enhancing immune response and preventing disease outbreaks. Altogether, both microalgae and

cyanobacteria represent powerful platforms for the delivery of recombinant proteins (Kwon et al., 2019), using either whole-cell strategies or cell-derived components (such as extracellular vesicles) as carriers for heterologous proteins (Matinha-Cardoso et al., 2022). Thus, because of their adaptability, unique metabolic capabilities and amenability to genetic engineering, cyanobacteria in particular are strategically positioned to become key players for the development of sustainable biotechnological applications in aquaculture (Berla et al., 2013).

1.7. Objectives

The aim of this study was to evaluate the effect of genetically engineered *Synechocystis* sp. PCC 6803 expressing heterologous chitinases, enzymes capable of deconstructing the chitin polymer, on the chitin digestibility capacity of ESB-fed IM-based diets.

Aiming to increase IM chitin digestibility, in addition to the wild type (WT), two previously genetically engineered *Synechocystis* sp. PCC 6803 strains (SR1 and SL9), available in the lab, each producing different amounts of a heterologous chitinase from *Nostoc punctiforme* PCC 73102, were cultivated and incorporated in ESB diets with high IM levels (30% inclusion, *Hermetia illucens*).

Ultimately, this work pretends to maximize the use of IM-based diets as fish-feed ingredients for aquaculture species through reducing chitin's indigestibility, thus increasing chitin's overall energy availability and accessibility to entrapped proteins and lipids by the presence of highly active chitinases.

In addition, the use of cyanobacterial biomass brings in the prospect of improving feed's antioxidant and radical scavenging activities.

2. Materials and Methods

2.1. Ethical statement

The trial was approved by the Portuguese National Authority for Animal Health (DGAV; reference ORBEA_CIIMAR_27_2019), directed by accredited scientists (following FELASA category C recommendations), and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes.

2.2. Cyanobacterial cultivation and biomass production

Cultivation of *Synechocystis* sp. PCC 6803 wild type (WT) was carried out in liquid BG11 medium (Stanier et al., 1971); for cultivation of genetically modified strains (SR1 and SL9), BG11 medium was supplemented with 50 µg kanamycin mL^{-1}, unless stated otherwise. Stock cultures were maintained either in 50 mL plastic culture-flasks or in 100 mL glass Erlenmeyer flasks (40 mL culture volume in both cases), under a 16-hour light (50 µmol photons m^{-2} s^{-1})/ 8-hour dark cycle, with continuous shaking at 200 rpm and a constant temperature of 30 ºC. Cyanobacterial growth was monitored by measuring optical density at 730 nm (OD_{730}) in a UV-Vis spectrophotometer. Briefly, a small sample (1 mL) was taken into an Eppendorf tube with a serological pipette under the laminar flow chamber, to guarantee sterility of the samples, and a 1:10 dilution was prepared (0.1 mL of sample for 0.9 mL of BG11 medium) in duplicate.

For culture scale up, 250 mL glass Erlenmeyer flasks, with a final volume of 120 mL of culture, were prepared with an initial OD_{730} of 0.1, using the stock cultures as inoculum. Cells were grown under a 16-hour light (50 µmol photons m^{-2} s^{-1})/ 8-hour dark cycle, with continuous shaking at 200 rpm and a constant temperature of 30 ºC. The procedure described above was carried out inside a laminar flow chamber, to guarantee the sterility of the samples. When cultures reached an OD_{730} of approximately 1.0, these were used as inoculum to initiate 0.6 L cultures, with an initial OD_{730} of 0.1, prepared in 1 L glass gas washing bottles, and agitation was guaranteed by sparging with air (approximately 1 L min^{-1}). Cultures were grown at 25 ºC, under a 16-hour light (50 µmol photons m^{-2} s^{-1})/ 8-hour dark cycle until it reached an OD_{730} of approximately 1.5. Then, 8 L cultures were initiated in 10 L Nalgene bioreactors (Thermo Scientific, USA), with an initial OD_{730} of 0.1, and maintained at 25 ºC, under a 16-hour light (50 µmol photons m^{-2} s^{-1})/ 8-hour dark cycle.

Figure 4: Cultivation of Synechocystis sp. PCC 6803 under different volumes (left to right: 250mL, 1L, 10L)

Cultures were agitated by sparging with air (approximately 1 L min^{-1} for 10 days, followed by 3 L min^{-1} for the remaining cultivation period), and no antibiotics were added to the cultures of the genetically modified strains, to guarantee that no antibiotics were present in the biomass when formulating diets. At the end of the cultivation cycle, the biomass was collected by centrifugation at 5000 *g* (10 minutes, 23 ºC), stored at -80 ºC, and later lyophilized. Separately, two samples of 50 mL were also collected by centrifugation and frozen; one was later lyophilized, the other was kept frozen. The goal was to assess chitinase activity of each processing method.

2.3. Genetic analysis of the cyanobacterial strains upon cultivation

As the cultivation of the biomass in the larger containers was conducted in the absence of antibiotics, the engineered strains were subjected to genetic analysis to make sure the plasmid harboring the construction including the chitinase gene was still present after the antibiotic-free cultivation. For that, genomic DNA was obtained by mechanical and chemical extraction. Firstly, a small sample (25 mL) of cyanobacterial culture was centrifuged for 10 minutes at 5000 *g*, and the resulting pellet was suspended in 200 µL of TE buffer Tris-EDTA (50 mM Tris-HCl and 10 mM EDTA, pH 8.0). The suspension was transferred to a screw-capped tube containing 0.35 g of 0.6 mm diameter glass beads using glass beads and 600 µL of a 1:1 mixture of phenol and chloroform. Then, with the help of a Precellys Homogenizer (Bertin technologies, France), a cell lysis protocol of carried out, followed by a centrifugation for 7 minutes at 17000 *g* at 4 ºC. Supernatant was collected into an Eppendorf and mixed by vortexing with 500 µL of chloroform and another centrifugation (17000 *g*) was carried out for 5 minutes at 4 ºC, with the aqueous phase carefully transferred to a new tube Then, 2M sodium acetate and ethanol 70% were added with a proportion of 1:10 and 2:1 of the supernatant volume, respectively, and after agitation the solution was stored at -20 ºC for 1 hour to precipitate the DNA. Then, Eppendorf's were centrifuged for 20 minutes at max speed (17000 *g*, at 4 ºC) to collect the DNA, supernatant was discarded and added 500 µL of ethanol 70%, to remove the excess of salt, followed by another 17000 *g* centrifugation at 4 ºC, for 5

minutes for DNA cleansing. Finally, the tube was left on ice for 10 more minutes, to allow any residual ethanol to evaporate and then the pellet was suspended in 100 µL of nuclease-free water and DNA concentration was measured using NanoDrop™ One (Thermo Fisher Scientific).

The DNA template of interest was amplified by PCR in a Veriti 96-well thermal cycler (Applied Biosystems). This reaction was done in a final volume of 20 µL and consisted of 2 µL of DNA template and 18 µL of the master mix. Master mix consisted of two specific primers forward and reverse (2 µL each) listed in Table 1, Taq DNA polymerase buffer (4 µL), magnesium chloride ($MgCl_2$; 1.6 µL), Deoxyribonucleotide triphosphates (dNTPs; 0.4 µL), H_2O (7.9 µL) and Go Taq DNA polymerase (0.1 µL). In the thermocycler the first step was an initial denaturation at 95 ºC for 2 minutes, followed by 35 cycles of 30 seconds at 95 ºC, 20 seconds of annealing at 56 ºC, and 72 ºC of extension for 3 minutes. The protocol concluded with a final extension step at 72 ºC for 7 minutes.

The electrophoresis run was done using an agarose gel (0.8%) with 1x Tris/Acetic acid/EDTA (TAE) as buffer (Fisher BioReagents) and stained with GreenSafe Premium (NZYTech). NZYDNA Ladder III (NZYtech) was used as molecular weight marker and PCR products were separated for 45 minutes at 120V. Identity of the DNA fragments was determined by Sanger sequencing (Eurofins).

Table 1: Oligonucleotide primers used in the PCR reaction.

Primer Name	Sequence 5’ → 3’	T_a (ºC)	Purpose
PS1	AGGGCGGCGGATTTGTCC	55	Confirmation of constructions in pSEVA251 plasmid
PS2	GCGGCAACCGAGCGTTC		

2.4. Protein extracts and protein determination

To test chitinase activity in cyanobacterial extracts, 50 mL of cyanobacterial culture was collected in a Falcon and centrifuged at 4500 *g* for 10 minutes (22 ºC), removing the supernatant. Cells were suspended in 500 µL of BugBuster® protein extraction buffer (Millipore, USA) and transferred to 2 mL screw-cap tubes, containing 0.35 g of 425-600 µm glass beads (Sigma-Aldrich, Germany) to promote mechanical lysis. Cell lysis was carried out on a Precellys Homogenizer (Bertin technologies, France). At the end, samples were centrifuged at 10000 *g* for 7 minutes (4 ºC), the supernatant recovered, and samples were kept on ice until further analysis.

To quantify total protein in samples using the Bradford method (Bradford, 1976), it was necessary to prepare a standard curve with bovine albumin serum (BSA) (2 mg mL^{-1}) at concentrations of 0.25, 0.5, 1.0 and 1.4 mg mL^{-1}. The cyanobacterial protein samples were diluted in deionized water to fit the standard line, at a dilution of 1:10. Samples and standards were incubated with Bradford reagent for 5 minutes in darkness. Activity was read on a spectrophotometer at a wavelength of 595 nm.

2.5. Chitinase activity quantification assays

For the quantification of chitinase, a chitinase determination kit (Merck, EM, EB, MAM 11/13-1, Germany) based on the enzymatic hydrolysis of 3 substrates with chitinase was used. Hydrolysis releases 4-methylumbelliferone (4MU), whose ionization at basic pH is measured by the release of visible color at 450 nm. The substrates were separated into solutions, together with the kit buffer, in a total volume of 500 µL per substrate. The plate reading was performed with the presence of the three substrates for each of the samples (WT, SL9, SR1), a blank with only the substrates, and a positive control with a 1000x concentrated chitinase solution, which was diluted 1000x in TBS buffer (Tris-buffered saline solution). The plate was incubated for 1 hour, followed by the addition of the STOP sodium carbonate solution. Reading was done using the Biotek Synergy HT plate reader (Marshall Scientific, USA). Chitinase activity in samples was expressed in arbitrary units (A.U.). This protocol was done in triplicate.

2.6. Experimental diets

Four experimental diets were formulated to be isoproteic (45% protein) and isolipidc (18% lipids). All diets contained 20% fish meal (FM) and 30% insect meal (IM). It was tested a control diet without cyanobacteria inclusion, an experimental diet containing *Synechocystis* sp. PCC 6803 wild type (WT), and 2 other diets including each genetically engineered *Synechocystis* strain (SR1 or SL9). The biomass of WT, SR1 and SL9 cyanobacteria were incorporated into the diets at 1.15% inclusion rate. Chromium oxide (Cr_2O_3) at 0.5% was used as an inert marker for digestibility assessments.

Ingredients were finely ground, well mixed, and dry-pelleted in a laboratory pellet mill through a 2 mm die. The pellets were dried in an oven at 40 ºC for 24 hours and stored at room temperature in airtight bags until used. Table 2 presents the ingredients and proximate composition of experimental diets.

Table 2: Ingredient composition and proximate analysis of experimental diets for the digestibility trial.

Ingredient inclusion (% dry weight)	**CTR**	**WT**	**SR1**	**SL9**
Fish meal LT[1]	20	20	20	20
Insect meal (*Hermetia illucens*)[2]	30	30	30	30
Corn gluten meal[3]	15	15	15	15
Soybean meal[4]	9.9	9.8	9.8	9.8
Whole wheat[5]	6.3	5.3	5.3	5.3
Fish protein concentrate (CPSP)[6]	2	2	2	2
Fish oil	11.2	11.2	11.2	11.2
Vitamins[7]	1	1	1	1
Minerals[8]	1	1	1	1
Binder[9]	0.5	0.5	0.5	0.5
Taurine[10]	0.5	0.5	0.5	0.5
Chromium oxide	0.5	0.5	0.5	0.5
Dicalcium phosphate	1.6	1.6	1.6	1.6
Choline chloride (50%)	0.5	0.5	0.5	0.5
Cyanobacteria	0	1.15	1.15	1.15
Proximate composition (% Dry Weight)	**CTR**	**WT**	**SR1**	**SL9**
Dry matter	93.1	93.1	93.1	93.1
Ash	9.8	9.8	9.8	9.8
Crude protein	44.7	44.7	44.7	44.7
Lipids	17.7	17.7	17.7	17.7
Chitin	1.4	1.4	1.4	1.4
Energy (kJ/g DM)	21.0	21.0	21.0	21.0

[1] Sorgal, S.A. Ovar, Portugal (Crude Protein (CP): 68.3% Dry matter (DM); Crude lipid (CL): 10.6% DM).

[2] Black soldier fly larvae meal, Entogreen, Santarém, Portugal (CP: 49.7% DM; CL: 13.2% DM).

[3] Sorgal, S.A. Ovar, Portugal (CP: 60.9% DM; CL: 2.4% DM).

[4] Sorgal, S.A. Ovar, Portugal (CP: 48.4% DM; CL: 1.7% DM).

[5] Sorgal, S.A. Ovar, Portugal (CP: 12.0% DM; CL: 1.1% DM).

[6] Sorgal, S.A. Ovar, Portugal (CP: 72.3% DM; CL: 7.8% DM).

[7] Vitamins (mg kg^{-1} diet): retinol, 18,000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); α- tocopherol, 35; menadione sodium bisulphate, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400

[8] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.44 (g kg^{-1} diet).

[9] Aquacube. Agil, UK.

[10] Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.

2.7. Digestibility trial

The digestibility trial was conducted in CIIMAR animal facilities (BOGA).

Twenty-three ESB with an initial body weight of 27 g, were distributed across a digestibility system consisting of a battery of 5 fiberglass tanks of 60 L capacity designed according to Cho et al. (1982) and with a settling column connected to the outlet of each tank for feces collection. Tanks were supplied with a continuous flow of seawater (35.0 ± 1.0 g L^{-1} salinity, 8 mg L^{-1} oxygen) thermo-regulated to 22.0 ± 2.0 °C and with a photoperiod of 12:12 hours light:dark. During the trial, fish were hand-fed to apparent visual satiation twice a day, 6 days a week, and feces were collected from the settling column once a day, before the morning meal.

Digestibility measurements were carried out in three consecutive periods (3 blocks) to obtain three replicas for each diet, with each diet being assigned to a different group of fish within each period. The first 5 days of each period were used for allowing fish to adapt to the diets and then feces were collected once a day during 10 consecutive days.

Immediately after the collection, feces from each tank were centrifuged at 3000 *g* for 10 minutes, pooled and stored at −20 °C. Before proximal analysis determinations, feces were dried at 40 °C for one day, grounded, and stored at room temperature until analysis.

Apparent digestibility coefficients (ADC) of diets components (dry matter, protein, lipids, chitin and energy) were calculated as follows:

$$(\%)\mathbf{ADC\ diet} = \left(\mathbf{1} - \frac{\mathbf{dietary\ Cr2O3\ level} \times \mathbf{feces\ nutrient\ or\ energy\ level}}{\mathbf{feces\ Cr2O3\ level\ x\ dietary\ nutrient\ or\ energy\ level}}\right) \times \mathbf{100}$$

2.8. Sampling

At the end of each digestibility period, 3 fish per tank were sampled 5 hours after the morning meal to ensure that feed was present in the intestine for digestive enzyme activities analysis. For that purpose, fish were euthanized using an overdose of ethylene glycol monophenyl ether and liver and whole-intestine of the fish were sampled and stored at -80 °C until analyses.

Whole-intestine was sampled to evaluate the activity of the digestive enzyme's total alkaline protease, α-amylase, lipase and chitinase. Fish oxidative state was evaluated in the liver by analyzing the activity of the antioxidant enzymes catalase, glutathione peroxidase, glutathione reductase and the concentration of the lipid peroxidation marker.

2.9. Bromatological analysis

The bromatological analysis of ingredients, diets and feces was realized according to the standard methods defined in AOAC (2000).

2.9.1. Dry matter and ash

Briefly, dry matter was assessed through drying the samples (ingredients and diets) at 105 ºC in an oven until constant weight. Incineration at 405 ºC in a muffle furnace overnight allowed to obtain the non-organic (ash) portion of the samples.

2.9.2. Protein quantification

The crude protein content of ingredients, diets and feces was determined by the quantification of the nitrogen (considering that proteins contain an average of 16% of N: N × 6.25) by the Kjeldahl Method, after acid digestion, using a Kjeltec digestion and distillation system (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively). Briefly, about 0.5 g of ingredients, diets and feces were weighed; added a catalyzer to accelerate the digestion (Kjeldahl tablet) plus 15 mL of concentrated sulfuric acid (H_2SO_4). Samples were digested at 420 ºC for 1 hour converting the sample nitrogen to ammonium sulfate and thereafter distilled in the distillation unit. Water and sodium hydroxide (NaOH) at 40% were added to each digestion tube, and saturated boric acid (H_3BO_3) was used for ammonium binding during distillation. Nitrogen (N) content was quantified by titration with hydrogen chloride (HCl, 0.5 N) using the pH indicator methyl orange (Merck KGaA, Germany).

2.9.3. Lipid quantification

The lipid content of the ingredients and diets was obtained by continuous extraction with petroleum ether using the SoxTec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046). Quantification was done using around 0.5 g of each sample, placed in a cartridge, and 50 mL of petroleum ether added to the extraction beakers. The extraction took place in the extraction unit where the samples were first boiled at 115 ºC for 45 minutes and then washed for 1 hour, with the lipids being extracted into the beakers. After evaporation of the solvent, the beakers were dried in an oven at 100 °C for 30 minutes. In the end, the beakers were weighed, and the lipid content was estimated from the difference in weight between the beakers before and after extraction.

2.9.4. Gross energy quantification

The gross energy was determined by direct combustion of the sample using an adiabatic pump calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261) by measuring the rise of temperature in the 2 L water surrounding stainless-steel bomb.

Briefly, a portion of the diets and feces (1 and 0.5 g, respectively) was condensed into a tablet and burned in a calorimetric bomb under a pressurized oxygen atmosphere (2.53x106 Pa). The rise in water temperature due to combustion was measured to calculate the energy content of the sample.

2.9.5. Chromium oxide quantification

Chromium oxide was quantified using a colorimetric method with perchloric acid as the oxidizing agent (Bolin et al., 1952). To prepare the oxidizing reagent, 10 g of sodium molybdate were dissolved in 150 mL of distilled water in a container that was then placed in a cold-water bath on a magnetic stirrer. Next, 150 mL of concentrated sulfuric acid (H_2SO_4) were slowly added under constant stirring. After cooling, 200 mL of perchloric acid ($HClO_4$, 70-72%) were added, and the mixture was homogenized.

Samples of diets, feces, and a chromium oxide standard were weighed (200, 100, and 50 mg, respectively), and 5 mL of the oxidizing reagent were added to each tube. The tubes were placed on a heating plate for a digestion cycle (230 °C for 1 hour). After cooling, 2 mL of $HClO_4$ (70-72%) were added to each tube, which was then reheated for 15 minutes at 230 °C. A filtration procedure was subsequently carried out for each tube into a volumetric flask: 50 mL for diet and feces samples, and 100 mL for the chromium standard (0.5 mg mL^{-1}). To obtain a calibration curve, different dilutions of the chromium oxide standard were prepared. Finally, samples were read at 440 nm, and absorbance values were recorded.

2.10. Chitin quantification

Chitin quantification was assayed in diets and feces following the protocol from (Guerreiro et al., 2020) and consisted of 3 steps: degreasing, deproteinization and chitin quantification. The first two steps are regarded as pre-treatments. Due to the low feces' lipid content, degreasing was only performed in diets. Briefly, 1 g of sample was mixed with acetone (1 g 10 mL^{-1}) and incubated with agitation (200 rpm) at room temperature (RT) for 1 hour. Then it was centrifuged at 19800 *g*, 4 °C for 15 minutes, and the supernatant discarded. Pellet was washed with 10 mL of distilled water, and the centrifugation process was repeated three times. At the end, pellets were left drying at

100 ºC until constant weight. Degreasing yield was calculated dividing the final for the initial weight of the samples.

For deproteinization, 0.5 g of the recovered pellet was mixed with 0.5 M NaOH (Sodium hydroxide) solution (0.5 g 10 mL^{-1}) and incubated with agitation (200 rpm) at room temperature for 4 hours. A washing protocol was performed as described above. The recovered pellets were dried in an oven at 100 °C until constant weight and the yield of the protocol was calculated dividing the mass after deproteinization and the initial mass of each sample before deproteinization.

For chitin quantification,10 mg of the recovered pellet was mixed with 6 M HCl (Hydrochloric acid) solution (10 mg 3 mL^{-1}) and incubated in a water bath at 100 °C for 6 hours. Later, a filtration with 0.2 µm cellulose acetate filters was done and each sample was neutralized with NaOH 12 M with the pH adjusted to 6.5 ± 0.05 and the final volume to 10 mL. Spectrophotometric determination was carried out using 3-Methyl-2-ben zothiazolinone hydrazone hydrochloride (0.5% MBTH) at 650 nm.

2.11. Digestive enzymes

Whole-intestine samples were homogenized (dilution 1:5 w/v) with a homogenization buffer (pH 7.8) with EDTA (0.1mM), Trizma base (100mM) and Triton (0.1%) using an Ultraturrax homogenizer. Thereafter, samples were centrifuged at 30000 *g* for 30 minutes at 4 °C. After this centrifugation, the supernatants were divided into aliquots and kept at -80 °C.

2.11.1. Total alkaline protease activity

Total alkaline protease activity was measured by the casein-hydrolysis method. The reaction mixture containing casein (1% w/v; 0.125 mL), buffer (0.1M Tris-HCl, pH 9.0; 0.12 mL) and homogenate supernatant (0.05 mL) was incubated for 1 hour at 37 °C. The reaction was stopped with the addition of 0.3 mL trichloroacetic acid solution (TCA) at 8% w/v. Samples rested for 1 hour at 4 ºC and then were centrifuged at 1 800 *g* for 10 minutes. Supernatant absorbance was measured at 280 nm in a UV plate. Control blanks for each sample were prepared adding the supernatants from the homogenates after the incubation period. A tyrosine (0.096% w/v) solution was used as a standard.

2.11.2. Lipase activity

Lipase (EC 3.1.1.3) activity was measured according to the instructions of the kit manufacturer (Spinreact, ref. 1001274, Spain). The formation rate of methylresorufin (with molar extinction coefficient, 60.65 mM^{-1} cm^{-1}) was assessed photometrically at 580

nm at 37 ºC. This rate is by convention proportional to the concentration of catalytic lipase present in the samples homogenate.

2.11.3. Amylase activity

Amylase activity (EC 3.2.1.1) was measured according to the instructions of the kit manufacturer (Spinreact, ref. 41201, Spain) by the rate of 2-chloro-4-nitrophenol formation (molar extinction coefficient, 12.9 $mM^{-1}cm^{-1}$) at 37 °C. Absorbance was read at 405nm.

All the enzyme activities (total alkaline proteases, lipase and amylase) were expressed as miliUnits per mg of hepatic soluble protein (specific activity). The protein concentration was determined utilizing bovine serum albumin as standard as described in Bradford (1976).

The amount of enzyme required to catalyze the hydrolysis of 1 µmol of substrate per minute under the assay conditions was considered as one unit of the enzyme.

All the measurements were performed in a Multiskan Go microplate reader (model 5111 920; Thermo Scientific, Nanjin, China).

2.12. Oxidative stress enzymes

Liver samples were homogenized (dilution 1:5) in ice-cold phosphate buffer (0.1M, pH 7.4), with the help of a homogenizer. The procedure was made on ice. Homogenates were centrifuged at 12000 rpm for 25 minutes (4 ºC). After centrifugation, the supernatant was collected, and the aliquots stored at -80 ºC.

2.12.1. Catalase activity

Catalase (EC 1.11.1.6, CAT) activity was determined as described by Claiborne (1985) and Giri et al. (1996) by measuring the decrease of hydrogen peroxide (H_2O_2) concentration at 240 nm over 3 minutes, in 10 second intervals, at 25 °C, due to the decomposition of H_2O_2 into O_2 and H_2O. CAT activity was expressed as H_2O_2 consumed/minute/mg protein (ε = 43.5 M^{-1} cm^{-1}).

2.12.2. Glutathione reductase activity

Glutathione reductase (EC 1.6.4.2, GR) activity was measured by monitoring the decrease in NADPH absorbance during the oxidation of NADPH into $NADP^+$, at 340 nm, for 6 minutes in 30 second intervals as described by Cribb et al. (1989). GR activity was expressed as nmol of NADPH oxidized/minute/mg protein using a ε = 6.22 × 10^3 M^{-1} cm^{-1}.

2.12.3. Glutathione peroxidase activity

The activity of glutathione peroxidase (EC, 1.11.1.9, GPx) was quantified by the oxidation of NADPH to $NADP^+$ at 340 nm, according to the method described by Paglia & Valentine (1967). The absorbance was monitored for 5 minutes at intervals of 30 second at 25 °C and expressed as nmol $NADP^+$/minute/mg protein ($\varepsilon = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

2.12.4. Lipid peroxidation

Concentration of thiobarbituric acid-reacting substances (TBARS) was determined according to Buege & Aust (1978). The reaction mixture consisted of the sample (50 µL) and a mixture of thiobarbituric acid (TBA) (0.375% p/v), Trichloroacetic acid (TCA) (15% p/v), Hydrochloric acid (HCl) 0.25N and Butylated hydroxytoluene (BHT) (0.01% p/v) prepared previously (250 µL). The mixture was heated in a 100 ºC bath for 15 minutes and centrifuged at 1500 *g* for 10 minutes. Absorbance in the supernatant was measured at 535 nm. Concentration was expressed as nmol malondialdehyde/g of tissue, calculated from a calibration curve (Malondialdehyde MDA 0.1 mM and mili-Q water were used for the standard slope).

2.13. Protein determination

To measure liver and intestine protein concentration to express digestive and oxidative stress enzymes per mg of protein (specific activity), the Bradford method was used (Bradford, 1976). Briefly, Bovine γ-globulin (1.4 mg mL^{-1}) was used to obtain the standard curve by serial dilution in distilled water. To each microplate well was added 250 µL of Bradford reagent and the 5 µL of standards or samples. Both samples and standards were incubated for 10 minutes, in the dark and absorbance was read at 595 nm.

2.14. Statistical analysis

Data are presented as mean values and standard deviations. Statistical evaluation of the data was carried out using a completely randomized experimental design Linear Mixed Model, with diet as fixed factor and block as random factor. The significance level of 0.05 was used for rejection of the null hypothesis. All the statistical analyses were carried out using SPSS 30.0 software package (IBM Corp.) for Windows.

3. Results and Discussion

3.1. Cyanobacteria growth and chitinase activity

For this work, we had three cyanobacterial strains of *Synechocystis* sp. PCC 6803 at our disposal: a wild type strain (WT) and two genetically modified strains, transformed with a replicative plasmid (pSEVA251) (Ferreira et al., 2018) containing constructs for the expression of the heterologous chitinase ChiA, from the cyanobacterium *Nostoc punctiforme* PCC 73102. The decision to use the chitinase from this cyanobacterium is related to the expression capacity of *Synechocystis* sp. PCC 6803, which is known to be limited when producing heterologous proteins from phylogenetically distant organisms. Therefore, to ensure stable expression, the chitinase from *N. punctiforme* PCC 73102 was selected.

The difference between the two genetically modified strains lies in the regulatory element controlling expression - the promoter; while strain SR1 uses the P_{rbcL} promoter of the rbcL gene (a native, medium-strength promoter under normal growth conditions that regulates the expression of the large subunit of the RuBisCO enzyme) (Till et al., 2020), strain SL9 uses the $P_{trc.x.lacO}$ promoter (a strong, well-characterized synthetic constitutive promoter) (Pinto et al., 2015; Ferreira et al., 2018). Thus, these strains enable constitutive production of the target enzyme in this study, although at notably different expression strengths. These genetically modified strains had previously been generated in the laboratory and were fully characterized and ready for cultivation.

To produce diets containing cyanobacterial biomass and to obtain a sufficient amount of cells for diet formulation, cultures had to go through a scale-up process to grow at the L scale. For this purpose, cultivation started at a scale of 40 mL (stock culture), then progressed to 120 mL, 0.6 L, and finally 8 L. We regularly monitored culture growth throughout the process by measuring the optical density (OD). OD steadily increased in all cultivation setups (Figure 5), meaning that cells were viable and actively dividing, irrespectively of its genetic background. However, differences could be observed across the different cultivation scales, with cells in the 8 L scale growing the slowest (OD_{730} of approximately 0.4 at day 10 of cultivation), followed by the 120 mL scale (OD_{730} of 0.8, day 10), and 0.6 L scale (OD_{730} of approximately 1.2 at day 10).

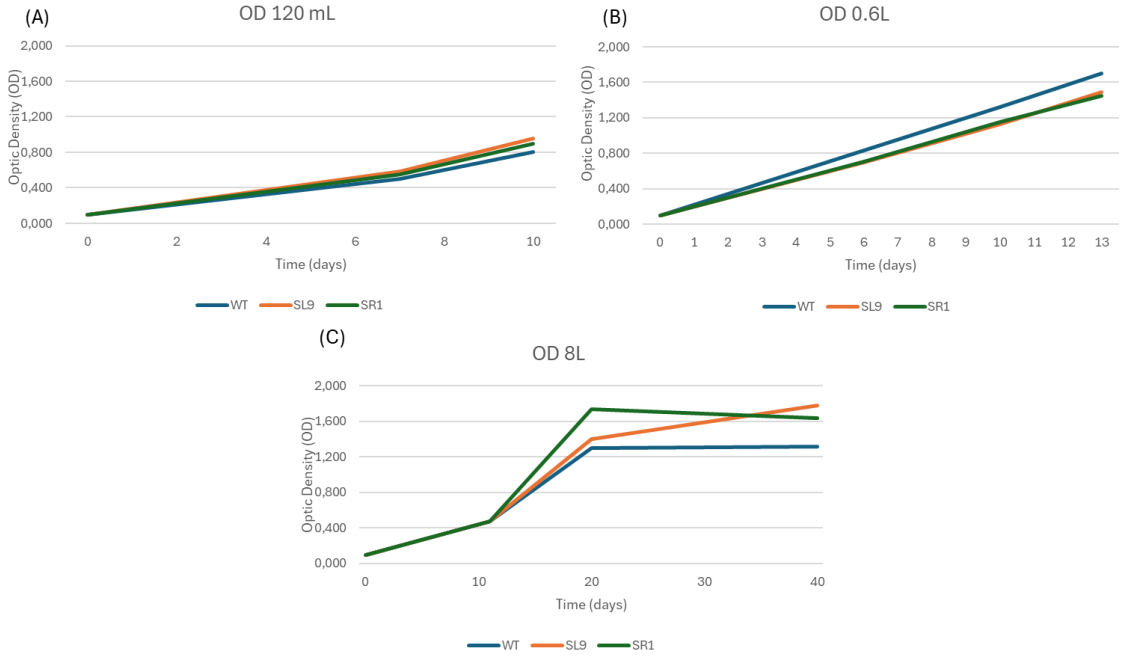


Figure 5: Optical density (OD_{730}) of S6803 during the production cycle in three different volumes: 120 mL (5A), 0.6 L (5B) and 8 L (5C). WT – S6803 wild type; SL9 – genetically modified strain with synthetic $P_{trc.x.lacO}$ promoter (highest expression); SR1 – genetically modified strain with native promoter P_{rbcL}.

These differences in growth are likely related to a combination of different factors, including flask geometry (Erlenmeyer flask, cylindrical gas-washing bottle, and cylindrical Nalgene bioreactor), materials of the recipient (glass vs polycarbonate), and mixing method (orbital shaking vs aeration with air sparging). In fact, even the air flow rate made a difference in growth, as the 8 L system changed its performance from day 10 to 20 as compared to the first 10 days of cultivation, since aeration was changed from 1 L min^{-1} to 3 L min^{-1} (Figure 5C). Nevertheless, in such cultivation scale, the growth seemed to arrest at day 20, since it remained constant throughout the remaining period of cultivation. These results show that long-term growth is not sustainable at 8 L volume in the polycarbonate bioreactors, mostly because of the design of the container, as this is a critical factor when producing cyanobacteria in a high yield production setting (Kossalbayev et al., 2024). That is because cyanobacteria are photoautotrophic organisms, with light being an important element for their growth. In such system, with high cell density, light is mostly absorbed by peripheral cells. Thus, light availability represents one of the constrains for growth, as with increasing cyanobacterial density light has more difficulty travelling through the reactor (Novoveská et al., 2023). On the other hand, the container design and agitation system may also impact gas distribution (Kossalbayev et al., 2024), a relevant factor as cyanobacteria are dependent on

inorganic carbon (mainly in the form of CO_2) to be fixed and converted into sugars (for energy) or structural components (for biomass). These parameters can help explain the slower growth of S6803 at the 8 L scale, and hypothesize that growth will have to be carried out in different systems (for example, air-lift column or flat-panel bioreactors) for improved growth rates and higher biomass production.

Chitinase activity was assayed during the biomass production cycle in three different moments. Firstly, during the volume of 0.6 L and in the beginning of the 8 L cycle growth, as to observe if chitinase maintained activity over time. Then, protocol was done a third time to check what type of processing method to use with S6803 – frozen or lyophilized – and involved quantification of chitinase, by the reading of the activity of each sample using a chitinase activity assay kit (Merck, Germany). Obtained values were normalized with S6803 total protein.

As expected, chitinase activity was higher in the two genetically engineered strains than in the wild type, since this cyanobacterium does not naturally produce chitinases. Chitobiosidase (Fig. 6A) and endochitinase (Fig. 6C) activities are the most predominant, especially in the SR1 strain.

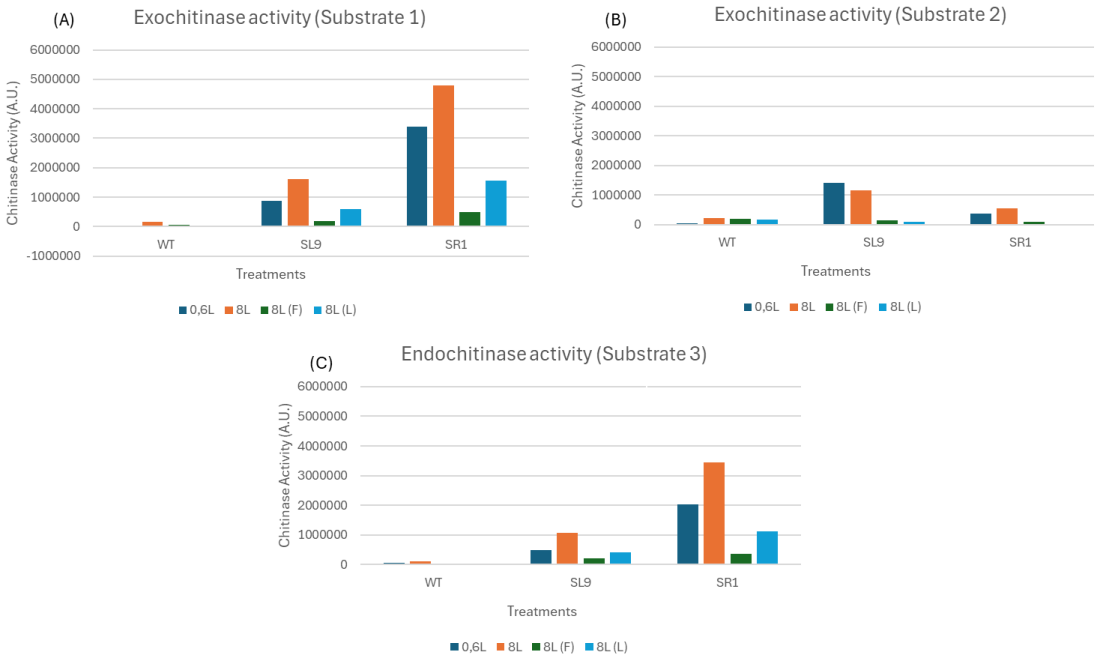


Figure 6: Chitinase activity in different treatments, according to growth conditions and biomass processing method. Each substrate represents a specific chitinolytic activity: chitibiosidase (Substrate 1), β-N-acetylglucosaminidase (Substrate 2) and endochitinase (Substrate 3). Values were normalized by Synechocystis total protein through Bradford Method.

Activity was measured on both lyophilized (8 L (L)) and frozen biomass (8 L (F)) at the end of the biomass production cycle, which shows chitinase activity is still present, despite the biomass processing method (Fig. 6). After determining that S6803 maintained chitinase activity after lyophilization, cyanobacteria were lyophilized and turned into powder to formulate the feeds.

Based on the analysis of the results (Fig. 6), it was observed that chitinase activity of SL9 was higher than SR1 regarding β-N-acetylglucosaminidase (corresponding to exochitinase activity) (Fig. 6B), which was expected given promoter strength. Still, both maintained a low activity. Despite that, it was noted that the chitinase activity of strain SL9 was lower than that of strain SR1 in both chitibiosidase (exochitinase activity) and endochitinase activity, which does not align with promoter strength and with what would be expected. In fact, previous activity-characterization assays of these strains had consistently shown significantly higher activity in SL9. Therefore, after a molecular analysis to confirm the presence of the chitinase gene (Fig. 7), the DNA fragments generated by PCR were sent for Sanger sequencing.

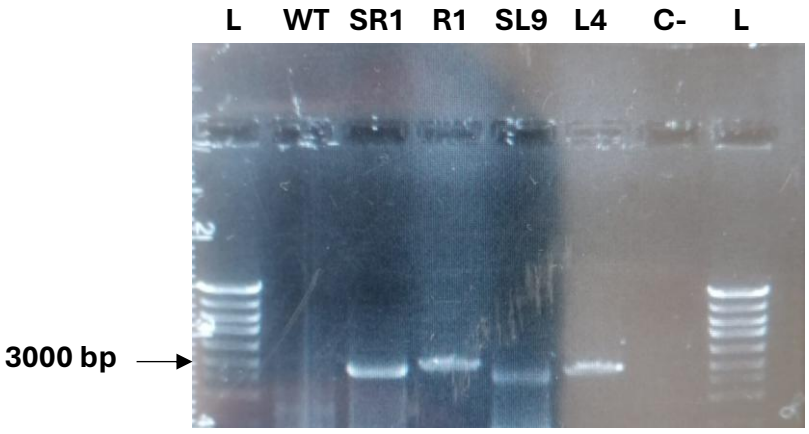


Figure 7: Genetic analysis of the cyanobacterial strains used in this work. Agarose gel showing the result of PCR amplification with primers PS1 and PS2 (specific for plasmid pSEVA251), ic modification (S6803 wild-type, WT), both DNA fragments with promoter (SR1) and its promoter P_{rbcL} (R1) and DNA fragments with promoter (SL9) and its promoter $P_{trc.x.lacO}$ (L4), separated by electrophoresis on agarose gel. Expected sizes: SR1 – 2935 bp; SL9 – 2771 bp; NZYDNA Ladder III (NZYtech) was used as the molecular weight marker (L). Negative control (CTR -) was done with water.

Interestingly, strain SL9 exhibited a deletion in the initial region of the $P_{trc.x.lacO}$ promoter, which may explain the loss of promoter activity and, consequently, the impaired induction of the *N. punctiforme chiA* gene. As a result, reduced expression could lead to lower protein accumulation and therefore lower enzymatic activity. This phenomenon of genetic instability has already been observed in the laboratory during attempts to express other

genes using this strong promoter, and it is also documented in the literature (Bentley et al., 1990; Jones, 2014; Ng et al., 2015). In large-scale cultivation, these mutations are particularly problematic because prolonged growth favors the accumulation of point mutations and genetic rearrangements (Gupta & Srivastava, 2021).

To overcome this problem, cells of the genetically modified SL9 strain could be recovered from the −80 °C frozen stock and cultivated again. However, due to time constraints, it was decided to proceed with the digestibility trial using the available biomass, even though it was known that the biomass did not display the maximum possible chitinase activity.

3.2. Diets digestibility

Insect meals are promising FM alternatives for aquafeeds (Henry et al., 2015; Nogales-Mérida et al., 2019). The current work was planned to assess the potential effects of inclusion of genetically modified chitinase degrading cyanobacteria S6803 on the improvement of chitin and overall diets digestibility.

Fish promptly accepted the experimental diets, and the apparent digestibility coefficients (ADC) of dry matter and protein (Table 3) were consistent with those reported in a previous study conducted with ESB also fed with *Hermetia illucens* at 30% inclusion (Rangel et al., 2024). In contrast, the chitin digestibility values of the CTR diet were higher than those observed in that study (38% versus 24%), though it represents a low digestible value.

Table 3: Apparent digestibility coefficients (%) of experimental diets.

	CTR	WT	SR1	SL9	*p*-value
Dry matter	65.3±1.2	66.4±1.2	64.7±1.2	68.4±1.2	0.190
Protein	86.8±0.6	87.9±0.6	86.2±0.6	88.3±0.6	0.910
Energy	75.7±0.8	76.8±0.8	75.3±0.8	78.4±0.8	0.093
Chitin	34.2±7.0	36.5±7.0	32.5±7.0	33.9±7.0	0.947

Mean and standard deviation (± SD) are presented for each parameter (n = 3). Data was analyzed using a Linear Mixed Model (LMM). Statistical significance was considered at $p < 0.05$.

Although several carnivorous fish species possess only vestigial chitin digestion capacity, or none at all (Guerreiro et al., 2021; Lindsay et al., 1984), some species, such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), have been shown to digest chitin to a considerable extent (Eggink et al., 2022; R. E.

Olsen et al., 2006). Nevertheless, despite evidence of endogenous chitinase expression and activity in these species (Eggink et al., 2022; Holen et al., 2023), the relative contributions of host-derived enzymes versus microbiota-mediated degradation remain unclear. In ESB, it is possible that the observed chitin digestibility results from *Hermetia illucens* induced modulation of the intestinal microbiota, as previously reported by (Rangel et al., 2022). A role for endogenous chitinolytic activity cannot be excluded, as ESB is known to express acidic mammalian chitinase in the intestine (Calduch-Giner et al., 2016). However, the genetically engineered S6803 strains SR1 and SL9 did not enhance chitin digestibility in ESB, and likely did not increase total gut chitinolytic activity (to be confirmed in future analyses), as similar chitin ADC values were observed in fish fed the CTR diet and the S6803 wild type (Table 3).

The lack of effect may be attributed to cyanobacteria's insufficient resistance to passage through the ESB gastrointestinal tract. Incorporating the spores of the chitinolytic fish isolate FI645 (closely related to *Bacillus licheniformis*) into a ESB diet containing 30% *Hermetia illucens* meal significantly increased chitin digestibility compared to the control diet (Rangel et al., 2022). Spores are highly robust structures that withstand extreme conditions (Nicholson et al., 2000; Nicholson & Setlow, 1990) such as the one of fish gastrointestinal tract.

Undigested chitin can also lead to a slight decrease in digestibility of protein and energy (Belghit et al., 2018; Guerreiro et al., 2021; Rangel et al., 2024). As chitin is an insoluble fiber, it can hinder enzyme action and lower the ability for enzymes to digest all other nutrients, entrapping proteins and lipids within the chitin matrix, creating a physical barrier between the feed and digestive enzymes and obstructing the access of proteases and lipases to the substrates, thus limiting their digestibility (De Marco et al., 2015). In the present study, the ADC values for dry matter, protein, and energy were similar among diets (Table 3), which were in line with similar digestive enzymes activities (Table 4). The lack of effect of the S6803 strains SR1 and SL9 in enhancing chitin digestibility did not improve enzymatic access to substrates.

Table 4: Specific activities (mU mg^{-1} protein) of α-amylase, lipase and total alkaline protease in the intestine of European sea bass fed the experimental diets.

	CTR	WT	SR1	SL9	*p*-value
α-Amylase	84.2±12.0	115.7±11.4	88.3±11.4	91.3±12.0	0.173
Lipase	16.0±1.7	14.2±1.7	10.8±1.7	10.8±1.7	0.099
Protease	1620.8±373.1	1461.2±359.2	1690.6±359.2	1702.5±365.6	0.79

Mean values and standard deviation (± SD) are presented for each parameter (n = 3). Data was analyzed using a Linear Mixed Model (LMM). Statistical significance was considered at $p < 0.05$.

3.3. Oxidative stress enzymes

Excessive generation of reactive oxygen species (ROS) leads to oxidative stress, which can induce a variety of deleterious effects in fish, including DNA damage, disruption of cellular membranes and proteins, and lipid peroxidation within membrane structures (Abdel-Daim et al., 2019). To maintain redox homeostasis, organisms rely on endogenous antioxidant defense systems, particularly the enzymatic activities of glutathione reductase (GR), catalase (CAT), and glutathione peroxidase (GPx) (Nordberg & Arnér, 2001).

Lipid peroxidation, a key indicator of oxidative stress, involves the oxidative degradation of biological membranes and other lipid- and protein-containing structures. This process is initiated by ROS, which generate highly reactive intermediates capable of further interacting with cellular proteins and membrane components, thereby impairing cellular damage (Grintzalis et al., 2013).

Overall, oxidative stress arises when ROS production exceeds the capacity of antioxidant defenses, resulting in a redox imbalance and increased susceptibility to cellular injury.

Insect meals can influence oxidative balance in marine fish, although the magnitude and direction of the response are highly dependent on species, tissue type, insect species, and inclusion level. In ESB, the replacement of 50% FM with a mixture of *Hermetia illucens* and *Tenebrio molitor* meals led to an increase in muscle oxygen radical absorbance capacity, indicating enhanced antioxidant potential (Costa et al., 2026). Similarly, another study on ESB reported elevated antioxidant activity in the heart, muscle, and digestive tract when fish were fed diets containing 50% *T. molitor* meal (Bousdras et al., 2022). In hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂), dietary inclusion of *H. illucens* meal replacing 40% of fishmeal improved hepatic antioxidant status and immune capacity compared to control diets (Huang et al., 2025).

Overall, the antioxidant potential of IM may be linked not only to their chitin content but also to their specific nutritional composition, particularly their lipid fraction, as lipids are highly susceptible to oxidative processes (Ngo & Kim, 2014).

The use of cyanobacterial biomass brings in the prospect of improving feed's antioxidant and radical scavenging activities (Ismaiel et al., 2013; Singh et al., 2017), as antioxidants are commonly found in these photoautotrophic microorganisms. The aim of the study by Ismaiel et al. (2013) was to evaluate antioxidant efficacy, including antioxidant content, activity, and enzyme levels. Among the 11 freshwater cyanobacterial species examined, *Spirulina platensis* exhibited the highest radical-scavenging activity and reducing power (524% and 244% higher than the control, respectively), while *Nostoc linkia* showed the greatest chelating activity (195%). The study also demonstrated a positive correlation between cyanobacterial biomass and the antioxidant activity of chlorophyll *a*, carotenoids, and phenolic compounds.

However, in the present study, liver oxidative stress enzymes were not affected by the diet composition (Table 5). While not statistically significant, CAT and GPx were higher in SL9 diet GPx also showed the lowest *p*-value out of all others (0.091). Here, the absence of an antioxidant effect played by S6803 cells in ESB metabolism may be related to a relatively low incorporation rate in fish diets (1.15%), as opposed to that reported for Coral trout (*Plectropomus leopardus*, Yu et al., 2018) and Rainbow trout (*Oncorhynchus mykiss*, Teimouri et al., 2019), in which the best antioxidant effect was observed when *S. platensis* was included at 10% in diets.

Table 5: Activity of oxidative stress in the liver of European seabass fed with experimental diets.

	CTR	WT	SR1	SL9	*p*-value
CAT	293.0±37.3	277.9±39.9	293.0±39,9	324.7±39.9	0.865
GPx	8.6±1.7	7.8±1.7	9.9±1.7	12.5±1.7	0.091
GR	12.0±2.0	10.8±2.0	11.4±2.0	9.9±2.0	0.753
LPO	12.8±8.0	26.4±7.7	33.0±7.7	21.1±7.4	0.183

Mean values and standard deviation (± SD) are presented for each parameter (n = 3). Data was analyze
Linear Mixed Model (LMM). Statistical significance was considered at $p < 0.05$.
Catalase (CAT) - µmol min^{-1} mg $protein^{-1}$; Glutathione peroxidase (GPx) - nmol $NADP^{+}$ min^{-1} mg $protein^{-1}$; G
reductase (GR) – nmol min^{-1} mg $protein^{-1}$; Lipid peroxidation (LPO) - nmol malondialdehyde g $tissue^{-1}$.

4. Conclusion

The present study evaluated the potential of *Synechocystis* sp. PCC 6803 as a biological vehicle to produce chitinases, with the goal of incorporating this cyanobacterium into IM-based diets for ESB. By enhancing chitin degradation within the gastrointestinal tract, it was hypothesized that genetically engineered, chitinase-producing S6803 could improve the digestibility of IM, as well as modulate digestive and antioxidant enzymatic activities.

Firstly, preliminary tests on chitinase activity showed that chitinolytic activity is maintained after freeze-drying, suggesting the reproducibility of the S6803 production method on a larger scale. During our cultivation, not all systems displayed the same growth performance. At the volume of 0.6 L, cyanobacteria had the fastest growth to reach the OD of 1. So, we can conclude that keeping them at 0.6 L during all production would allow for the fastest growth, despite being a small volume when it comes to large scale production. Optimization for large scale production should be taken into account.

Regarding the digestibility trial, however, the obtained results did not reveal significant differences between experimental treatments and the control diet regarding apparent digestibility coefficients. Similarly, no significant effects were observed on intestinal digestive enzymes or on hepatic antioxidant enzymes. These findings indicate that, under the conditions tested, the inclusion of S6803 did not elicit measurable improvements in digestion or oxidative status in ESB.

The results suggest that additional optimization may be required, either in the expression and stability of chitinase activity in S6803 or in dietary inclusion levels, before meaningful physiological responses can be achieved. Future research should therefore focus on enhancing and stabilizing chitinase expression, evaluating different inclusion percentages of S6803 and exploring alternative delivery approaches to fully realize the potential of microbial platforms in aquafeeds. Additionally, the lack of S6803 effects may also be attributed to cyanobacteria's insufficient resistance to passage through the ESB gastrointestinal tract. Future perspectives include testing S6803 under *in vitro* conditions that mimic the ESB stomach or intestinal pH to infer its resistance to the gut environment simulation.

Overall, this work contributes to the emerging field of microbial biotechnology applied to sustainable aquaculture nutrition, offering a foundation for future improvements in the use of engineered microorganisms to support the efficient utilization of insect-based ingredients.

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