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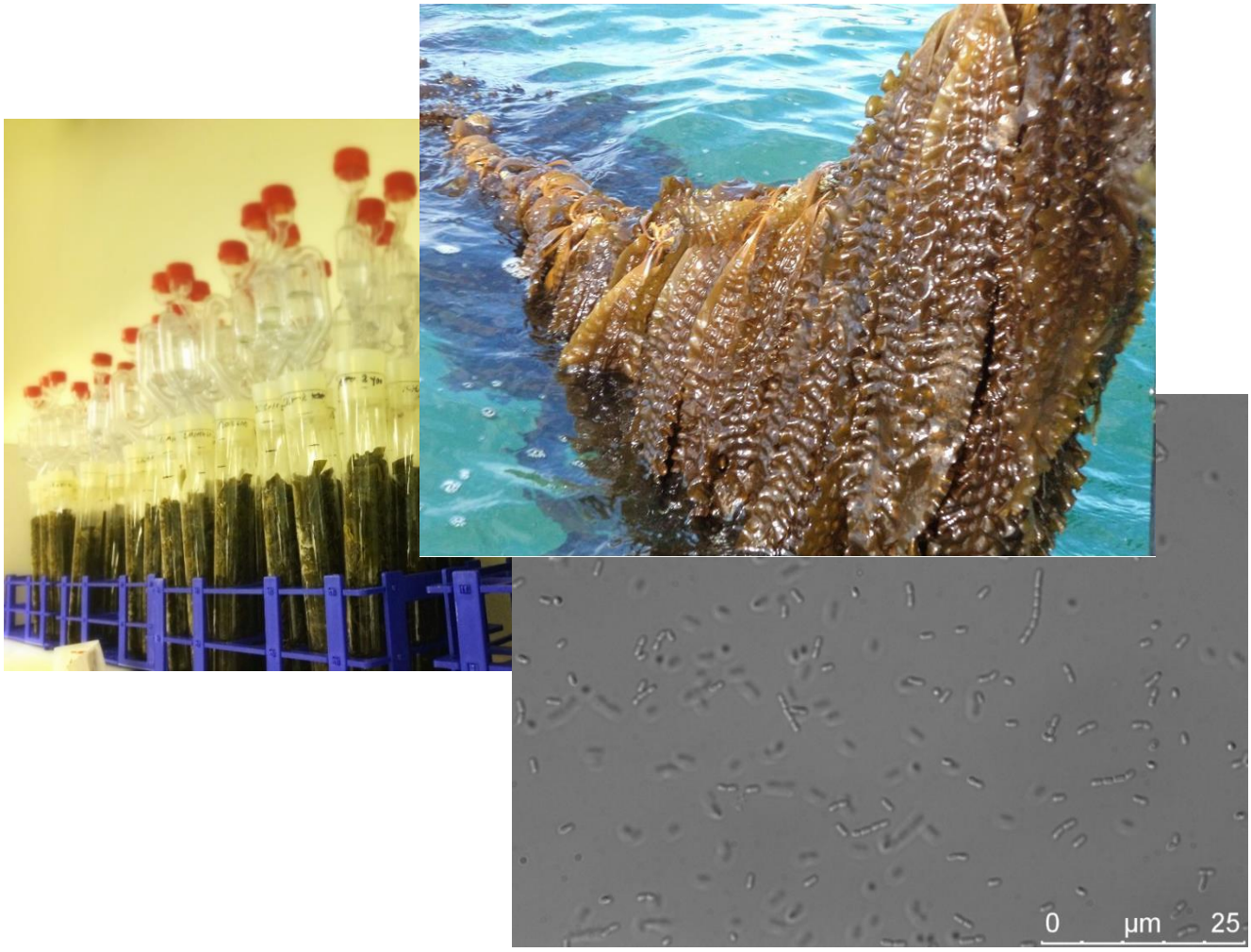

Targeted microbial ensilage additives for *Laminaria digitata* and *Saccharina latissima*

Master's thesis in Biotechnology

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Front page images: **Upper right:** *Saccharina latissima* cultivated on lines. **Lower right:** Microscopic image of bacteria isolated from *Laminaria digitata*. **Left:** Laboratory scale ensiling of S. latissima and L. digitata.

Abstract

Seaweeds are a potential feedstock for biofuel and biochemical production. However, seaweed biomass is subject to rapid degradation. This project aims at evaluating the prospect of using microbial additives in an ensiling process for the preservation of the brown seaweed *Laminaria digitata* and *Saccharina latissima*. Lactic acid bacteria (LAB) isolated from seaweed silage may be suitable as microbial ensilage additives as they thrive in that type of environment.

Seven bacterial strains were isolated from seaweed silage of the species *L. digitata,* and were determined to belong to the genus *Lactobacillus.* The bacterial isolates were screened for growth on the carbohydrates laminarin and mannitol, which are storage carbohydrates in the kelp species of interest. The results from the screening showed that the isolates grew on mannitol but not on laminarin.

Two strains were selected for further investigation of products formed during growth on mannitol. The main products formed for both strains were lactate and acetate. In continuation, these strains were tested as microbial ensilage additives for *S. latissima* and *L. digitata*. The effectiveness of the strains as ensilage additives was compared to a chemical additive and ensiling without additives. Biomass retention was monitored over the course of the ensiling assuming biomass loss as gases was due to unwanted fermentation. The isolates had a positive effect retaining 1 % more biomass, compared to ensiling without any additives. However, there was no significant difference between the two stains. Furthermore, the isolates were slightly outperformed by the chemical additive.

Acknowledgements

I would like to thank my two supervisors Joakim Olsson and Eva Albers for all their patience and guidance during this project. Also, much gratitude goes to Gunnar Cervin at Gothenburg University, the Sven Lovén Center Tjärnö, for his help with collecting the seaweed. Lastly, I would like to thank the Algae group and the entire Industrial Biotechnology group, everyone has been very welcoming and I very much enjoyed doing my master thesis project here.

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1 Introduction

The increasing world population and environmental demands have made it a necessity to escalate the production of sustainable commodities, such as biofuels and bioplastics. As a result of this escalation, a larger quantity of agricultural crops, like corn, rapeseed or sugar cane, are being farmed for use as feed stocks for these products. With this in mind, it has become questionable if production such as this is sustainable, as it is in competition over arable land with food production, resulting in higher food prices (1). In the US, around 38 % of the total corn crop was used for bioethanol production in 2015 (2). In addition, to further increase the crop production, ecosystems important for maintaining the balance of greenhouse gases, such as rainforest and grasslands, are being converted into farmlands which leads to an increased carbon debt (3). This has led to a shift in attention towards biomass of marine origin *i.e.* algae, which do not compete with food production over land area. The term "algae" comprises a wide variety of photosynthetic organisms, which harnesses sunlight to fix inorganic carbon, such as atmospheric carbon dioxide, by photosynthesis. Through this process, the algae produce carbohydrates, protein and lipids (4). Seaweed refers to a group of multicellular marine macroalgae, which is very diverse, both in ecology and physiology.

Besides not requiring arable land area, cultivation of seaweed in itself has several advantages. First of all, during growth the seaweed absorbs excess nitrogen and phosphorous released from the agricultural, resulting in bioremediation (5). Secondly, more biodiversity may be a result of seaweed cultivation, since the seaweed provide a suitable environment for several aquatic animals (6). Also, the cultivation of seaweed does not require any pesticides or fertilizers which can contaminate the environment. Furthermore, compared to land-based crops seaweed grows faster (7) and does not contain any lignin, whose recalcitrance is inhibiting the utilization of lignocellulosic material such as spruce.

1.1 Aim

The main goal of this master thesis project was to investigate whether or not indigenous microorganisms of the brown seaweed species *S. latissima* and *L. digitata* are suitable as microbial additives in ensilage of these species. To reach this goal a number of objectives had to be reached, which are presented below.

- Purification of microbial strains isolated from previous ensilage tests.
- Identification of said strains by microbiological and molecular biological means.
- Characterization of said strains to investigate growth on different carbon sources, their acid production as well as acid and temperature tolerance.
- Ensilage tests, inoculating the seaweed with a selection of the identified microorganisms.

1.1.1 Research questions

To achieve the set objectives of the project the following questions was necessary to answer:

- What characteristics do the microorganisms isolated from *S. latissima* and *L. digitata* exhibit?
- What species of microorganisms are there in *S. latissima* and *L. digitata* silage?
- Which source of carbon indigenous to *S. latissima* and *L. digitata* do they mainly utilize?

- What acids do the microorganisms produce?
- How are their tolerance to acids and temperatures?
- Do the selected purified strains have any effect on the preservation of *S. latissima* and/or *L. digitata* when used as microbial additives?

1.2 Scope of the project

Due to time and resource constraints, certain aspects were out scope for the project. Firstly, *S. latissima* and *L. digitata* were the only seaweed species investigated, which in turn limited growth characterization of the isolated bacteria to the carbohydrates laminarin and mannitol. Secondly, the acid tolerance was only studied for lactic, formic, acetic, and propionic acid, as these were the organic acids used in the ensilage experiments where the strain originated from. Thirdly, the examination of bacteria and yeasts will be limited to species that have been isolated from *S. latissima* or *L. digitata* silage and one *L. plantarum* strain previously isolated from an industrial fermentation plant. Lastly, analyses to determine the chemical composition of *S. latissima* or *L. digitata* after ensilage will not be conducted, instead it will be limited to measuring biomass retention and change in pH during the ensilage of the seaweed.

2 Background

2.1 Application, availability and potential uses of seaweed

Around 7.5-8 million tonnes is cultivated today, mainly for human food products and to obtain hydrocolloids like agar, carrageenan and alginate, but some is used as fertilizers and animal feed additives (8). However, it has become of interest to cultivate seaweeds to replace oil-derived products, as part of the growing need for sustainable of bio-based commodities, like fuel and materials.

There are around 10 000 species of seaweeds, which fall into one of three groups empirically based on pigmentation, red, green or brown (9). In Sweden, brown seaweeds of the species *L. digitata* and *S. latissima* are very common, these belong to the family of seaweeds called *Laminariaceae* or more commonly kelp. They are the sole group of seaweeds that have cells specialized for transport of nutrients, stored mainly as the polysaccharide laminarin and the sugar alcohol mannitol (10). In *L. digitata*, the content of mannitol and laminarin can reach up to 20 % and 18 % respectively and in *S. latissima* they can reach up 19 % and 33 %, depending on the surrounding environment and season (11). Laminarin mainly consists of β-1,3 linked glucose residues, hence it could potentially be used a carbon source in yeast fermentation, post-hydrolysis (7). Manntiol is the alcohol form of the sugar mannose, it is not readily fermented by regular baker's yeast, however, other microorganisms (12), like *Zymobacter palmae*, possess the metabolic functions to utilize it. In addition to these carbohydrates, the kelp contains high value nutritional compounds, like omega-3 fatty acids, vitamins, proteins with many of the essential amino acids and polyphenols with anti-oxidative activity (11). Due to the content of these compounds, seaweed of these species could potentially also be used to produce food or feed products. In an effort to take advantage of the seaweeds potential, a project called SEAFARM (www.seafarm.se) has been established in Sweden, researching how to cultivate seaweed and how to best make use of various valuable compounds.

2.1.1 Seafarm – Macroalgae for a biobased society

Seafarm is collaboration project between Chalmers University of Technology (Chalmers), Royal Institute of Technology (KTH), Gothenburg University (GU), Lund University (LU), and Linnæus University attempting to make use of seaweeds to further the goal of establishing a bio based-economy. Seaweeds of the species *S. latissima* and *L. digitata* are cultivated on the west coast of Sweden in a national park in Kosterhavet. As stated above, seaweeds contain several valuable compounds and it would be desirable to utilize them all. Therefore, Seafarm is divided into 5 focus areas, each working to evaluate different parts of the production chain.

- Focus area 1 (GU) – Establishing a pilot-cultivation of macroalgae as well as investigating the ecological and environmental effects of the cultivation.
- Focus area 2 (CTH) – Asses the preparation and preservation of macroalgae before they are sent to the biorefinery.
- Focus area 3 (CTH/KTH) – Evaluation of the biorefinery process *i.e*. how to extract the different compounds and make use of them in the production of environmentally sustainable products.
- Focus area 4 (Linnæus University) – Investigation of biogas production from the waste streams from the biorefinery.
- Focus area 5 (KTH/LU) – Sustainability analysis of the whole process, both ecological and economical aspects.

One of the challenges with having *S. latissima* and *L. digitata* as a starting point in a biorefinery is the post-harvest preservation, as the seaweed degrades rapidly if left untreated. One technique for preservation that is currently being investigated in Focus area 2 is ensilage.

2.1.2 Ensilage as preservation method for seaweed biomass

There are several approaches for preventing microbial activity in organic matter, such as heating, drying, salting, freezing or vacuum packing. Which technique to apply, depends on the material and what is desired in the end products. For instance, heat treating the material is very effective in preventing microbial activity, but it also affects the material itself and in the case of seaweed it might be too energy intensive due to high content of moisture (13).

The basic principle of ensilage, is to establish an anaerobic environment around the biomass to promote the growth of indigenous lactic acid bacteria (LAB). The LAB will in turn produce lactic acid, decreasing the pH, resulting in conditions too harsh to sustain microbial activity (14). However, this process is very complex and depends on several factors, for instance, the amount and the activity the LAB, the quantity of water-soluble-carbohydrates, as it affects the activity of the LAB and the buffering capacity of the material, since the pH decrease need to occur relatively fast. Temperature and moisture content also affect the ensiling, as it will affect the proliferation of the microorganism in the seaweed (15, 16).

To improve the quality of the silage, different additives have been developed to ensure successful ensiling. These additives can belong to one of three categories, chemical, bactericidal or microbial additives (14, 15). Chemical additives are commonly organic acids, like formic, propionic or acetic acid, this provides an instant decrease in pH, inhibiting microbial activity.

Bactericidal additives, contains selective or non-selective bacteriostatics, preventing specific or general growth of certain bacteria thereby indirectly promoting the growth of LABs. Microbial additives typically consist of LABs, and by adding them, the lactic acid production will increase resulting in a faster pH drop. The microbial additives are often accompanied by amylases, when ensiling agricultural crops, to increase the amount of water-soluble-carbohydrates.

In addition, there are specific factors that needs to be considered when ensiling seaweed, more specifically the time period in which the seaweed is harvested. Depending on the season, the chemical composition of the seaweed may favor the growth of certain microorganisms, for instance, peak laminarin content is found during the summer, hence laminarin-metabolizing microorganism may be more proficient in seaweed harvested during that period (17). Another seasonal variation that might affect the ensiling is the growth of epiphytes on the seaweed (18, 19).

2.1.3 Lactic acid bacteria and its role in a ensiling process

Lactic acid bacteria (LAB) are one of the most well-known group of microorganisms. They are used in various food and feed applications, and as mentioned above they play an important role in the ensilage process. LABs can readily be found in plant materials and guts of animals due to the fact that lactic acid bacteria lack most anabolic pathways, forcing them to require a nutrient rich environment. The bacteria belonging to the LAB group are Gram positive, aerotolerant anaerobes, non-sporulating rod or cocci shaped bacteria capable of fermentative production of primarily lactate from carbohydrates. The lactate fermentation can either be homofermentative, producing only lactate, or heterofermentative, producing mainly lactate as well as other organic acids (20, 21).

For a successful ensilage of seaweed, using microbial additives, certain characteristics would be useful for the LAB to be able to compete with other microorganisms residing in the seaweed. For instance the concentration of salt during the ensiling may be high, as the seaweed is cultivated in seawater, therefore higher halo tolerance may be necessary. The temperature during the ensiling may be difficult to control, hence, a LAB that grow over a wide range of temperatures is suitable. Furthermore, the LAB needs to possess the metabolic functions for fermenting the carbohydrates that are present in the seaweed, with a relatively fast acid production (16).

Diverse LABs have been isolated from marine origins. For example, there is a *Carnobacterium* isolated from an Antarctic lake which has optimal growth in neutral pH conditions (22). On the other hand, the novel *Marinilactobacillus psycrotolerans* grows in alkaline to acidic conditions (23). Due to this diversity of LAB of marine origin, there might exist a strain suitable for use as microbial additive for seaweed ensiling.

3 Materials and methods

The project was divided into three parts, identification, characterization and application of microbial additives for ensilage of macroalgae. Identification was performed by selective culturing, microscopic studies and phylogenetic studies. Characterization focused on growth screening on two carbohydrates predominant in S. *latissima* and *L. digitata*. Finally, the identified and characterized bacteria were used as additives in seaweed ensilage tests.

3.1 Identification of bacterial isolates

The isolation and identification of bacteria from *Laminaria digitata* silage consisted of several steps. Firstly, single species was obtained by plating and restreaking. Secondly, the morphology of the bacteria was observed microscopically. Thirdly, a fragment of the 16S ribosomal RNA gene was sequenced and aligned to determine species identity. Lastly, a screening for growth on xylose was performed to determine if the bacteria belonged to the *Lactobacillus pentosus* species.

3.1.1 Growth media

All media used for the bacterial growth experiments were based on the MRS media developed by de Man *et al*. for growth of *Lactobacilli* (24). All liquid media were made by dissolving each component in natural seawater, hence the media contained (g l^{-1}): peptone 10, meat extract 8, yeast extract 4, ammonium acetate 0.19 citric acid monohydrate 1.8, manganese sulfate monohydrate 0.04, magnesium sulfate heptahydrate 0.2, dipotassium hydrogen phosphate 2 and well as Tween 80 1 µl ml^{-1} of media. Depending on the experiment the media was made with different contents regarding carbon and energy source. The last component added to media was Tween 80 (polysorbate 80) 1 µl/ml of final media. All liquid media was dissolved in natural seawater and sterile filtered through 0.2 µm cellulose filter. In the case of agar plates, MRS obtained from Oxoid (Basingstoke, Hampshire, England) or Merck (Merck, Darmstadt, Germany) was supplemented with 12 g l^{-1}agar and dissolved in artificial seawater. When screening for growth on xylose, the plates were made from the liquid media stock solutions supplemented with agar and xylose.

3.1.2 Isolation and cultural morphology

Seven MRS-plates with crude isolates of potential acidifying bacteria originating from *L. digitata* silage were provided as a starting point for this project. To obtain pure species isolates, single colonies were picked and re-streaked on new MRS-plates, which were incubated in anaerobic conditions at room temperature (~20 °C) for 2 days. Following the incubation, single colonies were picked from each plate and re-streaked on MRS-plates, followed by incubation in aerobic conditions at room temperature. The re-streaking procedure was repeated 3 times. Colonies were picked from the final plates and suspended in artificial seawater on glass slides and the cellular morphology was studied with light microscopy (Leica Microsystems DMI 4000B). Following the microscopic observations, the isolates were cultured in MRS media (supplemented with glucose 20 g l^{-1}) and glycerol stocks prepared.

3.1.3 16S ribosomal RNA gene sequencing

The extraction of DNA, was performed using the PowerSoil ™ DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the provided protocol, but instead of using a

soil sample in step 1, cells were picked from plates with a loop and added to the PowerBead tubes. Gene fragments of the 16S rRNA gene was amplified by PCR, with the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1494-R (5'-GCTCTAGAGCTGACTGACTGAGGYTACCTTGTTACGACTT-3') obtained from Eurofins (MWG GmbH, Martinsried, Germany) and Phusion High-Fidelity DNA polymerase (Thermo Scientifc). The PCR-product was purified with Genejet purification kit (Thermoscientific) according to provided protocol. The purified PCR-product was run on a 1 % agarose gel, alongside Generuler 1 kb (thermoscientific) to determine that a DNA-fragment of the correct length had been obtained. The amplified 16S rRNA gene was sent for sequencing (Eurofins MWG GmbH, Martinsried, Germany) and the obtained sequence was aligned by BLASTn searches in the NCBI database to identify species of the isolated bacteria.

3.2 Characterization of bacterial isolates

For characterization of the isolates, three characteristics were investigated. Firstly, the temperature range of the isolates was examined. Secondly, a carbon source screening was performed, using the carbohydrates mannitol and laminarin. Thirdly, the acid tolerance of the bacteria was investigated. Finally, the production of organic was investigated.

3.2.1 Determination of temperature range by plate culturing

To elucidate in which temperature range the bacteria grew, they were plated on MRS-plates, which in turn was put in 12, 15, room temperature (RT) (approximately 20 °C), 30 and 37° C. In order to rule out whether or not the bacteria belonged to the *Lactobacillus pentosus* species, the bacteria were plated on MRS-xylose plates and MRS-plates absent of a carbon source as control.

3.2.2 Carbon source screening of the bacterial isolates

To elucidate whether or not the isolated bacteria had the ability to metabolize the carbohydrates laminarin and mannitol, which are indigenous to *Laminaria digitata* and *Saccharina latissima*, a screening was performed applying the Microbiology Reader Bioscreen C (Oy Growth Curves) growth monitor. In addition to the strains isolated from *Laminaria digitata* silage, a known *Lactobacillus plantarum* (CCUG 30503, from the culture collection at the University of Gothenburg) strain was included in the screening.

Cells were pre-cultured in 5 ml MRS-media (supplemented with glucose 20 g l^{-1}) overnight. 0.5 ml of each pre-culture were centrifuged at 20000 x g at room temperature for 1 min, the supernatant was removed and the cells were re-suspended in 0.5 ml natural seawater. This was repeated twice in order to wash the cells. The washed cultures were diluted in seawater to obtain an optical density at 600 nm ($OD_{600\ nm}$) of 0.36.

20 µl of the washed pre-cultures were added to 125 µl with MRS-media in triplicate cultures, resulting in a starting $OD_{600\ nm}$ of 0.05. The media in each well were supplemented with either laminarin 5 g l^{-1}, mannitol 5 g l^{-1}, mannitol 20 g l^{-1}, glucose 5 g l^{-1}or glucose 20 g l^{-1}. The culturing was done under semi-aerobic conditions at 20 °C with continuous shaking. $OD_{wideband,\ 420\text{-}580\ nm}$ was measured every 15 min, OD_{max} was determined as the highest value during the culturing, and μ_{max} was determined by calculating the slope of the exponential growth phase.

3.2.3 Acid toxicity screening of the bacterial isoaltes

Acid toxicity of lactic, acetic, formic and propionic acid was also investigated for the isolates and *L. plantarum*, CCUG 30503, using the same procedure as described in section 3.2.2, except the MRS-media was supplemented with mannitol 20 g l^{-1} and 50 mM, 0.5 M or 1 M of either lactic, acetic, formic or propionic acid.

As two different growth patterns could be observed in the initial screening, two out of the seven strains, one for each growth pattern, as well as the *Lactobacillus plantarum* (CCUG 30503) strain were selected for a second screening. The culturing was performed as previously described, but the pre-culturing was done with mannitol (20 g l^{-1}) as carbon source instead of glucose and the organic acid concentration was decreased to 50 mM, 100 mM and 150 mM, also a mix of propionic (17.5 mM) and formic acid (32.5 mM) was included. Furthermore, it was investigated if the two strains could metabolize the acids, by using a MRS-media containing lactic, acetic, formic or propionic acid as sole carbon source at a concentration of 50 mM.

3.2.4 Product formation investigation by shake flask cultures

Two growth patterns could be observed between the seven silage isolates during cultivation in the Bioscreen, hence, two isolates (EO20 and EO22) were selected for shake flask culturing as well as the *Lactobacillus plantarum* (CCUG 30503) strain. During the shake flask cultivation samples were taken with regular intervals for analysis by HPLC.

The same pre-culture procedure was used as above, except that the carbon source in the MRS-media was mannitol 20 g l^{-1}. After washing, the cells were diluted to an $OD_{600\ nm}$ of 1.275. 1 ml of washed pre-culture was used to inoculate a shake flask containing 50 ml MRS-media (mannitol 20 g l^{-1}), resulting in an initial $OD_{600\ nm}$ of 0.025. The cells were cultured in triplicates at 25 °C, 150 rpm (IKA® 4000i Control) under both aerobic and anaerobic conditions respectively. OD was measured every one and half-hours, during the exponential phase and samples were taken to analysis of mannitol consumption and acid production. The samples were centrifuged at 20000 x g and the supernatant was transferred to clean tube and stored in -20 °C freezer.

Mannitol, lactic and acetic acid content were analyzed by HPLC (Ultima 3000, Dionex) coupled to a refractive index detector (Shodex) and a UV-detector (Ultima 3000, Dionex). Prior to analysis samples were diluted in 5 mM sulfuric acid and filtered through a 0.2 µm nylon membrane. Samples were analyzed using an Aminex 87HPX column (Dionex) held at 45 °C, with a flow rate of 0.6 ml min^{-1}, using an isocratic eluent composed of 5 mM sulfuric acid. Mannitol, lactic and acetic acid were identified according to their retention times. External standards with acetate, glycerol, pyruvate and succinate, mannitol and lactate were used at a concentration between 0.025 g l^{-1} - 20 g l^{-1}.

3.2.5 OD relation to CFU

As the bacteria were going to be used as additives during ensilage of seaweed, it was necessary to relate the optical density of the shake flask cultures to the amount of viable cells. Cultures were grown aerobically in the same conditions as described in section 3.2.4. Samples were taken when $OD_{600\ nm}$ had reached 1.0, 2.0 and 3.0. The samples were diluted 10^6, 10^7 and 10^8 times in natural

seawater and plated on MRS-agar plates in triplicates. Plates with between 25 and 250 colonies were counted, and CFU ml^{-1} at the specific OD was determined.

3.3 Ensilage of kelp using microbial additives

Wild kelp of the species *L. digitata and S. latissima* were collected in April 2015 off the Swedish West coast in Kosterhavet. The seaweed was kept on ice at all time between collection and preparation for ensiling. The Seaweed was cut into 1x1 cm pieces and subjected to 1 of 5 treatments: 1) Control (No additives) 2) Addition of *L. plantarum* (CCUG 30503) (10^6 CFU g^{-1} seaweed) 3) Addition of silage isolate EO22 (10^6 CFU g^{-1} seaweed) 4) Addition of silage isolate EO20 (10^6 CFU g^{-1} seaweed) and 5) Addition of an acid mixture of 65 % formic and 25 % propionic acid (2 ml kg^{-1} seaweed). The seaweed was stirred with spoon in a beaker for 5 min after the additives had been added. The same procedure was used for the control even though nothing was added. Following the stirring the seaweed was packed in pre-weighed tubes, approximately 100 ml in volume, leaving a head space of 2 cm between the seaweed and the cap. The cap was comprised of a silicon/rubber stopper with an attached airlock filled with glycerol, providing anaerobic conditions and allowing the pressure to be monitored. Furthermore, a reference tube was made, containing 1 ml water to monitor the weight change due to moisture absorption. The tubes were weighed regularly over the course of 84 days, and a percentage biomass retention was calculated, in order to evaluate each treatment, assuming a decrease in weight as gases passed out of the tube due to degradation.

The pH of the seaweed was also measured directly after the treatment, after 3 days and 84 days. In order to measure the pH, a small portion of seaweed (2.5-5 g) was stirred with 1 ml of water per gram seaweed, and the pH was measured with pH-meter on the resulting liquid.

3.4 Statistics

Statistical analysis was done by performing one-factor ANOVA followed by a post-hoc test using Tukey's HSD method, with a significance level of 0.05, using the software SAS JMP 11.

4 Results

4.1 Bacterial morphology

As a preliminary identification of the isolates, the micromorphology was studied. From the microscopic observations it was possible to deduce that the cells were rod-shaped, forming rows *i.e*. streptobacilli. Micromorphology of this type is a typical characteristic of LAB of the *Lactobacillus* genus. An example observation of one of the isolates is shown in Figure 1. Furthermore, the colonies formed on a MRS-plate had a raised, milky-white appearance, also indicative of the *Lactobacillus* genus.

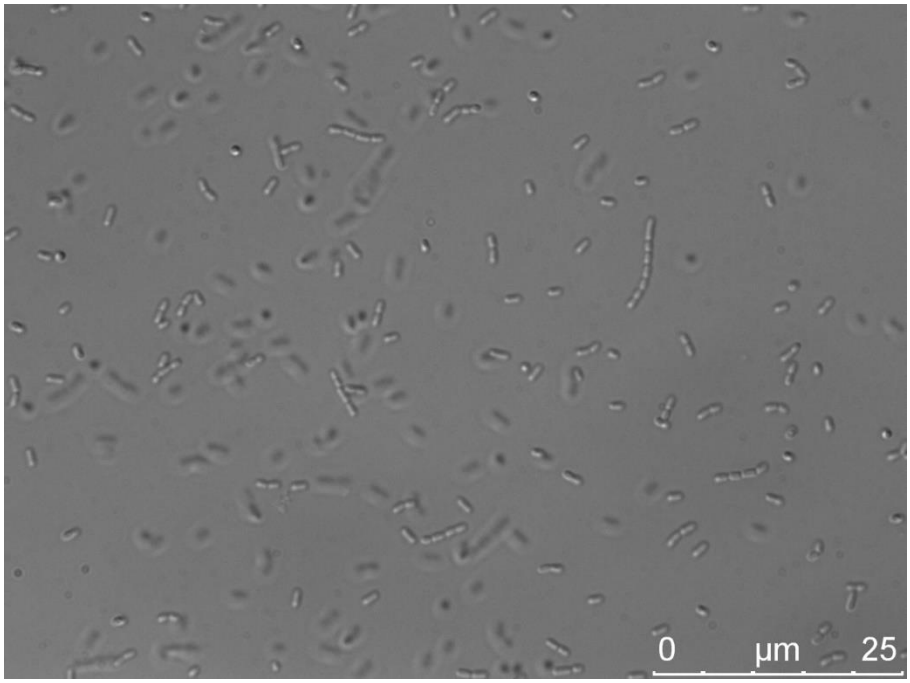

Figure 1. Microscopic image of one isolate from L. digitata silage.

4.2 Phylogenetic analysis

The length of the PCR-amplified 16S rRNA gene fragment can be observed in Figure 2. Sequences of the fragments were determined for all isolates and through comparison using BLASTn, all seven isolates had 100 % identity to *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus fermentum*. Additionally, three of the strains had 100 % identity with *Lactobacillus helveticus*. Length of the determined sequences and species with 100 % identity, for each isolate, can be found in Table 1 (full sequences are shown in Appendix 1). As can be seen in Table 1, for isolates EO18, EO19 and EO24, a slightly longer DNA fragment was successfully sequenced. These were the ones that had 100 % identity to *L. helveticus*. In addition, when the isolates was aligned to each other, they all had 100 % identity to one another. Hence, it is possible that the increased length of EO18, EO19 and EO24 is the only reason why they had 100 % identity to *L. helveticus*, since the shorter length of the other strains would rule out certain homologs in the database.

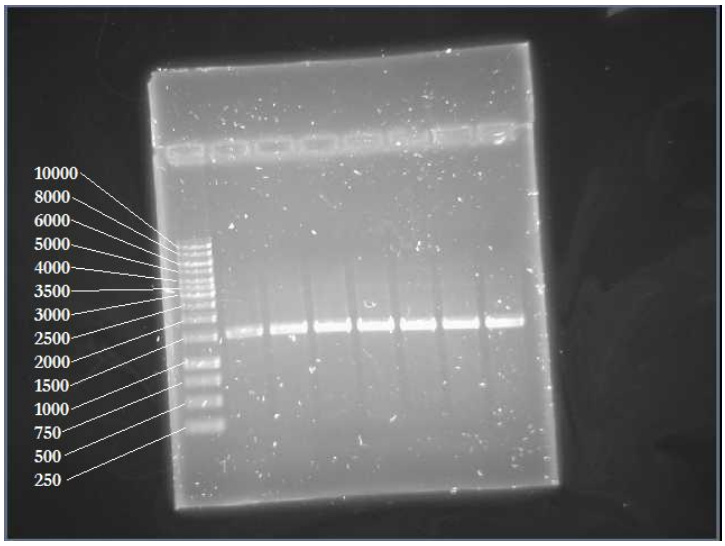

Figure 2. Fragments of the 16S rRNA gene, isolated from the silage. From left to right: EO25, EO24, EO23, EO22, EO20, EO19, EO18.

Table 1. 16S rRNA gene fragment length and the Lactobacillus sp. with 100 % similarity by alignment in the NCBI database using BLASTn.

Strain	Length (bp)	Lactobacillus species with 100 % similarity
EO25	1397	*L. plantarum, L. pentosus, L. fermentum*
EO24	1420	*L. plantarum, L. pentosus, L. fermentum, L. helveticus*
EO23	1407	*L. plantarum, L. pentosus, L. fermentum*
EO22	1397	*L. plantarum, L. pentosus, L. fermentum*
EO20	1408	*L. plantarum, L. pentosus, L. fermentum*
EO19	1410	*L. plantarum, L. pentosus, L. fermentum, L. helveticus*
EO18	1416	*L. plantarum, L. pentosus, L. fermentum, L. helveticus*

4.3 Temperature range

To investigate in which temperature range the isolates could grow, they were plated in MRS and incubated in either 12, 15, RT, 30 37 °C. Observing the plates over two weeks, colonies could be observed after 2 days at RT, 30 and 37 °C. At 15 °C colonies were observed after 5 days and no colonies formed at 12 °C within the two weeks. As for the plates where glucose had been replaced with xylose, very small colonies could be observed after 2 days. However, colonies of same size could be seen on plates absent of a carbon source as well, hence, it was assumed that the growth that had occurred was due to the other components of the media. Furthermore, it could be concluded that none of the strains were of the species *L. pentosus*.

4.4 Carbon source screening of the bacterial isolates

The 7 isolates and *L. plantarum* (CCUG 30503) were screened for growth on mannitol and laminarin, to investigate which storage carbohydrate in *L. digitata* and *S. latissima* they could utilize (control replicates were grown on glucose). Growth rate μ_{max} and OD_{max} are shown in Table 2. The, largest difference, with regards to growth on mannitol or laminarin could be seen in

final OD when grown on mannitol 20 g l^{-1}. During this fermentation, four strains CCUG 30503, EO25, EO22 and EO18 reached a final OD between 2.15-2.27, while the other strains, EO24, EO23, EO20 and EO19 only had a final OD of 1.40-1.57. Furthermore, there were also some slight differences in growth rate μ_{max}, with CCUG 30503 having the fastest growth and EO24 the slowest. None of the strains seemed to be able to metabolize laminarin with all strains reaching a final OD between 0.24 and 0.35. Growth curves of CCUG 30503, EO22 and EO20 on all carbon sources investigated (laminarin 5 g l^{-1}, mannitol 5 g l^{-1}, mannitol 20 g l^{-1}, glucose 5 g l^{-1}or glucose 20 g l^{-1}) can be seen in Figure 3. All growth curves can be found in Appendix 2.

*Table 2. Growth data with standard deviations during Bioscreen cultivation in saline MRS-media. Values with different letters in superscript along columns are significantly different ($p < 0.05$). Experiment was run in triplicates ($n = 3$). * No significant difference between the strains *, **1 replicate for mannitol 5 g l^{-1}, ***2 Replicates in laminarin 5 g l^{-1}.*

Strain	Carbon Source									
	Mannitol 5 g l^{-1}		Mannitol 20 g l^{-1}		Laminarin 5 g l^{-1}		Glucose 5 g l^{-1}		Glucose 20 g l^{-1}	
	μ_{max} (h^{-1})	OD_{max}	μ_{max} (h^{-1})	OD_{max}	μ_{max} (h^{-1})	OD_{max}*	μ_{max} (h^{-1})*	OD_{max}*	μ_{max} (h^{-1})*	OD_{max}
CCUG 30503	0.155±0.006^{a}	1.49±0.01^{abc}	0.147±0.016^{a}	2.27±0.03^{a}	0.166±0.039^{a}	0.34±0.04	0.233±0.008	1.44±0.02	0.247±0.003	1.73±0.00^{d}
EO25	0.129±0.008^{a}	1.48±0.03^{abcd}	0.092±0.003^{b}	2.15±0.20^{a}	0.119±0.034^{ab}	0.26±0.08	0.233±0.016	1.53±0.29	0.247±0.024	2.17±0.04^{a}
EO24**	0.120±0.000^{b}	1.53±0.00^{ab}	0.019±0.000^{c}	1.54±0.03^{b}	0.144±0.033^{ab}	0.32±0.07	0.225±0.028	1.66±0.13	0.259±0.007	1.40±1.19^{ab}
EO23	0.124±0.003^{a}	1.51±0.02^{a}	0.083±0.004^{b}	1.54±0.03^{b}	0.134±0.024 ^{ab}	0.36±0.02	0.250±0.014	1.57±0.21	0.245±0.011	2.04±0.02^{abc}
EO22	0.121±0.009^{a}	1.43±0.02^{d}	0.076±0.005^{b}	2.25±0.05^{a}	0.163±0.050^{a}	0.35±0.03	0.224±0.017	1.44±0.01	0.23±0.014	1.89±0.10^{c}
EO20	0.090±0.001^{ab}	1.45±0.01^{bcd}	0.076±0.002^{b}	1.40±0.09^{b}	0.160±0.017^{a}	0.35±0.04	0.249±0.019	1.73±0.04	0.255±0.003	2.09±0.07^{a}
EO19***	0.105±0.002^{ab}	1.48±0.03^{abcd}	0.078±0.001^{b}	1.57±0.05^{b}	0.083±0.039^{b}	0.26±0.13	0.259±0.007	1.73±0.04	0.252±0.011	2.09±0.02^{a}
EO18	0.122±0.004^{a}	1.43±0.02^{cd}	0.082±0.002^{b}	2.26±0.02^{a}	0.098±0.023^{ab}	0.25±0.05	0.246±0.015	1.49±0.02	0.235±0.007	1.91±0.05^{bc}

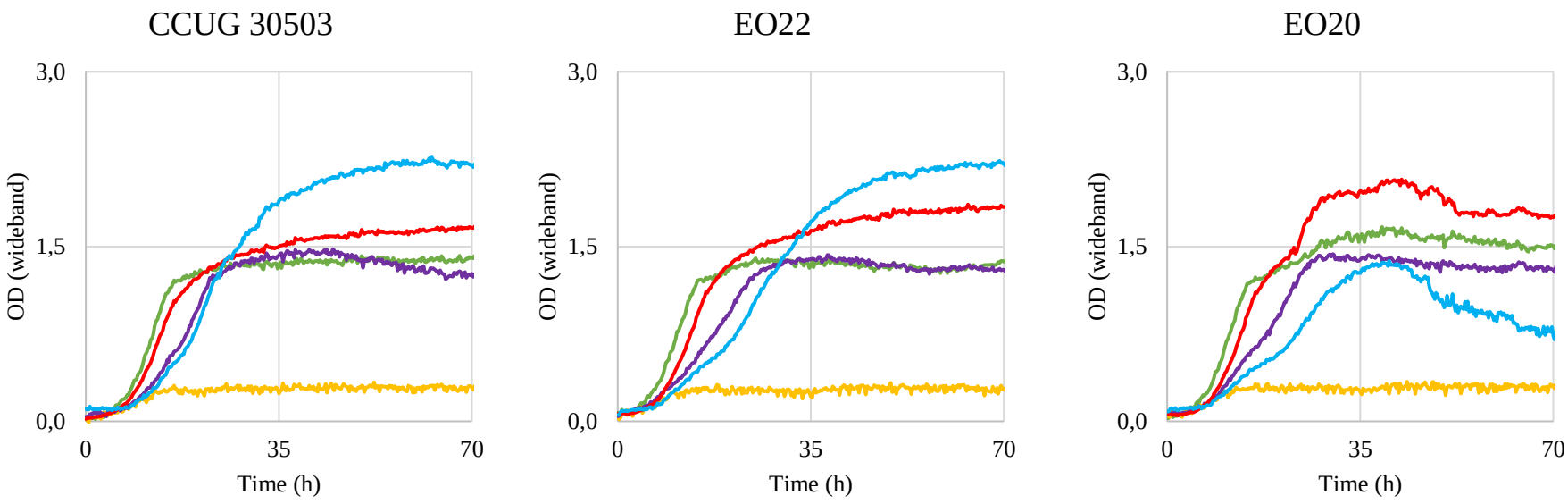

Figure 3. Bioscreen cultivation of CCUG 30503, EO22 and EO20 in with saline MRS-media with mannitol 5 g l^{-1} (–), laminarin 5 g l^{-1} (–), glucose 5 g l^{-1} (–), mannitol 20 g l^{-1} (–) and glucose 20 g l^{-1} (–) as carbon source. The graphs presented are average of 3 replicates.

4.5 Acid toxicity screening of bacterial isolates

To determine the acid tolerance of the isolates they were cultured in the Bioscreen with the MRS-media supplemented with lactic, acetic propionic or formic acid. As described in section 3.2.3 a preliminary screening was performed with the concentrations 50 mM, 0.5 M or 1 M using all strains that previously had been screened for growth on different carbon sources, however, no growth could be observed for any of the strains in any of the acids with the two higher concentrations. Growth rate μ_{max} and OD_{max} for the preliminary screening are shown in Table 3. When observing the growth in the presence formic acid (50 mM) it is clear that all strains are inhibited, only reaching final OD between 0.19 and 0.36 compared to the other acids. One can also see that EO24, EO23, EO20 and EO19 cultured in the presence of lactic acid 50 mM are now the group of strains attaining higher final OD with values between 2.05-2.29, while they had a lower final OD during the carbon source screening. Growth curves obtained during the initial acid toxicity screening can be found in Appendix 3.

*Table 3. Growth data with standard deviation during the first acid toxicity screening in Bioscreen using saline MRS-media with mannitol 20 g/l as carbon source supplemented with an organic acid. Values with different letters in superscript along columns are significantly different ($p < 0.05$). Experiment was run in triplicates (n = 3). N.D. = no data. *2 Replicates in propionic acid 50 mM, **2 replicates in formic acid 50 mM.*

Strain	Organic Acid							
	Lactic acid 50 mM		Acetic acid 50 mM		Propionic acid 50 mM		Formic acid 50 mM	
	μ_{max} (h^{-1})	OD_{max}	μ_{max} (h^{-1})	OD_{max}	μ_{max} (h^{-1})	OD_{max}	μ_{max} (h^{-1})	OD_{max}
CCUG 30503	0.093 ± 0.001^{b}	1.91 ± 0.01^{bcd}	0.076 ± 0.003^{abc}	0.76 ± 0.06^{b}	0.033 ± 0.003^{e}	1.64 ± 0.1^{ab}	N.D.	N.D.
EO25	0.113 ± 0.003^{ab}	1.74 ± 0.01^{d}	0.08 ± 0.001^{abc}	1.74 ± 0.01^{a}	0.089 ± 0.001^{c}	1.2 ± 0.03^{c}	0.033 ± 0.012^{b}	0.24 ± 0.01^{ab}
EO24	0.102 ± 0.01^{ab}	2.05 ± 0.22^{abc}	0.058 ± 0.005^{c}	1.81 ± 0.31^{a}	0.067 ± 0.008^{d}	1.46 ± 0.25^{bc}	0.018 ± 0.007^{b}	0.19 ± 0^{b}
EO23*	0.121 ± 0.006^{a}	2.23 ± 0.04^{a}	0.067 ± 0.019^{bc}	1.89 ± 0.23^{a}	0.097 ± 0.013^{bc}	1.49 ± 0.24^{abc}	0.024 ± 0.004^{b}	0.27 ± 0.07^{ab}
EO22**	0.117 ± 0.004^{ab}	1.78 ± 0.05^{cd}	0.073 ± 0.012^{abc}	2.04 ± 0.2^{a}	0.099 ± 0.002^{bc}	1.51 ± 0.01^{bc}	0.026 ± 0^{b}	0.34 ± 0.02^{a}
EO20	0.118 ± 0.003^{ab}	2.29 ± 0.01^{a}	0.095 ± 0.002^{ab}	2.09 ± 0.1^{a}	0.121 ± 0.007^{a}	1.93 ± 0.07^{a}	0.059 ± 0.007^{a}	0.36 ± 0.1^{a}
EO19	0.105 ± 0.023^{ab}	2.11 ± 0.16^{ab}	0.076 ± 0.013^{abc}	2.04 ± 0.21^{a}	0.108 ± 0^{ab}	1.79 ± 0.07^{ab}	0.034 ± 0.007^{b}	0.3 ± 0.02^{ab}
EO18	0.096 ± 0.009^{ab}	1.75 ± 0.02^{d}	0.096 ± 0.008^{a}	2.12 ± 0.01^{a}	0.111 ± 0.006^{ab}	1.64 ± 0.21^{ab}	0.028 ± 0.005^{b}	0.33 ± 0.00^{a}

The second toxicity screen was performed with lower concentrations of the organic acids and it also included a media lacking carbon source, to investigate whether or not the isolates could grow solely on the organic acids. As can be seen in Table 4, there was no positive significant difference in OD_{max} for any of the strains when grown solely on an organic acid compared to when grown in media absent mannitol and organic acid. Furthermore, an increase in organic acid concentration from 50 mM only has negative effects on growth, however, 50 mM propionic acid seemed to have had a positive effect in final OD for, CCUG 30503.

Another observation that can be made during the second acid toxicity assay is that none of the strains could grow in presence of 150 mM lactic acid as well as 100 and 150 mM formic acid, which is further illustrated in Figure 4. In addition, CCUG 30503 which had not shown any growth in the presence of 50 mM formic acid in first acid toxicity screen, did so during the second acid toxicity screen. All growth curves obtained during the second acid toxicity screening can found in Appendix 4.

Table 4. Growth data, μ_{max} and OD_{max}, with standard deviation during the second acid toxicity screening in Bioscreen using saline MRS-media with mannitol 20 gl^{-1} as carbon source supplemented with indicated organic acid. Values with different letters in superscript along columns indicate a significant statistical difference ($p<0.05$) between media composition for a specific strain. Values with different letter in superscript and parentheses along rows indicate a significant statistical difference ($p<0.05$) between strains for the specific media composition. N.D. = no data.

Media	**Strain**					
	μ_{max} (h^{-1})			**OD_{max}**		
	CCUG 30503	**EO22**	**EO20**	**CCUG 30503**	**EO22**	**EO20**
Mannitol 20 g l^{-1}	0.299±0.024$^{a\ (a)}$	0.263±0.004$^{a\ (ab)}$	0.224±0.011$^{a\ (b)}$	1.76±0.03$^{b\ (a)}$	1.92±0.28$^{a\ (a)}$	1.85±0.03$^{a\ (a)}$
No carbon source	0.266±0.034$^{ab\ (a)}$	0.246±0.001$^{ab\ (a)}$	0.292±0.045$^{b\ (a)}$	0.4±0.01$^{ef\ (a)}$	0.31±0.02$^{fgh\ (b)}$	0.27±0.00$^{gh\ (c)}$
Mannitol 20 g l^{-1} Lactic acid 50 mM*	0.141±0.006$^{d\ (a)}$	0.135±0.003$^{e\ (a)}$	0.136±0.005$^{d\ (a)}$	1.72±0.32$^{b\ (a)}$	1.64±0.12$^{ab\ (a)}$	1.66±0.07$^{ab\ (a)}$
Mannitol 20 g $l^{-1.}$ Lactic acid 100 mM	0.063±0.002$^{e\ (a)}$	0.051±0.001$^{h\ (a)}$	0.063±0.005$^{f\ (a)}$	0.48±0.03$^{ef\ (a)}$	0.3±0.04$^{gh\ (b)}$	0.35±0.01$^{fgh\ (b)}$
Mannitol 20 g $l^{-1.}$ Lactic acid 150 mM	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Lactic acid 50 mM	0.125±0.017$^{d\ (a)}$	0.135±0.005$^{e\ (a)}$	0.13±0.002$^{d\ (a)}$	0.34±0.00$^{f\ (a)}$	0.33±0.02$^{fgh\ (a)}$	0.28±0.01$^{gh\ (b)}$
Mannitol 20 g $l^{-1.}$ Acetic acid 50 mM	0.231±0.005$^{c\ (a)}$	0.172±0.004$^{d\ (a)}$	0.178±0.001$^{c\ (a)}$	2.26±0.00$^{a\ (a)}$	1.53±0.03$^{b\ (b)}$	1.85±0.20$^{a\ (c)}$
Mannitol 20 g $l^{-1.}$ Acetic acid 100 mM	0.136±0.005$^{d\ (a)}$	0.098±0.002$^{fg\ (b)}$	0.087±0.003$^{ef\ ©}$	1.62±0.24$^{b\ (a)}$	1.04±0.01$^{c\ (ab)}$	1.46±0.25$^{b\ (b)}$
Mannitol 20 $gl^{-1.}$ Acetic acid 150 mM	0.086±0.003$^{e\ (a)}$	0.067±0.011$^{h\ (b)}$	0.064±0.003$^{f\ (b)}$	0.79±0.03$^{d\ (a)}$	0.55±0.17$^{efg\ (a)}$	0.75±0.02$^{cde\ (a)}$
Acetic acid 50 mM	0.201±0.012$^{c\ (a)}$	0.235±0.013$^{b\ (a)}$	0.208±0.016$^{bc\ (a)}$	0.31±0.01$^{f\ (a)}$	0.25±0.01$^{h\ (b)}$	0.22±0.01$^{gh\ (c)}$
Mannitol 20 g $l^{-1.}$ Propionic acid 50 mM	0.200±0.006$^{c\ (a)}$	0.177±0.009$^{cd\ (b)}$	0.178±0.004$^{c\ (b)}$	1.30±0.02$^{c\ (a)}$	1.54±0.01$^{b\ (a)}$	1.69±0.16$^{ab\ (a)}$
Mannitol 20 g $l^{-1.}$ Propionic acid 100 mM	0.130±0.005$^{d\ (a)}$	0.113±0.003$^{f\ (b)}$	0.115±0.009$^{de\ (a)}$	0.96±0.04$^{d\ (a)}$	0.87±0.14$^{cd\ (a)}$	0.92±0.10$^{c\ (a)}$
Mannitol 20 g $l^{-1.}$ Propionic acid 150 mM	0.069±0.003$^{e\ (a)}$	0.062±0.001$^{h\ (a)}$	0.067±0.004$^{f\ (a)}$	0.69±0.01$^{de\ (a)}$	0.59±0.05$^{def\ (b)}$	0.59±0.01$^{def\ (b)}$
Propionic acid 50 mM	0.222±0.013$^{c\ (a)}$	0.192±0.004$^{h\ (b)}$	0.189±0.005$^{bc(\ b)}$	0.29±0.00$^{f\ (a)}$	0.28±0.02$^{gh\ (b)}$	0.24±0.01$^{gh\ (b)}$
Mannitol 20 g l^{-1} Formic acid 50 mM	0.086±0.001$^{e\ (a)}$	0.097±0.001$^{fg\ (a)}$	0.101±0.012$^{def\ (a)}$	0.39±0.04$^{ef\ (a)}$	0.56±0.01$^{efg\ (b)}$	0.48±0.01$^{efg\ (c)}$
Mannitol 20 g l^{-1} Formic acid 100 mM	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Mannitol 20 g l^{-1} Formic acid 150 mM	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Formic acid 50 mM	0.08±0.001$^{e\ (a)}$	0.092±0.008$^{g\ (a)}$	0.086±0.012$^{ef\ (a)}$	0.20±0.01$^{f\ (a)}$	0.18±0.01$^{h\ (a)}$	0.14±0.01$^{h\ (b)}$
Mannitol 20 g l^{-1} Mix	0.077±0.002$^{e\ (a)}$	0.091±0.006$^{g\ (b)}$	0.08±0.001$^{ef\ (b)}$	0.92±0.01$^{a\ (a)}$	0.83±0.02$^{cde\ (b)}$	0.81±0.05$^{cd\ (b)}$

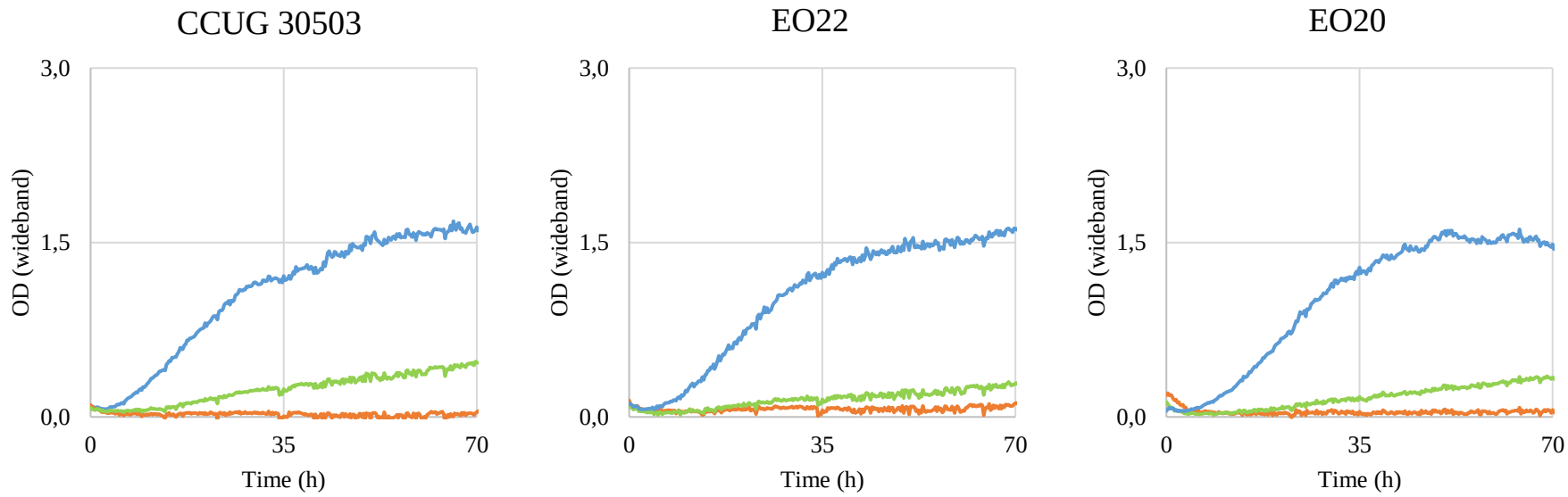

Figure 4. Bioscreen cultivation of CCUG 30503, EO22 and EO20 in saline MRS-media with mannitol 20 g/l, supplemented with lactic acid 50 mM (-), 100 mM (-) and 150 mM (-). The graphs presented are average of 3 replicates.

4.5 Product formation analysis

Two isolates, EO20 and EO22, as well as *L. plantarum* (CCUG 30503) were selected for further growth studies on mannitol, in 50 ml shake flask cultures under anaerobic and aerobic conditions. The cell growth as well as mannitol, lactate and acetate concentrations was measured over a 23 hour period. As can be seen in Figure 5, there was not a large difference between the different strains during anaerobic conditions. However, EO20 had slightly higher total production of lactate at ~11 g l^{-1}.The final OD was higher for EO22 and EO20 compared to CCUG 30503. Table 5 presents the corresponding specific growth rate, OD_{max}, rate of mannitol consumption, rate of lactate and acetate production as well as yield of OD, lactate and acetate on mannitol.

During aerobic conditions (Figure 6), the growth of CCUG 30503 was more inhibited, with a final OD and total mannitol consumption of ~2.2 and ~5.5 respectively, less than half of that of EO22 and EO20. However, there was no difference in lactate or OD yields on mannitol between the strains, but CCUG 30503 had almost twice as high acetate yield on mannitol than EO22 and EO20. Growth data during aerobic conditions are given in Table 6. Furthermore, between the two culture conditions, the yield of acetate on mannitol was higher during aerobic conditions compared to anaerobic.

*Table 5. Growth data during anaerobic shake flask culturing, with standard deviations. Values with different letters in superscript along rows indicate a significant statistical difference ($p<0.05$). Experiment were run in triplicates. *No significant difference between the strains.*

Anaerobic	**Strains**		
	CCUG	EO22	EO20
$\mu_{max,1\ (8\text{-}12\text{-}5\ h)}$ (h^{-1})*	0.282±0.008	0.277±0.008	0.309±0.030
$\mu_{max,2\ (12.5\text{-}21.5\ h)}$ (h^{-1})*	0.064±0.009	0.058±0.008	0.056±0.003
OD_{max} (600 nm)	5.87±0.12[a]	6.91±0.29[b]	6.39±0.12[c]
Total consumed mannitol (g l^{-1})*	10.13±0.95	11.20±0.53	11.38±0.54
Total produced lactate (g l^{-1})	10.06±0.5[a]	10.14±0.25[a]	11.01±0.17[b]
Total produced acetate (g l^{-1})*	0.58±0.1	0.58±0.1	0.58±0.1
$Y_{lac/man}$ (g g^{-1})*	1.00±0.05	0.91±0.06	0.97±0.06
$Y_{ace/man}$ (g g^{-1})*	0.06±0.02	0.05±0.01	0.05±0.01
$Y_{OD/man}$ (OD g^{-1})*	0.58±0.05	0.61±0.03	0.55±0.03
r_{man} (g l^{-1} h^{-1})*	-0.78±0.15	-0.76±0.01	-0.74±0.01
r_{lac} (g l^{-1} h^{-1})*	0.63±0.02	0.62±0.02	0.63±0.02
r_{ace} (g l^{-1} h^{-1})*	0.06±0.01	0.06±0.01	0.05±0.01

*Table 6. Growth data during aerobic shake flask culturing, with standard deviations. Values with different letters in superscript along rows indicate a significant statistical difference ($p<0.05$). Experiment were run in triplicates. *No significant difference between the strains within the variable*

Aerobic	**Strains**		
	CCUG	EO22	EO20
$\mu_{max,1}$ (h^{-1})	0.219±0.009[a]	0.256±0.009[b]	0.293±0.005[c]
$\mu_{max,2}$ (h^{-1})	0.046±0.002		
OD_{max} (600 nm)	2.22±0.04[a]	5.17±0.09[b]	5.12±0.15[b]
Total consumed mannitol (g l^{-1})	5.53±1.3[a]	10.21±0.33[b]	9.62±0.55[b]
Total produced lactate (g l^{-1})	6.17±0.20[a]	10.45±0.65[b]	10.02±0.08[b]
Total produced acetate (g l^{-1})*	0.98±0.11	1.15±0.10	1.09±0.02
$Y_{lac/man}$ (g g^{-1})*	1.17±0.35	1.03±0.09	1.04±0.06
$Y_{ace/man}$ (g g^{-1})*	0.19±0.07	0.11±0.01	0.11±0.01
$Y_{OD/man}$ (OD g^{-1})*	0.41±0.1	0.5±0.01	0.53±0.04
r_{man} (g $l^{-1}h^{-1}$)	-0.33±0.08[a]	-0.73±0.05[b]	-0.7±0.06[b]
r_{lac} (g $l^{-1}h^{-1}$)	0.36±0.00[a]	0.58±0.02[b]	0.58±0.02[b]
r_{ace} (g $l^{-1}h^{-1}$)*	0.06±0.00	0.06±0.01	0.05±0.01

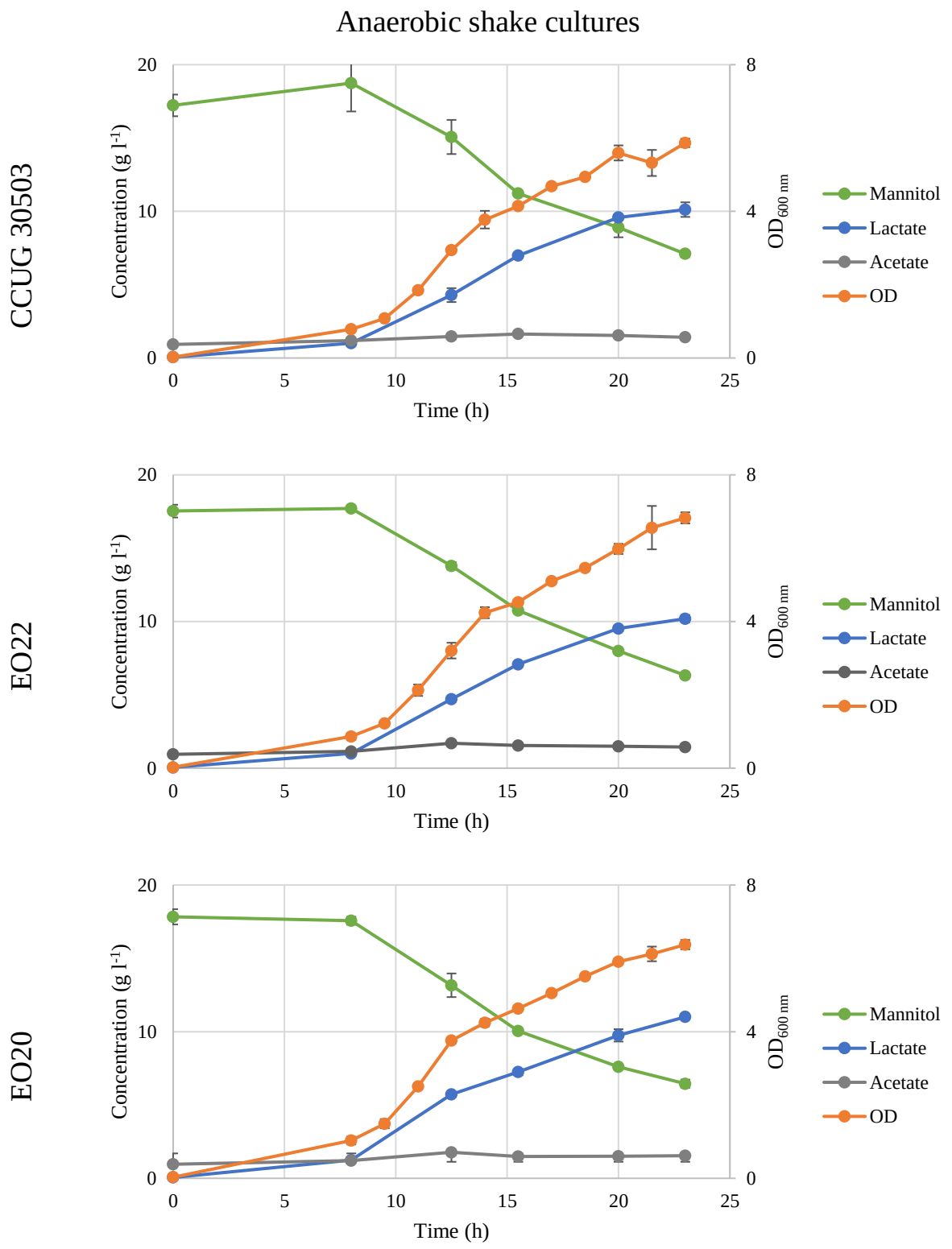

Figure 5. Cell growth as well as mannitol, lactate and acetate concentrations during culturing in saline MRS-media under anaerobic conditions. Error bars are standard deviations.

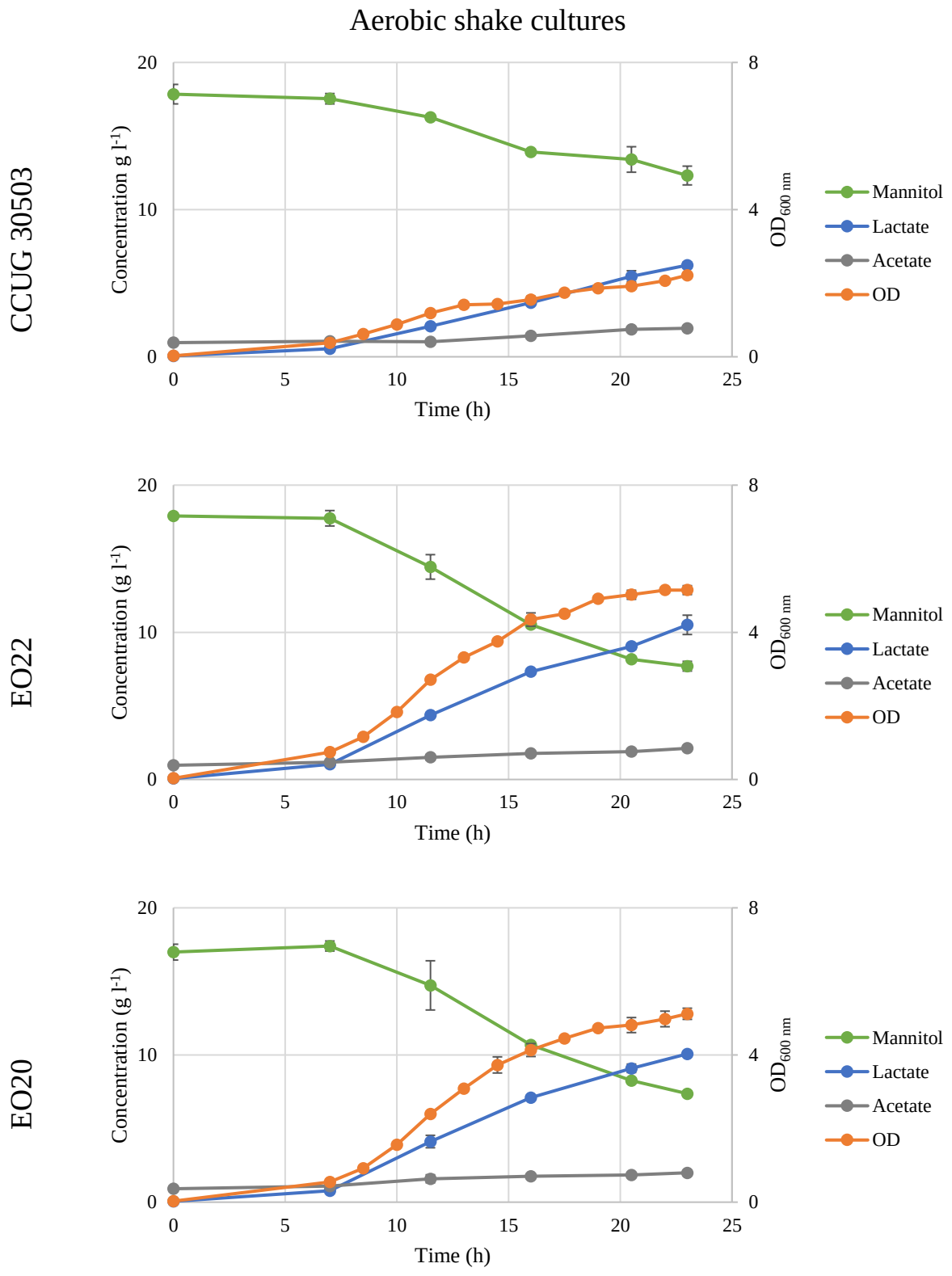

Figure 6. Cell growth as well as mannitol, lactate and acetate concentrations during culturing in saline MRS-media under anaerobic conditions. Error bars are standard deviations.

4.6 Biomass retention and pH during ensiling

Seaweed of the species *S. latissima* and *L. digitata* were ensiled for 84 days using the bacterial strains as additives, *i.e.* CCUG 30503, EO22 and EO20 as well as a mixture of formic and propionic acid. The effectiveness of each treatment was evaluated by measuring biomass retention, shown in Figure 7. As can be seen, the biomass was almost completely retained in all cases, with the exception of the control for *S. latissima*, which decreased with approximately 1 % in 84 days.

The pH was measured immediately after each treatment, then again after 3 days and finally at the end of the ensiling, shown in Table 7. The decrease in pH at the 3 day point was very similar between the microbial additives in the case of *S. latissima.* The addition of CCUG 30503 resulted in the largest decrease in pH, from 6.29 to 4.89, while EO22 with the smallest change, decreases pH from 6.47 to 5.37. However, in the *L. digitata* silage the difference in pH decrease was more evident, with EO20 decreasing the pH from 6.38 to 4.11, while CCUG 30503 decreased the pH from 6.37 to 5.14. In addition, the microbial additives decreased the pH even further, resulting in a pH of ~3.5 measured the final day. The pH in acid mixture remained stagnant at ~4.3.

Table 7. The pH of seaweed silage measured directly after treatment (0 days), after 3 days and after 84 days of enisling.

Seaweed species	Treatment	pH 0 days	pH 3 days	Final pH (84 days)
S. latissima	Control	6.38	5.17	5.47
S. latissima	CCUG 30503	6.29	4.89	3.60
S. latissima	EO22	6.47	5.37	3.56
S. latissima	EO20	6.23	4.93	3.58
S. latissima	Acid Mixture	4.15	4.32	4.16
L. digitata	Control	6.56	5.59	N/A
L. digitata	CCUG 30503	6.37	5.14	3.52
L. digitata	EO22	6.42	4.50	3.52
L. digitata	EO20	6.38	4.11	3.49
L. digitata	Acid Mixture	4.38	4.27	4.28

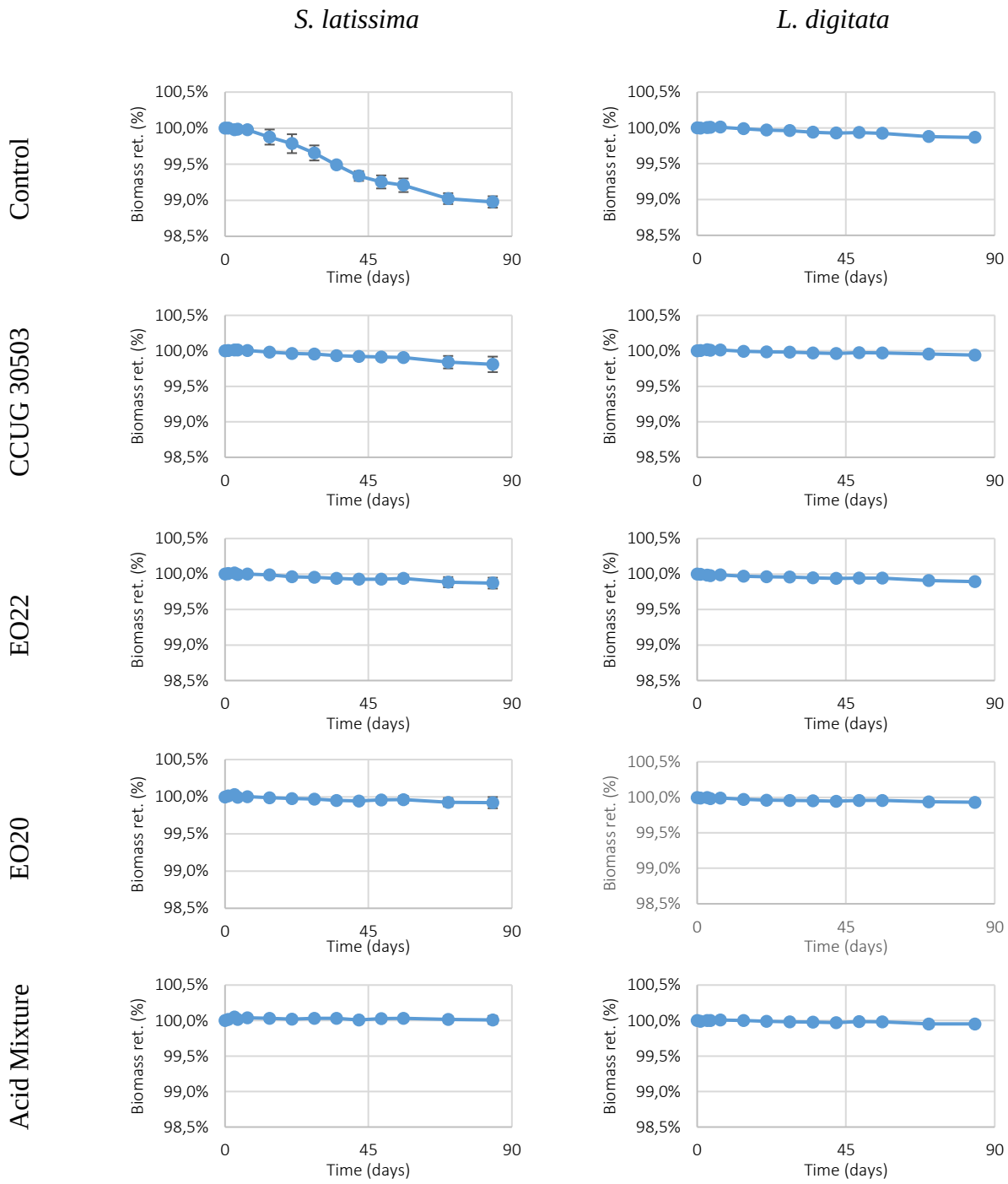

Figure 7. Biomass retention during the 84 days of the ensilage. Time in days is on x-axis and on the y-axis there is the percentage of seaweed biomass retained.

5 Discussion

5.1 Identification

The microscopic observation showed that all the isolates were rod-shaped, row forming bacteria, which is a typical characteristic of the *Lactobacillus* genus. As MRS-media is selective for *Lactobacillus* this was expected. However, the media is also positive for *Pediococcus*, *Streptococcus* and *Leunostoc,* but the micromorphology ruled out any bacteria from these genera due to them being cocci shaped (25-27). None of the strains grew on MRS-media supplemented with xylose, hence, *L. pentosus* may be excluded as species candidate as it should possess the ability to metabolize xylose (28).

As described in section 3.1.3, the 16S rRNA was sequenced in order to identify species identity of the isolates. When aligned to homologs in the NCBI database, a number of different species had homologs with 100 % identity. This is a recurring problem for several species of *Lactobacillus* as the 16S rRNA is very similar within the genus (29). To overcome this problem another highly conserved gene could possibly be sequenced. For instance, as described by Naser et al. (30), sequencing the genes rpoA (RNA polymerase alpha subunit) and pheS (phenylalanyl-tRNA alpha subunit), combined with the use of more sophisticated software, is one method that could be used to further discriminate between *Lactobacillus* species.

5.2 Characterization

As described in section 3.2.1, the temperature range of the isolates was investigated by plating, and from the results it can be concluded that the minimum temperature necessary for growth lies between 12 and 15 °C. The plating did not provide any information on how the growth rate depended on temperature, except that growth at 15 °C was substantially slower (5 days until colonies formed compared to 2 days for the other temperatures tested). Growth at low temperatures might be necessary for a successful ensilage in the Swedish climate, hence, these results strengthen that the isolates could be suitable as ensilage additives.

From the carbon source screening it could be assumed that the isolates did not possess the metabolic functions to metabolize laminarin. The little growth that had occurred can most certainly be attributed to the MRS-media, as the non-carbon source components of the media also could support some growth, as can be seen in Table 4.

From the Bioscreen experiments it was observed that mannitol and glucose could readily be metabolized by all the isolates and the *L. plantarum* (CCUG 30503) strain. Furthermore, two different growth patterns could be observed between the isolates when grown on mannitol (20 gl^{-1}) and glucose (20 gl^{-1}). The difference being that some of the strains grew more readily glucose than on mannitol while for other strains the pattern was reversed. Due to this difference, two isolates were selected, EO20 representing the strains that grew more readily on glucose and EO22 representing the strains that grew more readily on mannitol. These two strains were further characterized and also used as microbial ensilage additives during the ensiling. It was expected that the ensiling would be more or less effective depending on which of the strains were used as ensiling additve, this is further discussed in section 5.3.

As mentioned above two growth patterns could be observed in the Bioscreen, hence it was expected that the shake-flask cultures would behave in a similar fashion, however, this was not the case. As can be seen in Figure 5, the growth of the isolates as well as that of the *L. plantarum* (CCUG 30503) strain were similar in most regards when grown in anaerobic conditions. Nonetheless, there were some differences regarding OD_{max} and total production of lactate, with EO22 reaching the highest OD and EO20 producing the highest amount of lactate. However, during aerobic conditions, the isolates clearly outperformed the *L. plantarum* (CCUG 30503) strain in growth, which could be an indicator that the two isolates would proliferate faster in the beginning of an ensilage process before all the oxygen has been consumed, thereby leading to a faster drop in pH and inhibiting possible degrading microorganisms.

Another important observation is that none of the strains, cultured in shake flasks, completely consumed the mannitol, but instead their growth may have been halted due to the lactate production or another inhibiting factor resulting from either the proliferation or the lactate production. This can also be seen during the acid toxicity screening, where none of the strains were able to grow in the presence of 150 mM lactic acid. Hence, it is possible that a portion of the mannitol will remain after the ensiling, when using these strains as microbial additives.

5.3 Ensilage

The data from the shake flask culturing indicated EO20 would be most suited as a microbial additive, based on the higher production of lactate during anaerobic conditions, resulting in a larger decrease in pH. In addition, EO20 and EO22 was not as heavily impaired in aerobic conditions as *L. plantarum* (CCUG 30503) were, suggesting EO20 and EO22 may have higher activity in the beginning of the ensilage process when oxygen is still present. As can be seen in Table 7 the pH decrease after 3 days was the largest for *L. digitata* when EO20 was added, but for *S. latissima* the largest decrease in pH after 3 days was when *L. plantarum* (CCUG 30503) was used. However, this still provides little information regarding the efficiency of the microbial additives, as the critical pH level for a successful ensilage of seaweed is still unknown. The final pH is relatively similar for all treatments using microbial additives, which coincides with the similarity in growth and lactic acid production of the 3 strains during anaerobic conditions (Figure 5, Table 5).

Biomass retention barely changed at all during the course of the ensiling. All treatments of the *L. digitata* silage has remained close to 100 %, hence, it is not possible to say if the additives have had any effect. The control for *S. latissima* silage decreased the most, with ~1 %, hence it seems that the additives had some effect in this case. One reason why the ensilage seemed to work well, in the sense of biomass retention, can be due to the season it was collected. As stated in section 3.3, these seaweeds were harvested in mid-April, which might not be an optimal harvest period, but it may be the case that this specific collection of seaweed are more easily preserved by means of ensiling. For instance, the epiphytic growth is less widespread during this time period likely due to the cold temperature of the water (18, 19), and these epiphytes may cause complications in the ensiling process. Also, the chemical composition may have been favorable for LAB compared to other microorganism, since the laminarin concentration could have been quite low while there is still sufficient amounts of mannitol for the LAB to proliferate (17).

5.4 Benefits and drawbacks of using microbial additives in ensilage

As previously stated, this project did not entail a chemical composition analysis of the seaweed silage, however, regarding the results that has been produced, it can be speculated that the mannitol content of seaweed will have decreased. This needs to be seen as a trade-off in case of biofuel production from seaweed, as the mannitol otherwise could be fermented by yeasts to produce ethanol, even though ethanol produced in this manner is still low-yielding (31, 32). Hopefully, the laminarin content will remain unchanged, hence, the seaweed will still have value regarding biofuel production or other fermentation processes. Another implication that may arise downstream in a biofuel production process, is that lactic and acetic acid produced by the microbial additives may in fact inhibit the yeast from fermenting the biomass into bioethanol (33). The amount of lactic acid produced during the shake-flask cultivation in MRS-media was approximately 10 g l^{-1}. Yeast have been shown to be able to grow at 8 g l^{-1} (34), so this may not be a major drawback. Still, another preparation step, in which the organic acids are removed may be necessary. Separation of the organic acid from the biomass may prove to have a value in itself. If the lactic acid can be extracted it may be possible to convert it into polylactic acid, a biodegradable plastic. In addition, the final pH of the silage with microbial additives is quite low and this could have implications in downstream processing, hence limiting certain applications which the seaweed might otherwise be used for.

A process that have been shown to benefit from biomass ensiled with LAB as additives is biogas production. Biogas production from corn silage increased by 22 % when using microbial silage additives compared to spontaneous ensiling (without additives) (14). However, the microbial additive in this case was a complex mixture different microorganisms. In the same study, it was also shown that a microbial additives could have an adverse effect.

The microbial additives, may also have a positive effect concerning nutritional value, it may be the case that ensiled seaweed will have probiotic effect as other fermented foods have (35). Furthermore, laminarin have been shown to have beneficial health effects (36). These two factor in conjunction suggests that seaweed ensiled with microbial additives potentially could be beneficial feed or food products.

5.1 Conclusion

As shown by the ensiling results, the strains used as additives had a positive effect concerning biomass retention in the case of *S. latissima*. For *L. digitata*, all treatments resulted in nearly 100 % biomass retention, implying that the additives were superfluous. Since all strains used as additives produced very similar results, it is difficult to see a correlation with using a strain that produces more lactic acid leading to higher biomass retention. However, some relation can be seen in pH decrease and rate of lactic acid production in the *L. digitata* samples, with faster lactic acid production leading to more prompt pH drop. Despite the lack of correlation, it can still be concluded that ensiling with the aid of microbial additives is a suitable technique for retaining seaweed biomass of the species *S. latissima* and *L. digitata*. Ensiling with microbial additives may further improve the quality of seaweed as it has for other crops like corn. Bolsen et al. summarized the results of several studies from Kansas State University and found that microbial

additives improved the dry matter recovery in 19 corn silage by an average of 1.3 % compared to silages without additives (37). Dry matter content recovery was not measured in this project. However, the biomass retention in the *S. latissima* silage was improved by ~1 % when inoculating with LAB, based on wet weight. As such, the retention of dry matter would be even higher, if it is assumed that weight loss is mostly due to the degradation of biomass and not due to moisture loss.

Furthermore, the seaweed silage needs to be analyzed to determine the change in biochemical composition, and additional experiments has to be conducted on seaweed harvested at different time periods to establish how the intrinsic seasonal variation of the seaweed biochemical composition affects both ensiling and downstream processing. In addition, as ensiling is a very complex process depending on many factors, it may also be of interest to compare more complex mixtures microorganism as additives. In the end, the efficiency of the preservative measure has to be weighed against the final application.

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Appendix 1 – 16S ribosomal RNA gene sequences

Presented below are the sequences generated from sequencing the 16S rRNA gene of the seven isolates. Species with homologs found using BLASTn in the NCBI database can be found in Table 1.

>E25 16S ribosomal RNA gene, 1397 bp

ACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACT
GGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCT
AATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCAC
TTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATG
ATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCC
GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGA
GAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCG
CAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGT
AGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGG
CTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATG
AATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCC
GCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACT
ATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTA
TCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG
GATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGT
TCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTT
TGTAACACCCAAAGTCG

>E24 16S ribosomal RNA gene, 1420 bp

ACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACT
GGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCT
AATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCAC
TTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATG
ATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCC
GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGA
GAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCG
CAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGT
AGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGG
CTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATG

AATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCC
GCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACT
ATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTA
TCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG
GATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGT
TCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTT
TGTAACACCCAAAGTCGGTGGGGTAACCTTTTAGGAACCA

>E23 16S ribosomal RNA gene, 1407 bp

GAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGG
TGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAA
TACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTT
TTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGAT
ACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGC
GTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGA
GTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCA
GGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAAC
TGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCT
CGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAA
TGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGC
CTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTAT
GCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATC
AGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGA
TGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTC
GGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCA
TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTG
TAACACCCAAAGTCGGTGGGGTAACCT

>E22 16S ribosomal RNA gene, 1397 bp

CGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTG
GTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTA
ATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACT
TTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGA
TACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCG
CGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAG

AGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGC
AGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAA
CTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGC
TCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGA
ATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCG
CCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTA
TGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTAT
CAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
ATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTT
CGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGC
ATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTT
GTAACACCCAAAGTCGG

>E20 ribosomal RNA gene, 1408 bp

ACGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACT
GGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCT
AATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCAC
TTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATG
ATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCC
GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGA
GAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCG
CAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGT
AGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGG
CTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATG
AATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCC
GCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACT
ATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTA
TCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATG
GATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGT
TCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTT
TGTAACACCCAAAGTCGGTGGGGTAACC

>E19 16S ribosomal RNA gene, 1410 bp

TGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTA

ACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGC
ATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGAT
GGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAG
CCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGT
GAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACT
GTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGT
TTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAA
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GGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAG
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AACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTG
TAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACAC
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>E18 16S ribosomal RNA gene, 1416 bp

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CGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTA
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GGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCG
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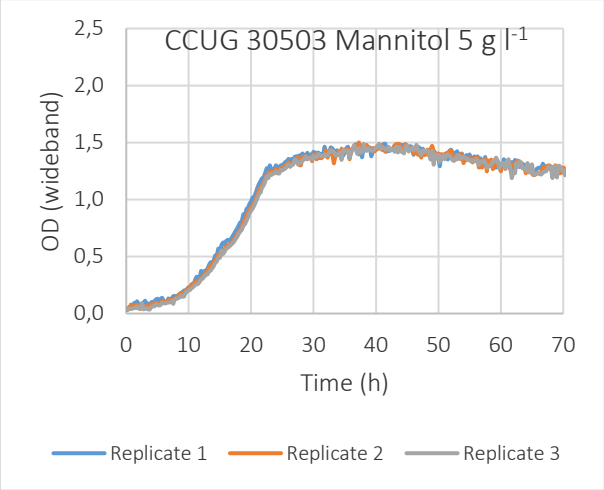
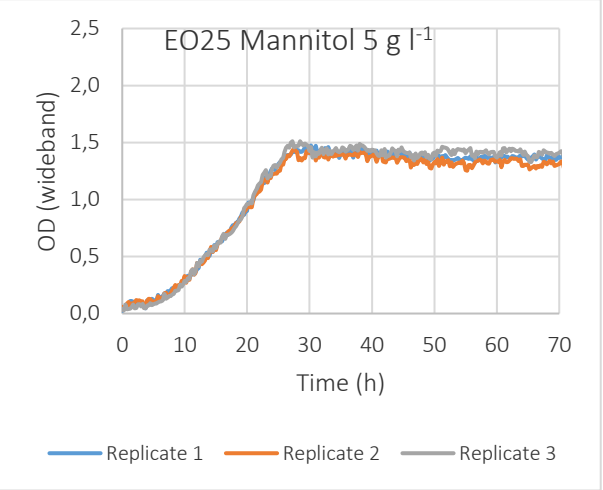
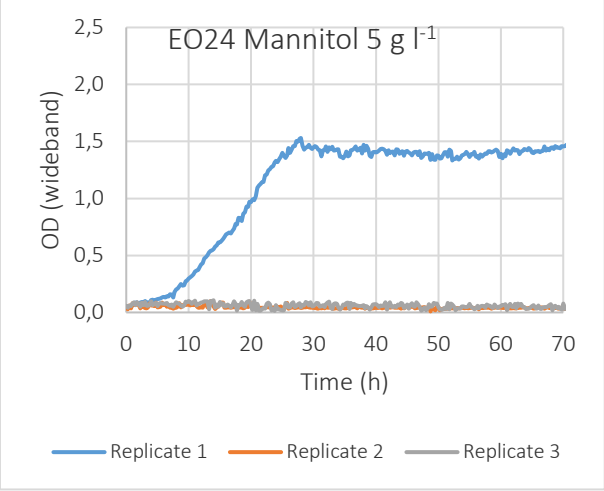
GCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGT
AACACCCAAAGTCGGTGGGGTAACCTTTTAGGAACC

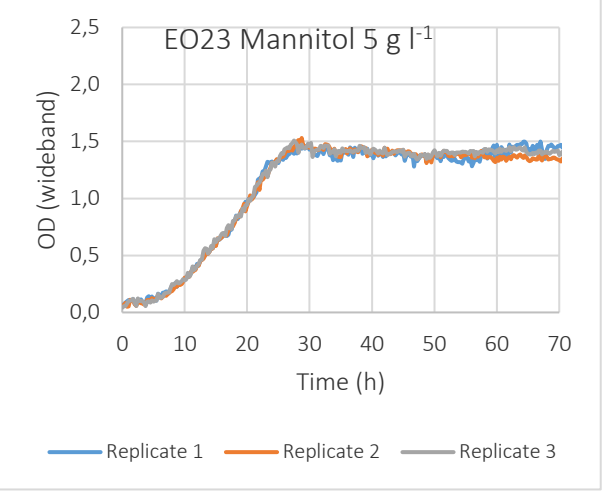
Appendix 2 – Growth curves of carobon source screening in Bioscreen cultivations

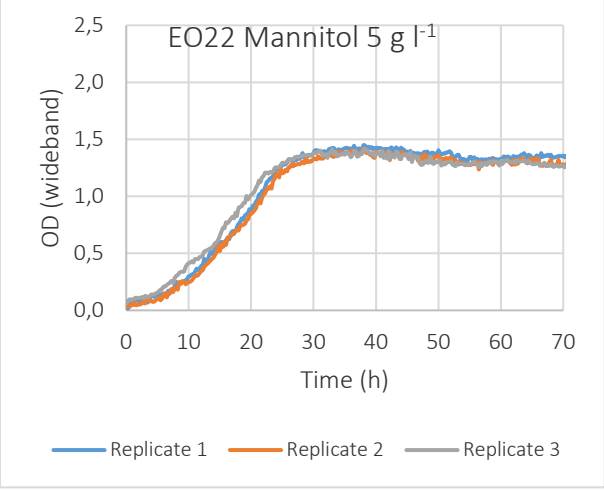
The corresponding growth curves to the values in Table 2, section 4.4. The seven isolates was screened for growth on mannitol (5 and 20 g l^{-1}), laminarin (5 g l^{-1}) and glucose (5 and 20 g l^{-1})

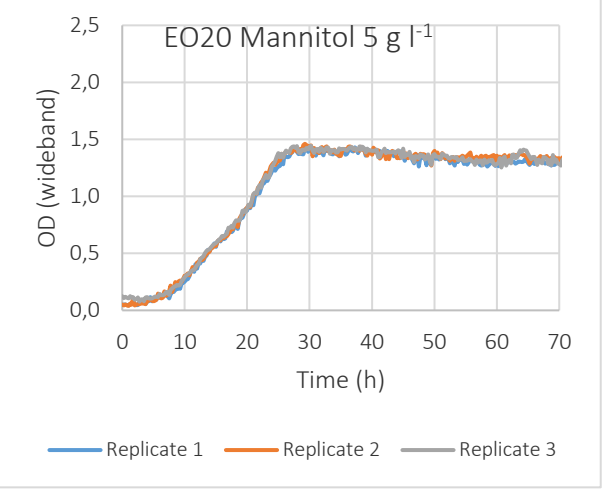


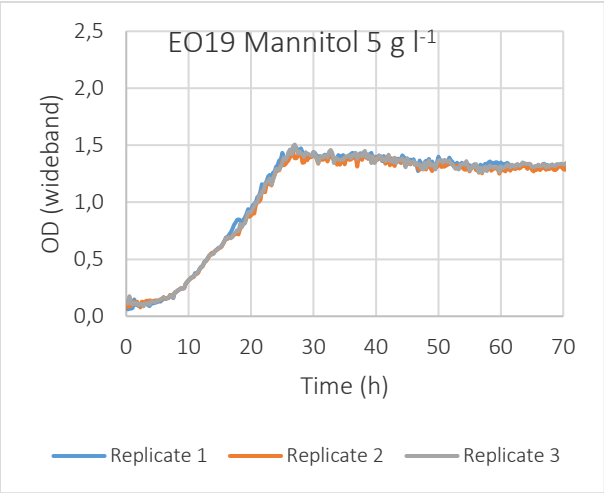








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OD (wideband)
Time (h)
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Replicate 2
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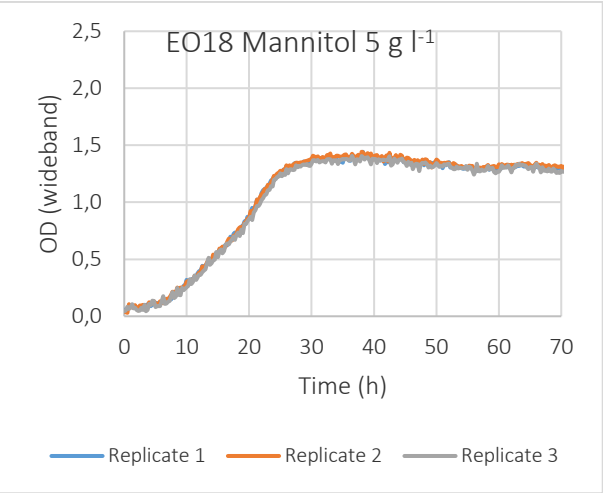
EO18 Mannitol 5 g l-1
OD (wideband)
Time (h)
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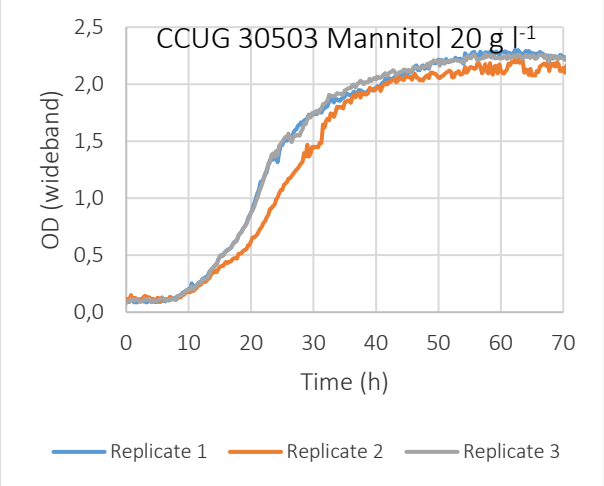
Replicate 2
Replicate 3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OD (wideband)
Time (h)
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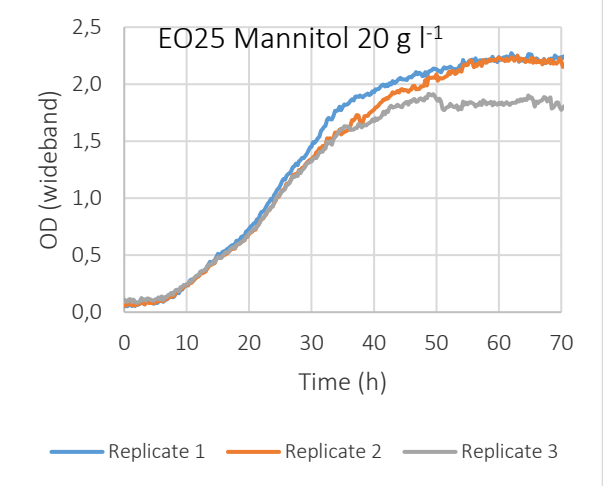
Replicate 3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OD (wideband)
Time (h)
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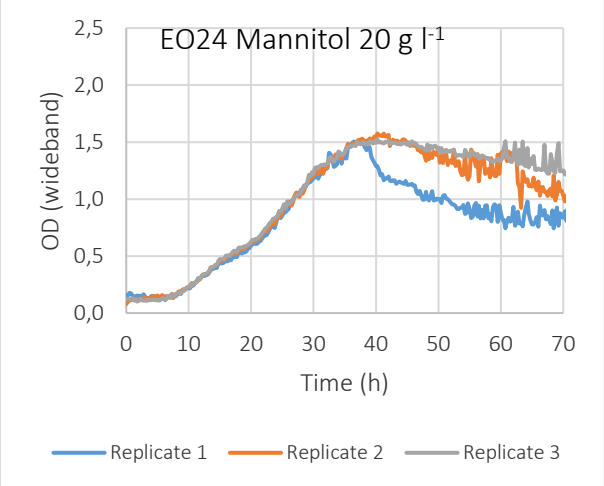
Replicate 3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OD (wideband)
Time (h)
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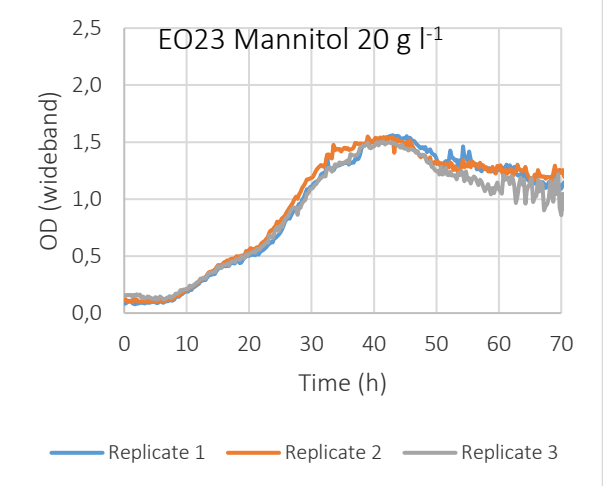
Replicate 3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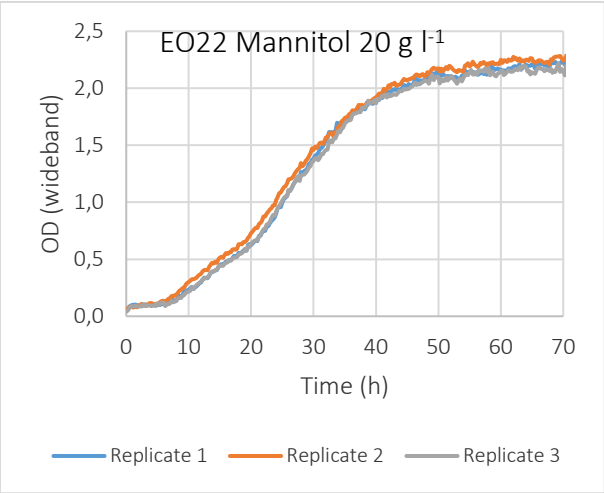
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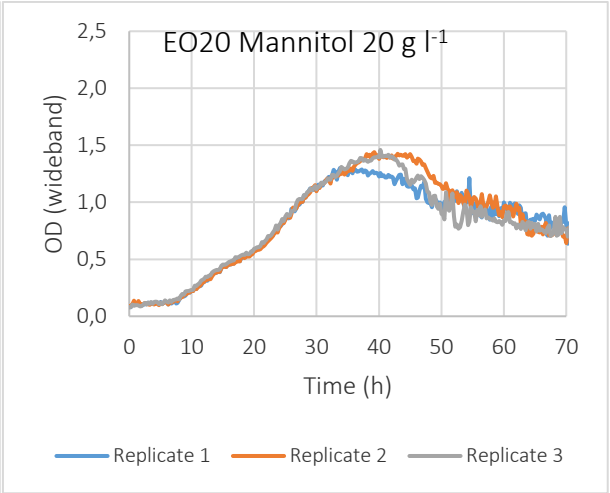
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3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Time (h)
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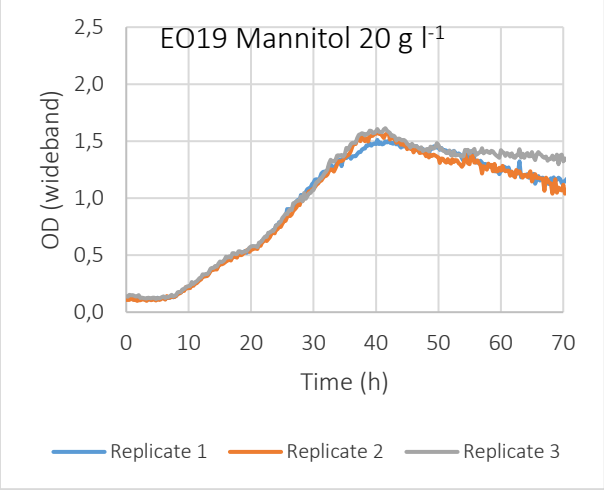
Replicate 2
Replicate 3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Time (h)
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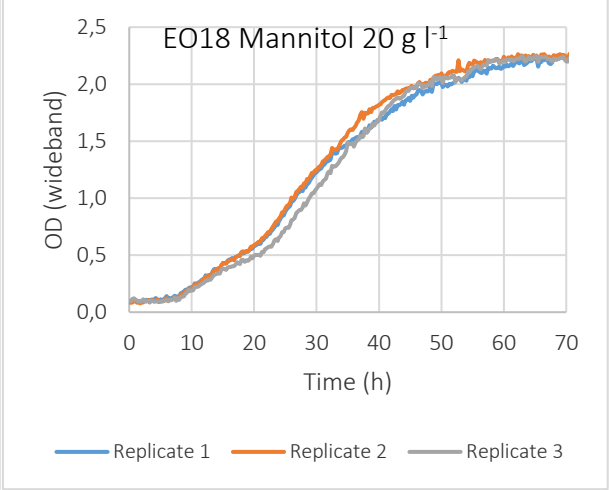
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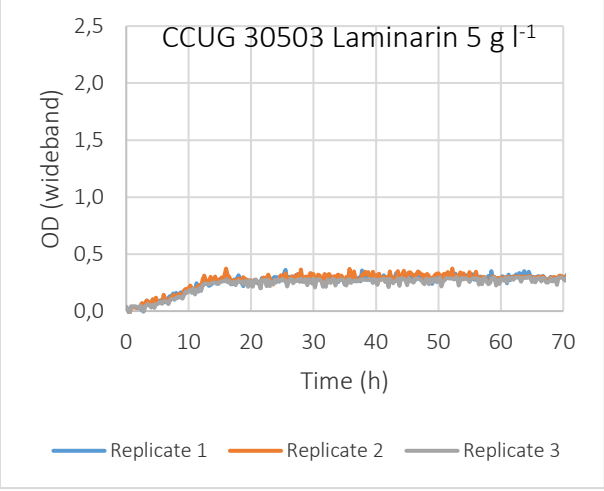
Time (h)
Replicate 1
Replicate 2
Replicate 3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Time (h)
Replicate 1
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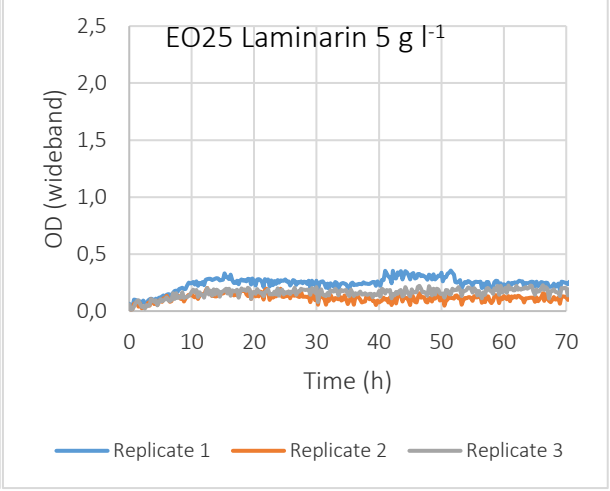
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OD (wideband)
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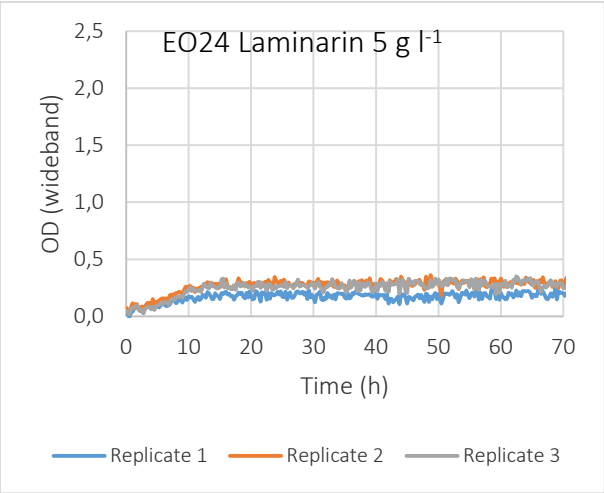
Time (h)
Replicate 1
Replicate 2
Replicate 3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OD (wideband)
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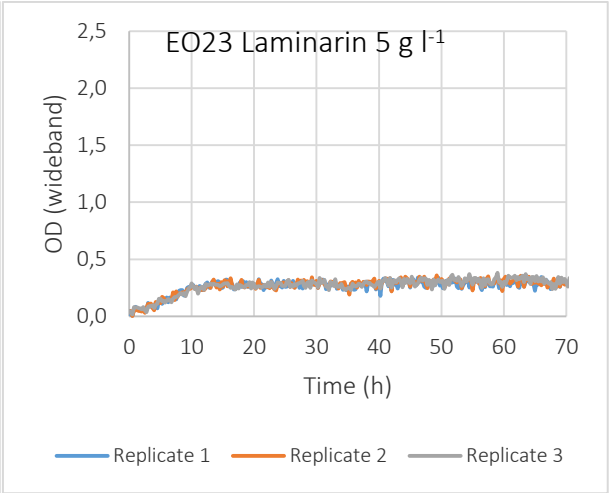
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO23 Laminarin 5 g l-1
OD (wideband)
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Time (h)
Replicate 1
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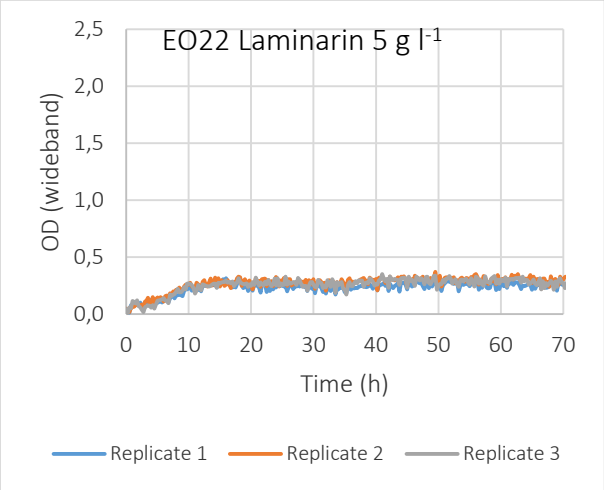
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OD (wideband)
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Time (h)
Replicate 1
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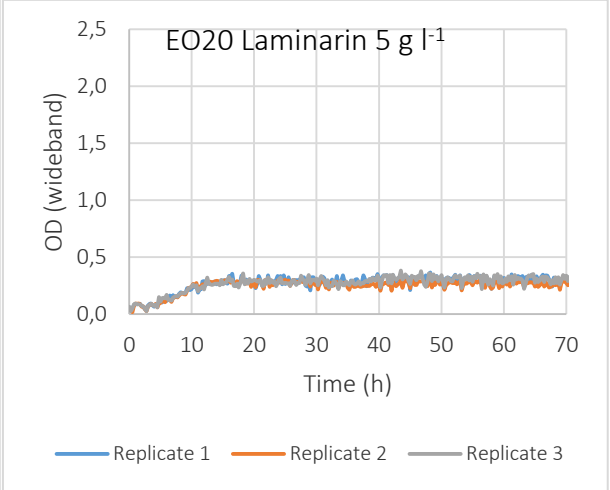
Replicate 3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OD (wideband)
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1,0
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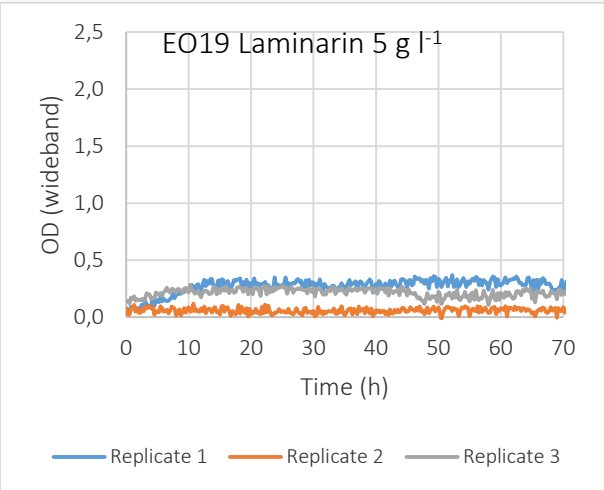
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3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OD (wideband)
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1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
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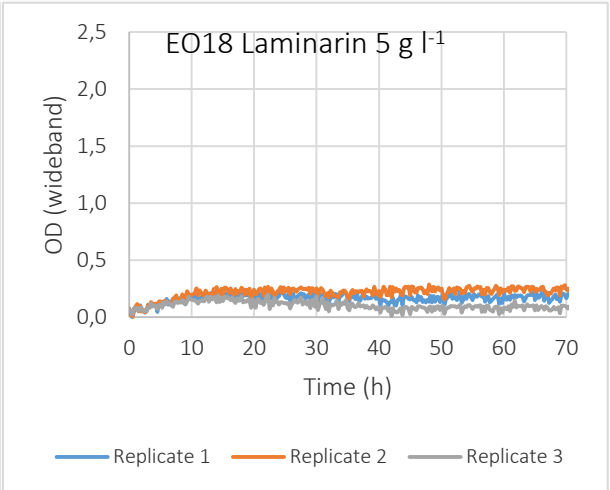
EO18 Laminarin 5 g l-1
OD (wideband)
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1,5
1,0
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0,0
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Time (h)
Replicate 1
Replicate 2
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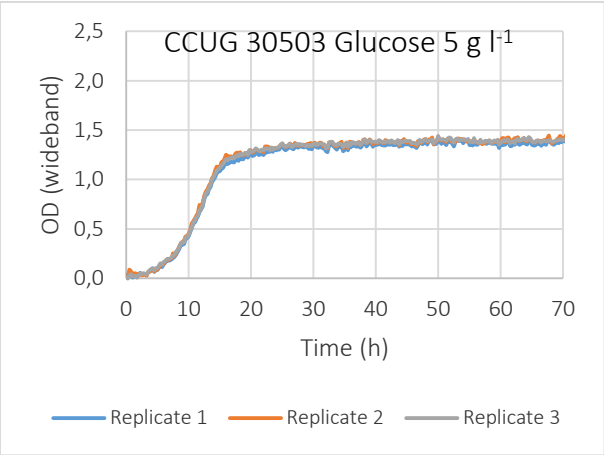
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OD (wideband)
Time (h)
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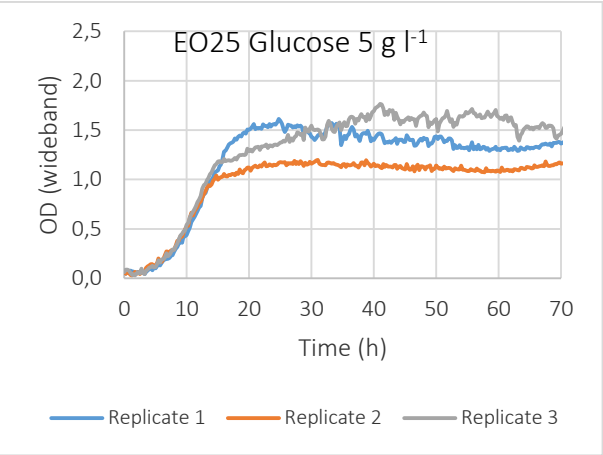
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OD (wideband)
Time (h)
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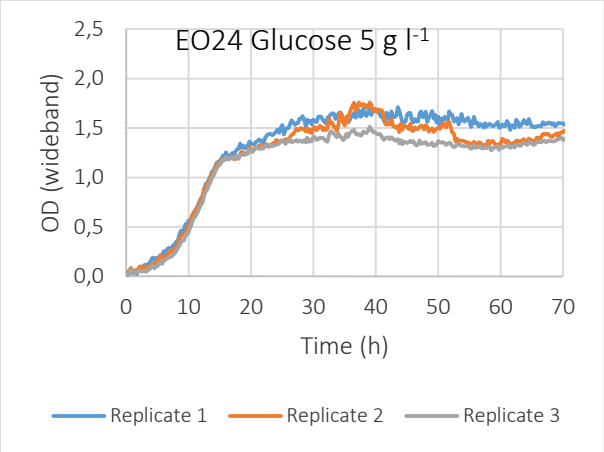
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OD (wideband)
Time (h)
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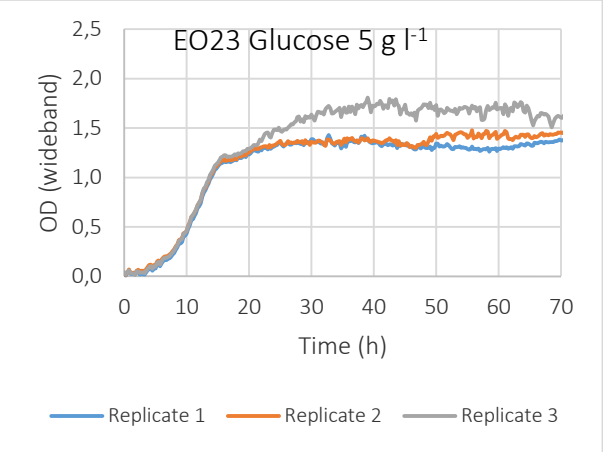
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OD (wideband)
Time (h)
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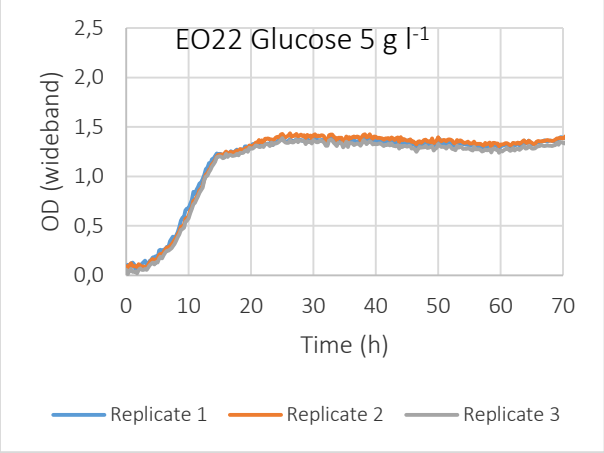
Replicate 3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Time (h)
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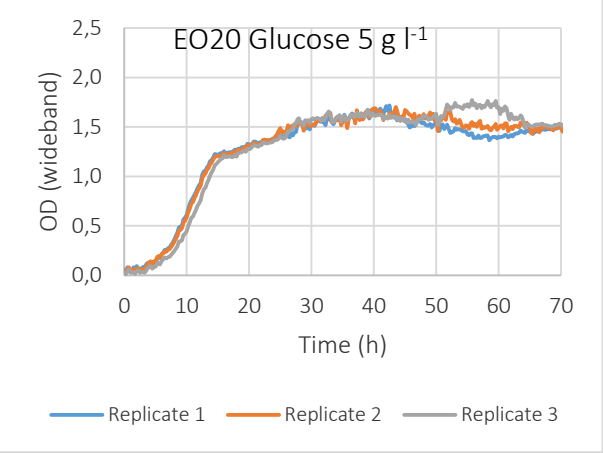
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OD (wideband)
Time (h)
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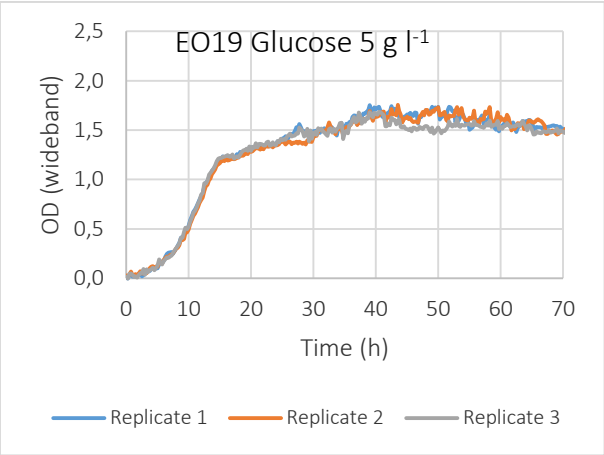
Replicate 2
Replicate 3


EO19 Glucose 5 g l-1
OD (wideband)
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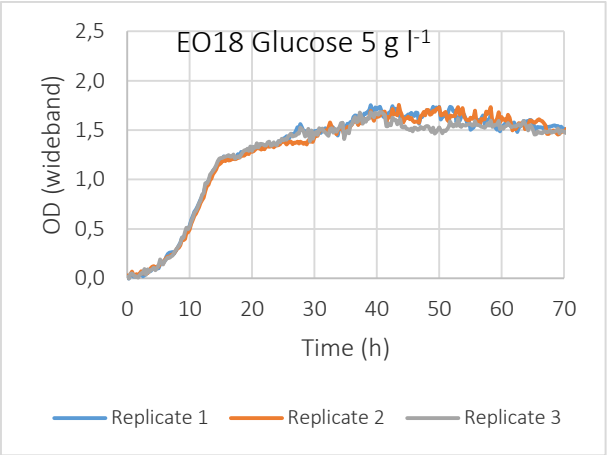
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3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1,0
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Time (h)
Replicate 1
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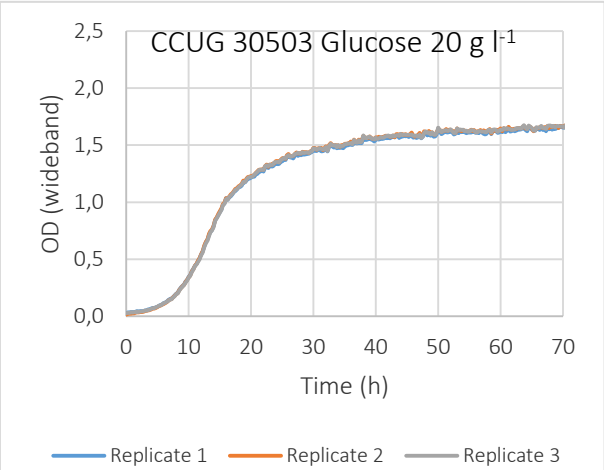
Replicate 3


CCUG 30503 Glucose 20 g l-1
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1,0
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0 10 20 30 40 50 60 70
Time (h)
Replicate 1
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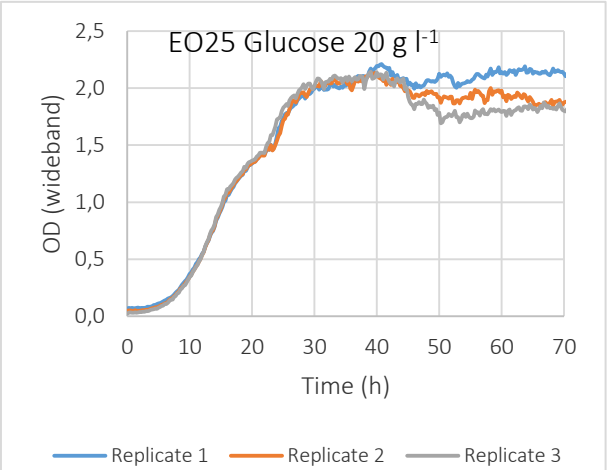
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OD (wideband)
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1,0
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0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
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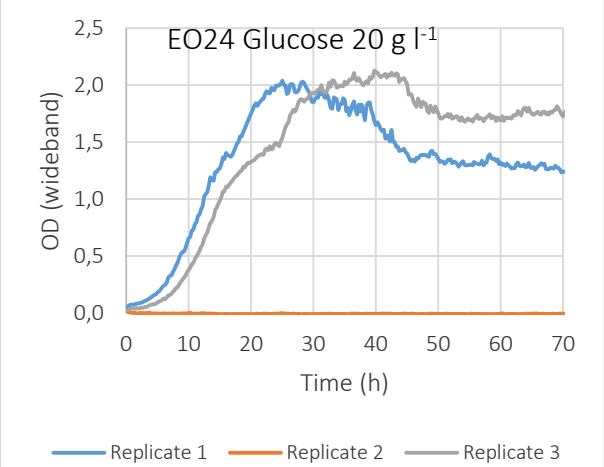
EO24 Glucose 20 g l-1
OD (wideband)
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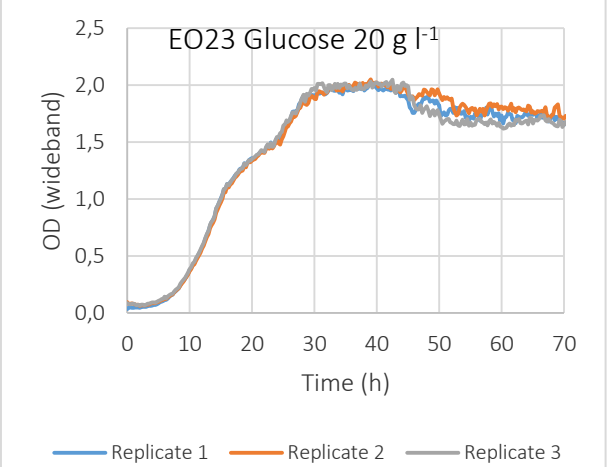
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO23 Glucose 20 g l-1
OD (wideband)
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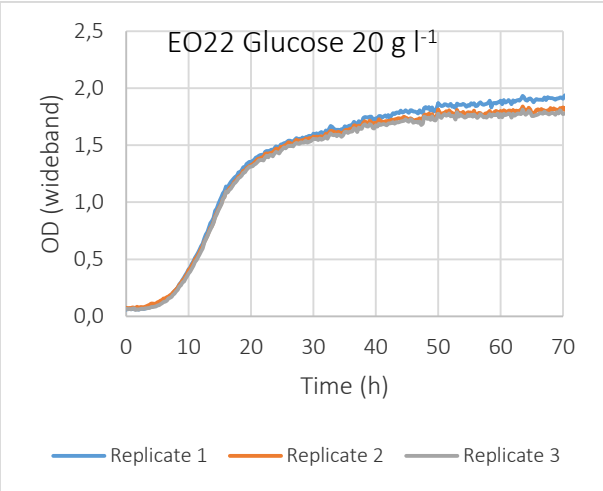
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO22 Glucose 20 g l-1
OD (wideband)
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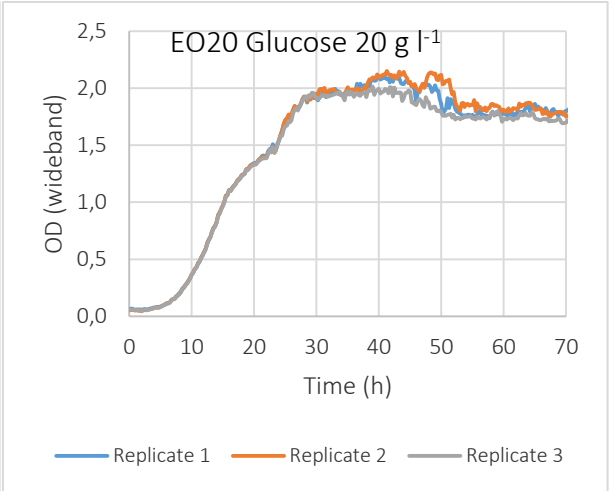
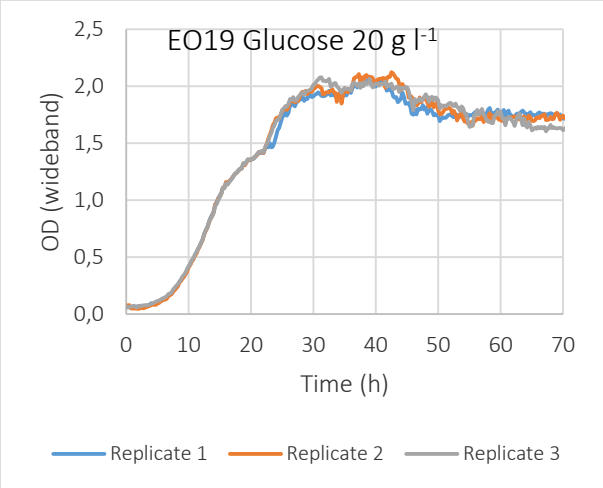
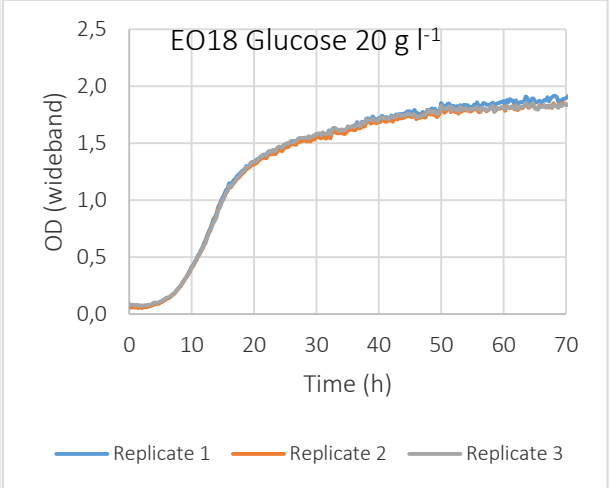
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

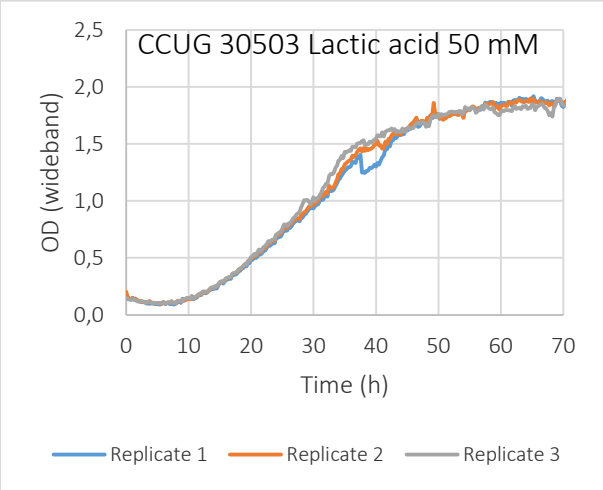

EO20 Glucose 20 g l-1
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

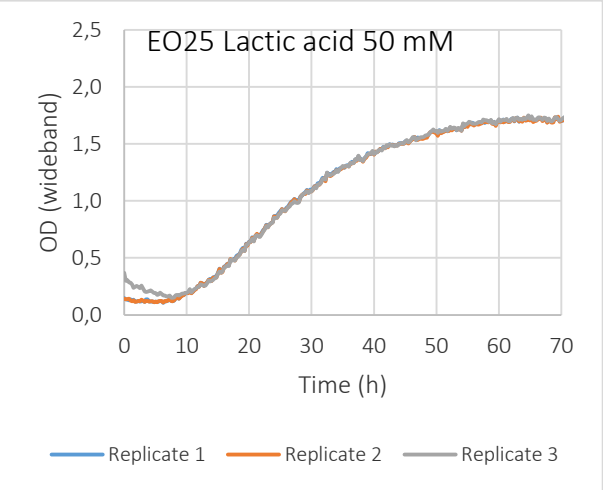

EO19 Glucose 20 g l-1
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

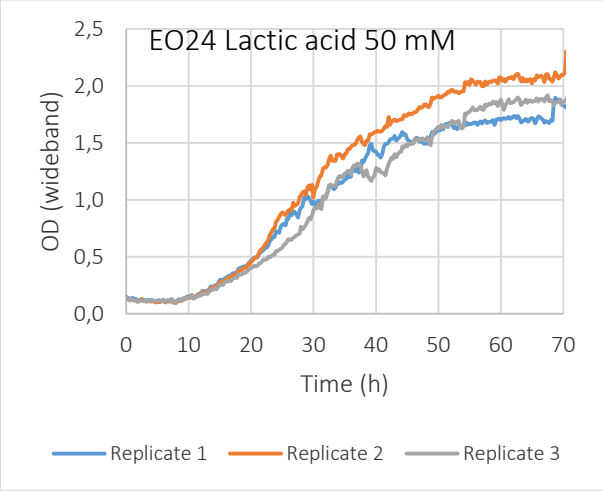

EO18 Glucose 20 g l-1
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

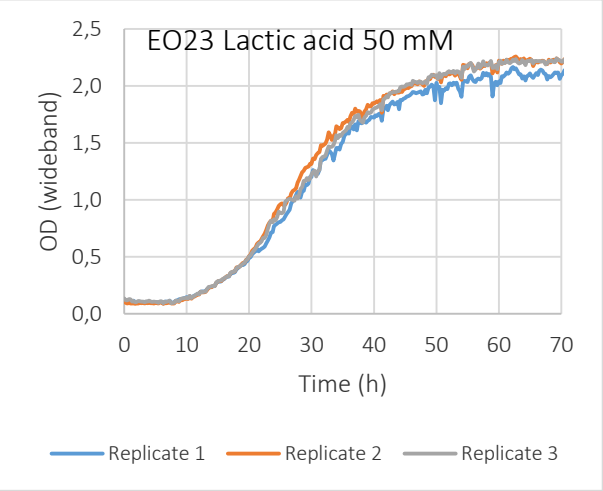
Appendix 3 – Growth curves obtained during the initial acid toxicity screening in Bioscreen cultivation

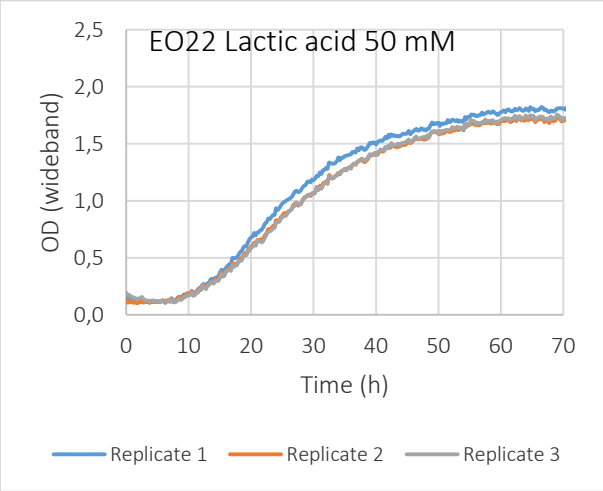
The corresponding growth curves to the values in Table 3Table 2, section 4.5. The seven isolates was screened for growth on mannitol (20 g l^{-1}), in presence of lactic, acetic, propionic or formic acid at a concentration of 50 mM, 0.5 M or 1 M.

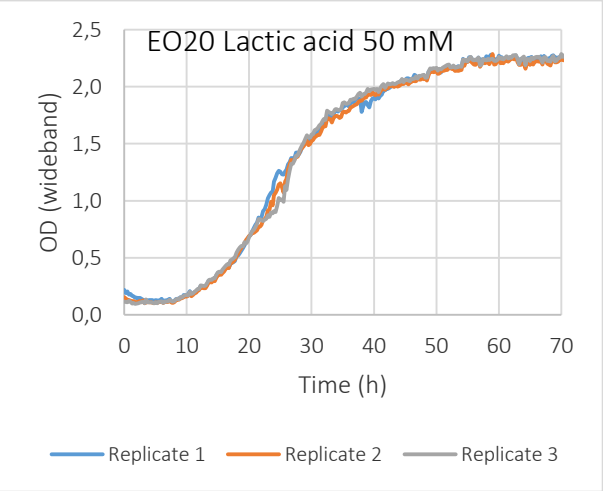


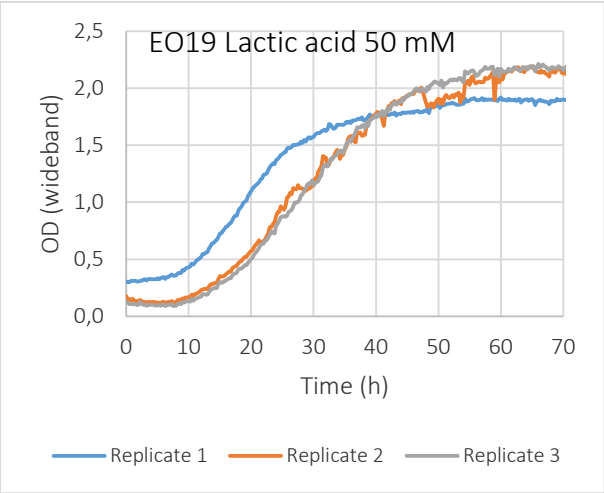


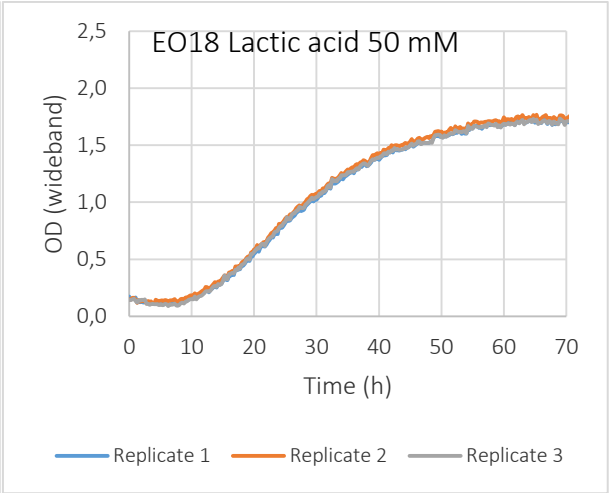


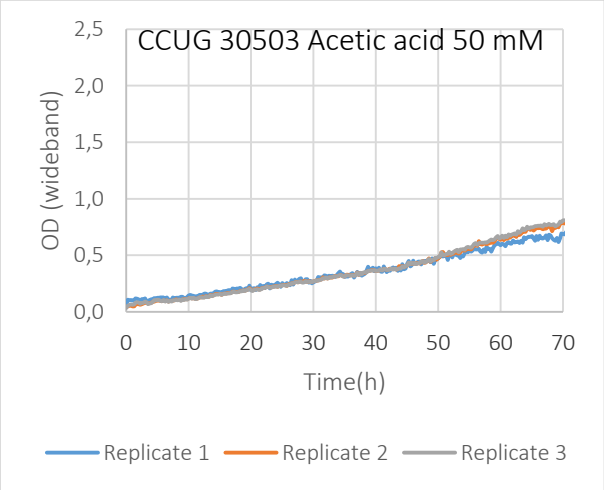


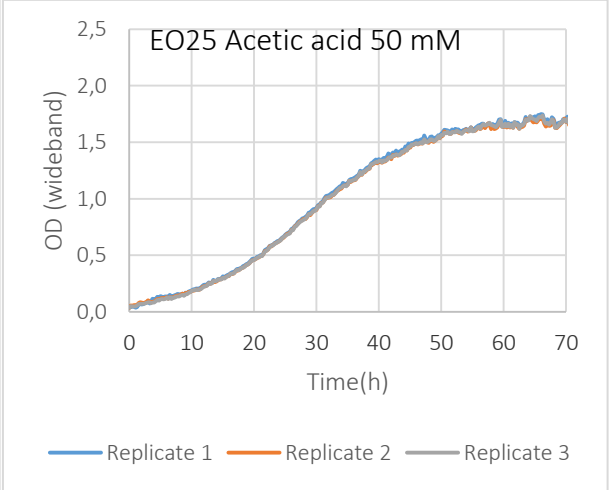


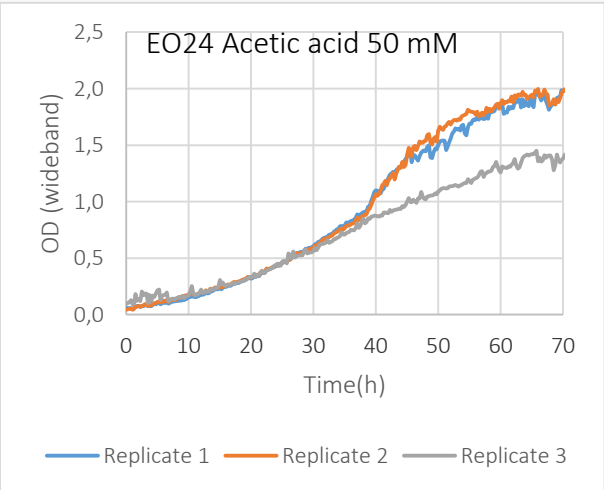


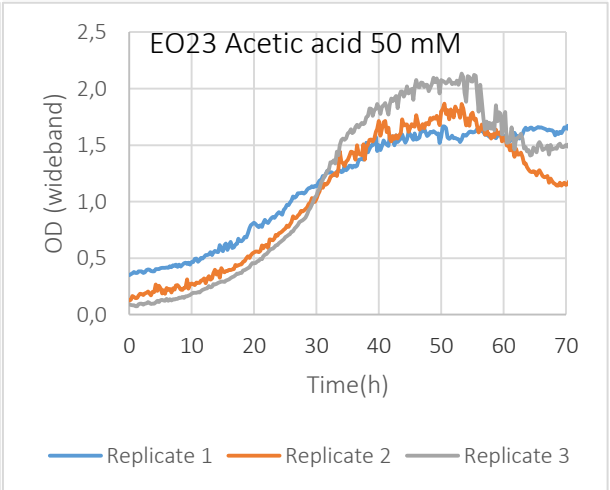

EO19 Lactic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

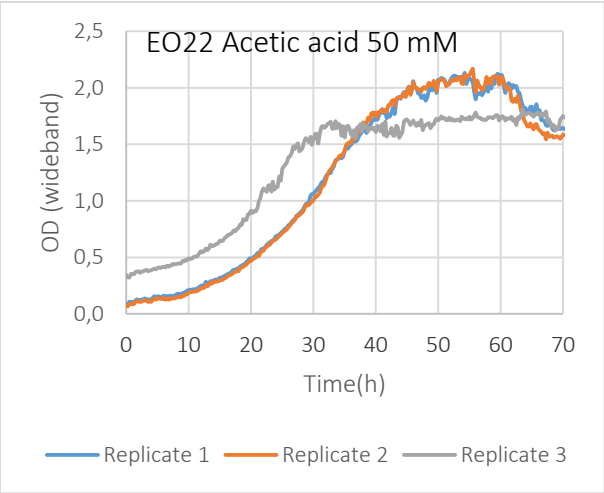

EO18 Lactic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

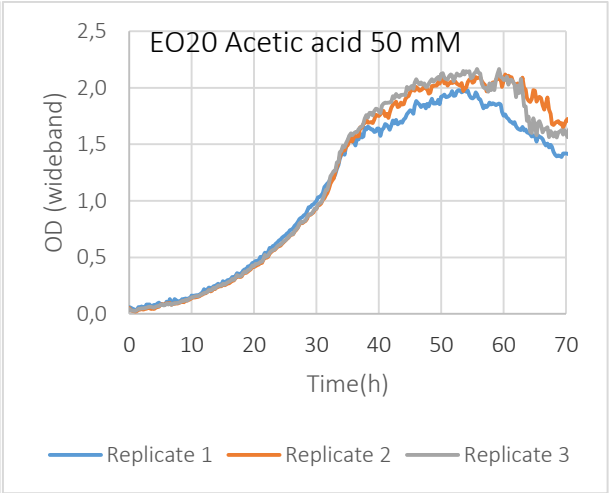

CCUG 30503 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

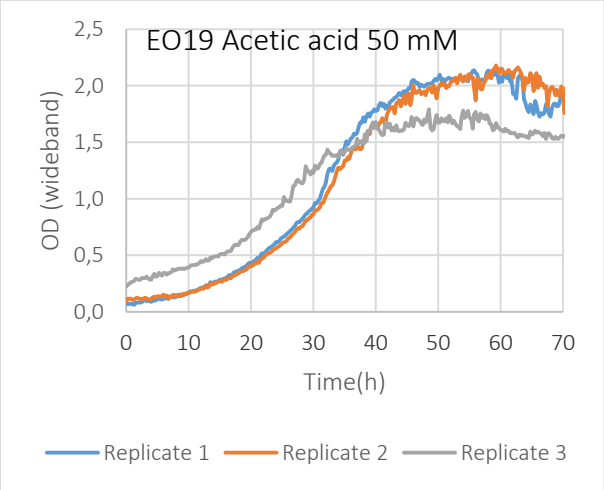

EO25 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

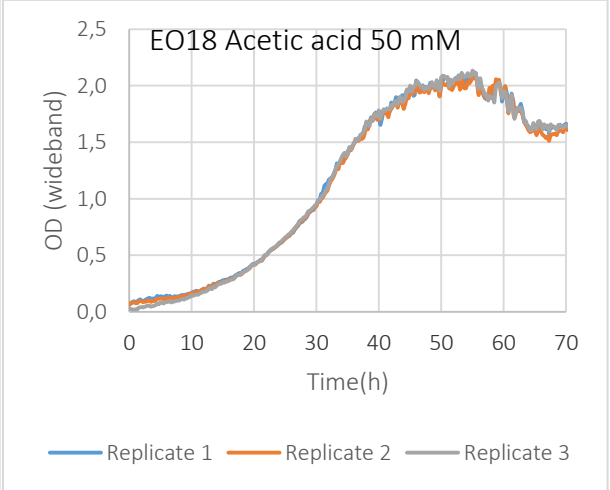

EO24 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

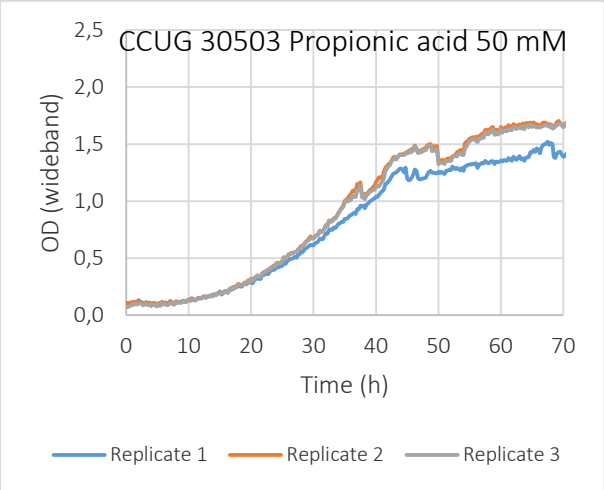

EO23 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

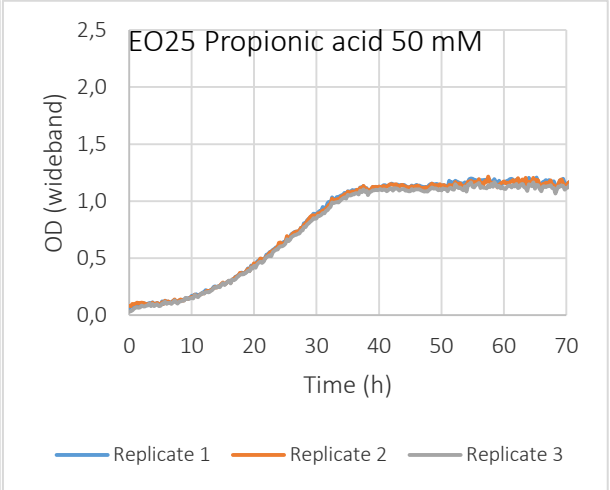

EO22 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

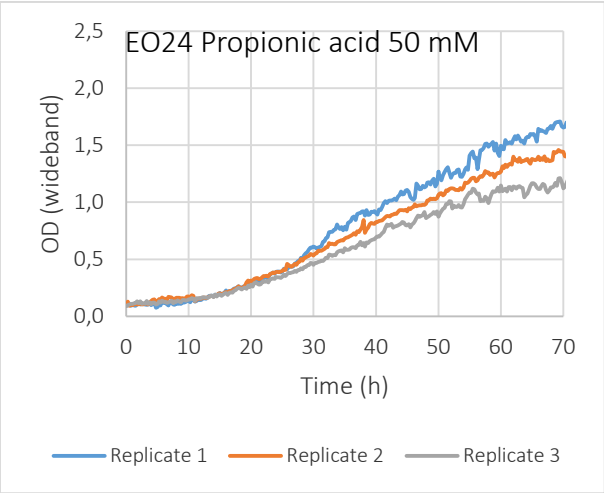

EO20 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

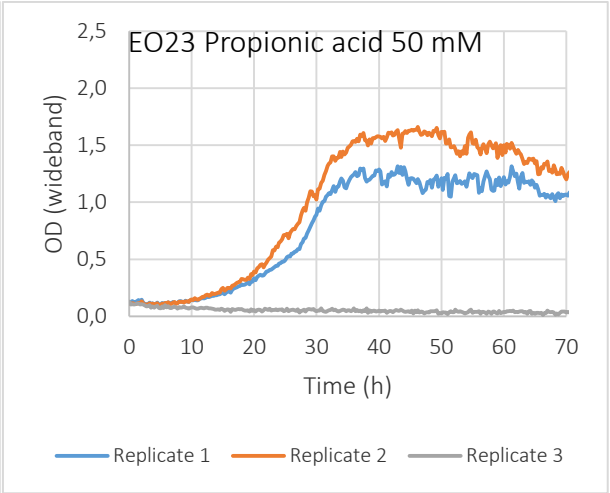

EO19 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

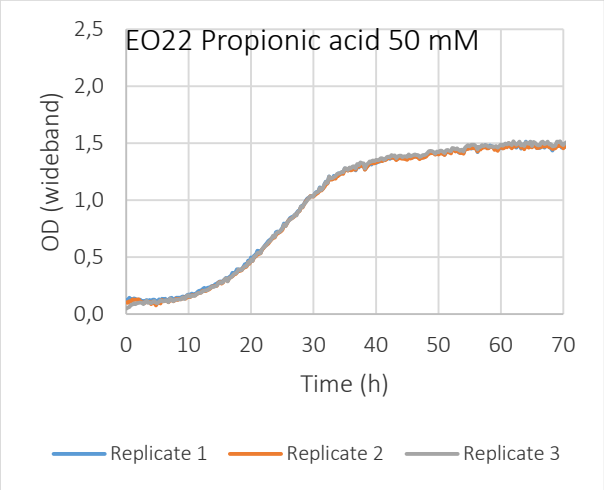

EO18 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

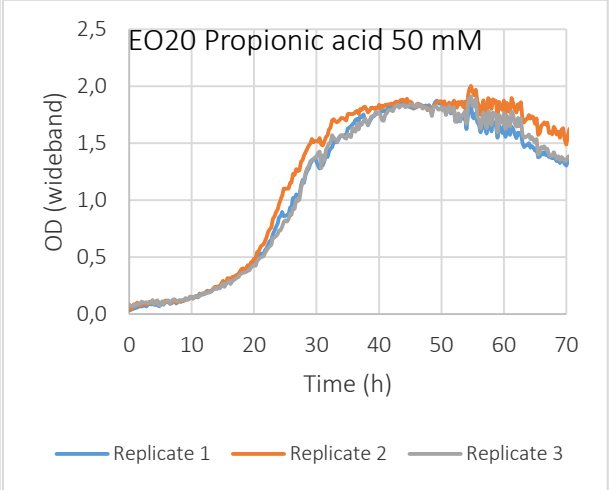

CCUG 30503 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

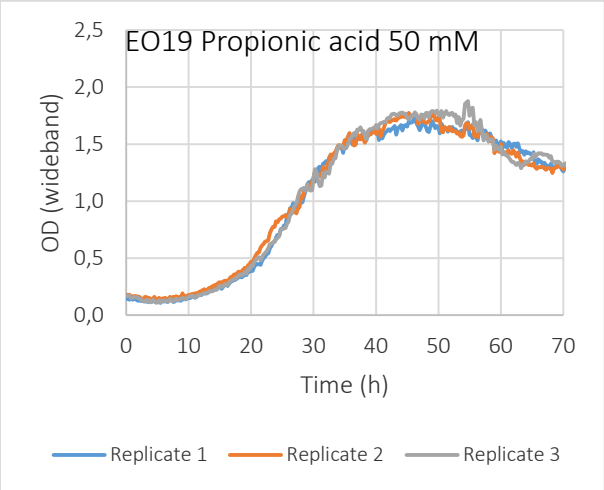

EO25 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

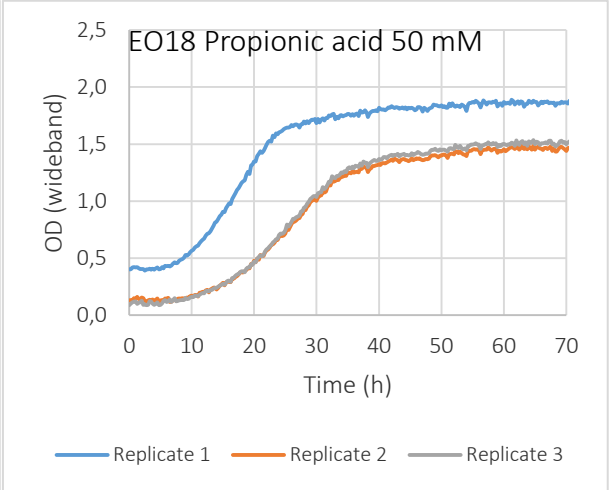

EO24 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

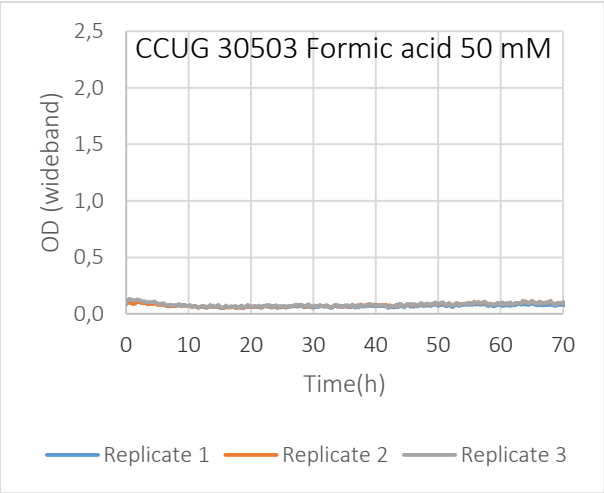

EO23 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

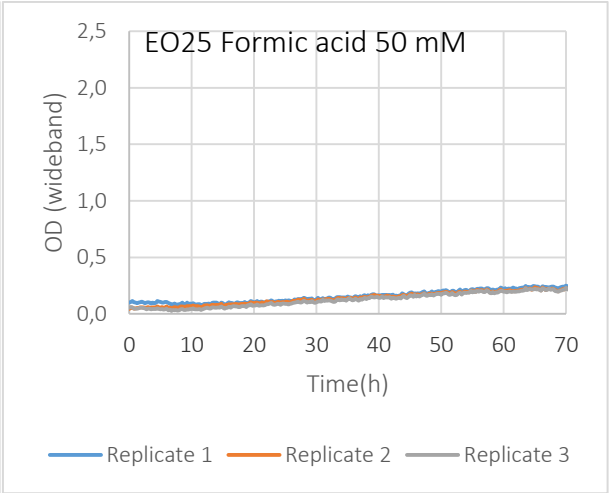

EO22 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

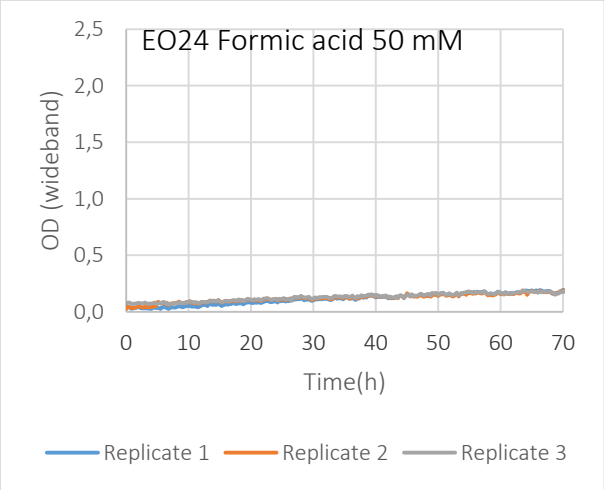

EO20 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

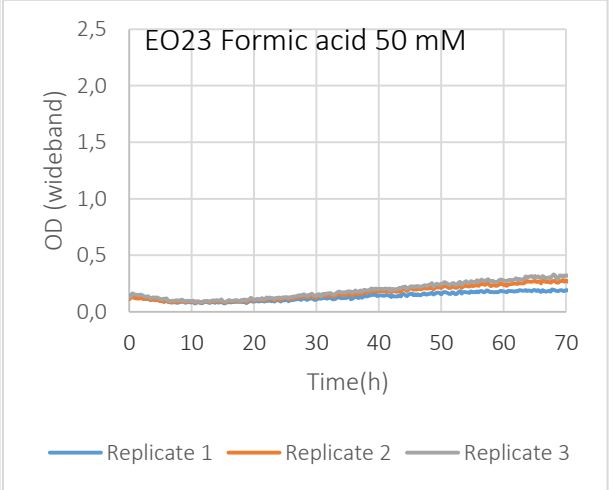

EO19 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

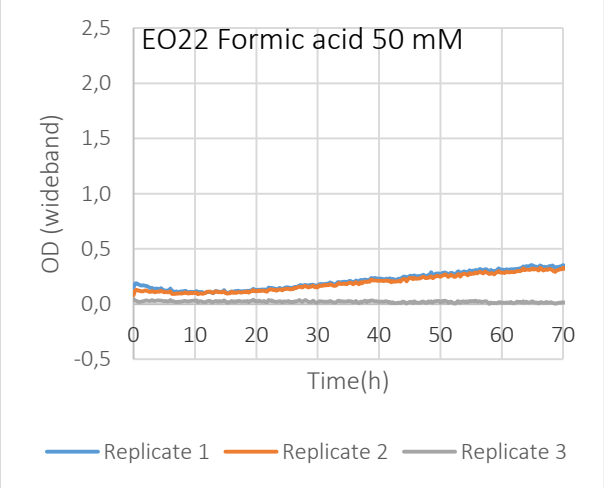

EO18 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

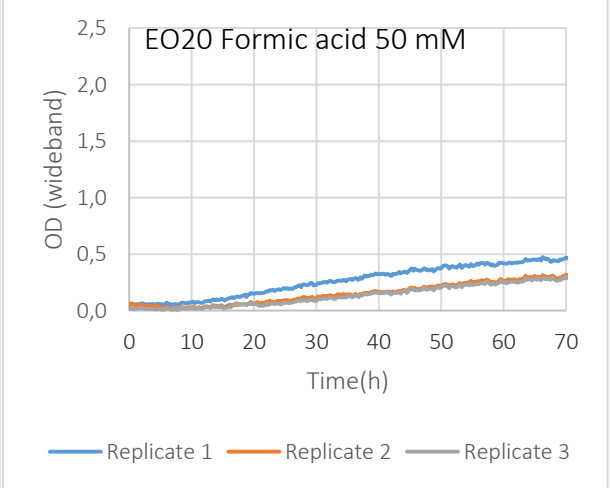

CCUG 30503 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time(h)
Replicate 1
Replicate 2
Replicate 3

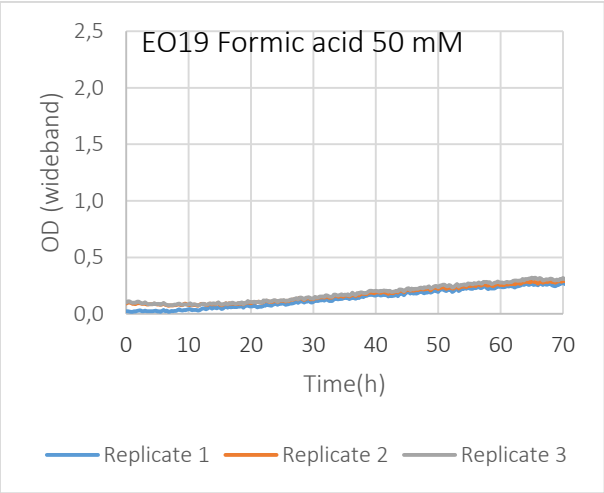

EO25 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time(h)
Replicate 1
Replicate 2
Replicate 3

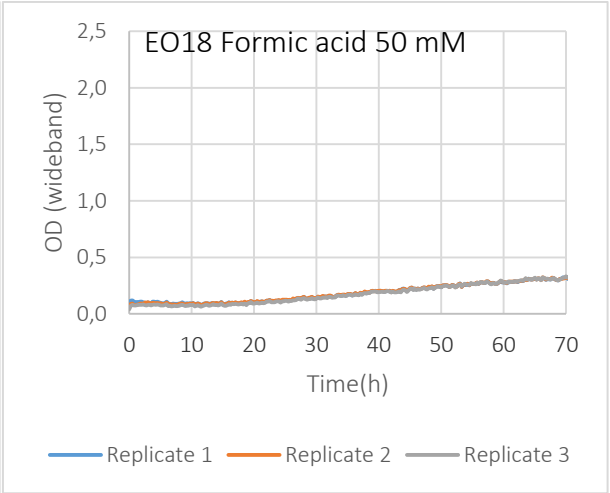

EO24 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time(h)
Replicate 1
Replicate 2
Replicate 3

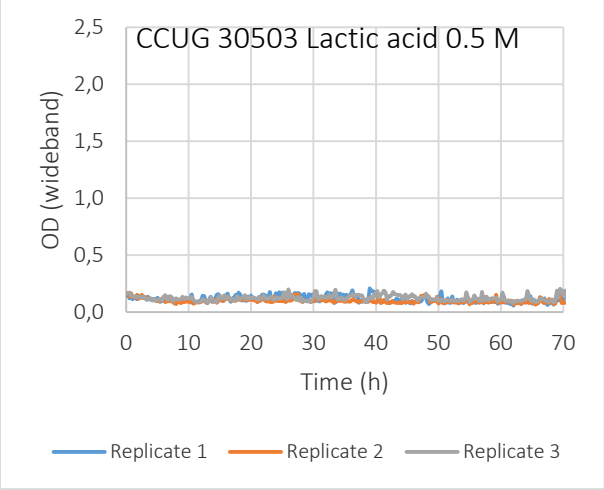

EO23 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time(h)
Replicate 1
Replicate 2
Replicate 3

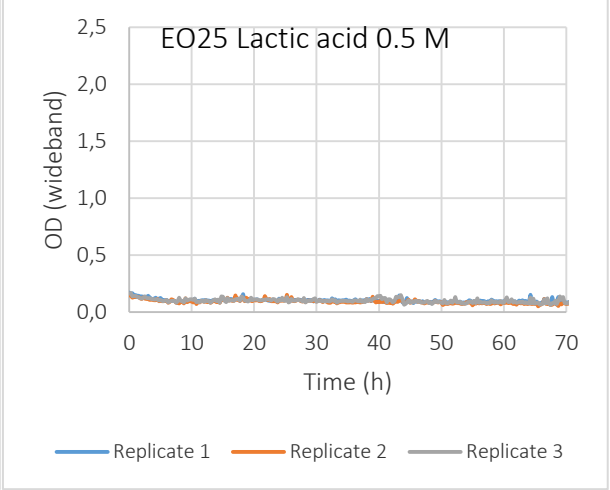

EO22 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
0
10
20
30
40
50
60
70
Time(h)
Replicate 1
Replicate 2
Replicate 3

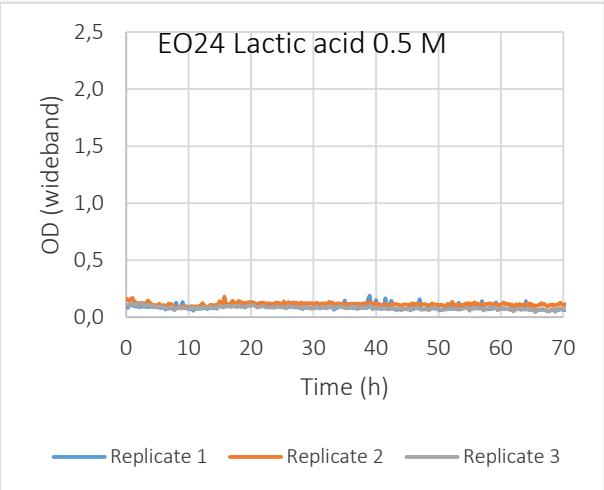

EO20 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time(h)
Replicate 1
Replicate 2
Replicate 3

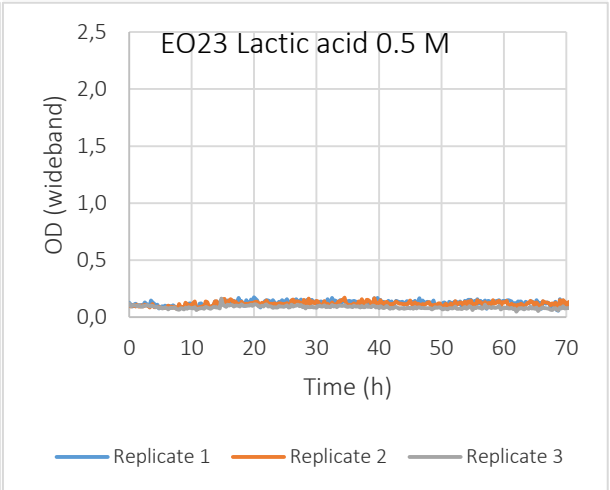

EO19 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

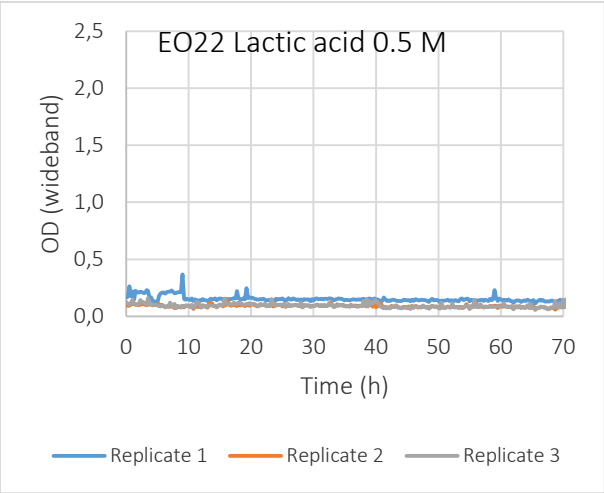

EO18 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

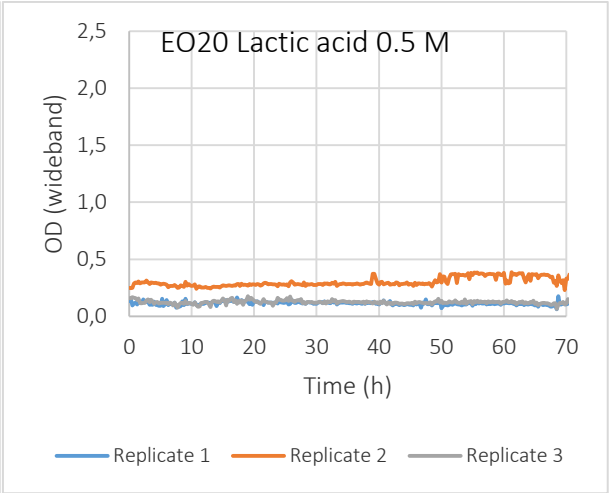

CCUG 30503 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

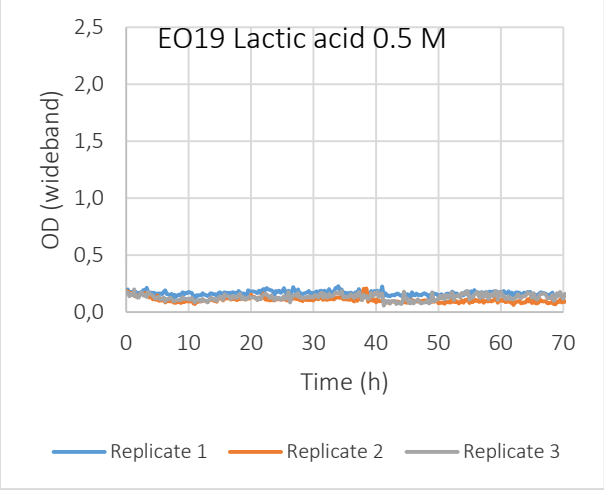

EO25 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

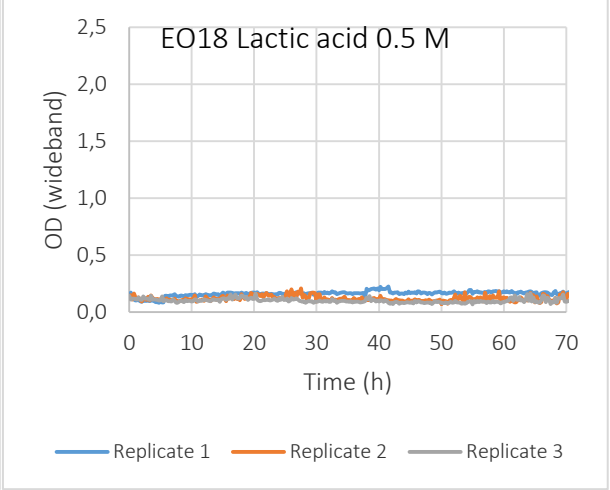

EO24 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

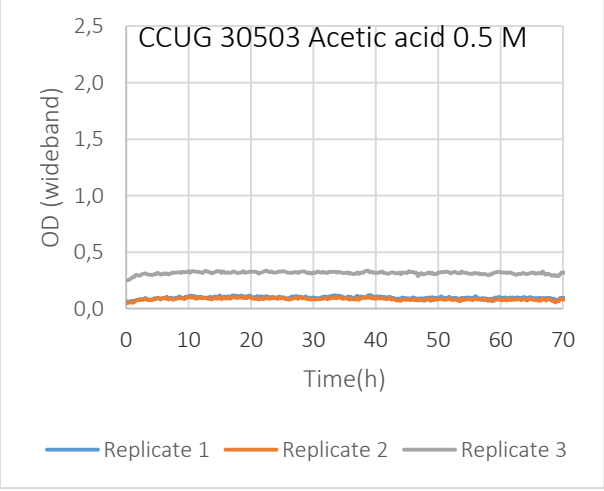

EO23 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

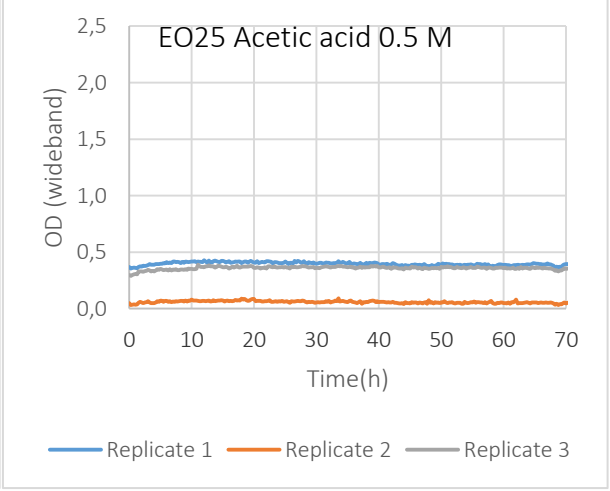

EO22 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO20 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO19 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO18 Lactic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


CCUG 30503 Acetic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3


EO25 Acetic acid 0.5 M
OD (wideband)
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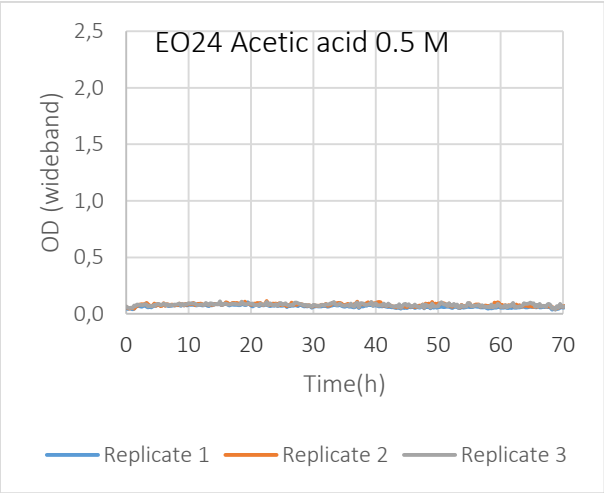
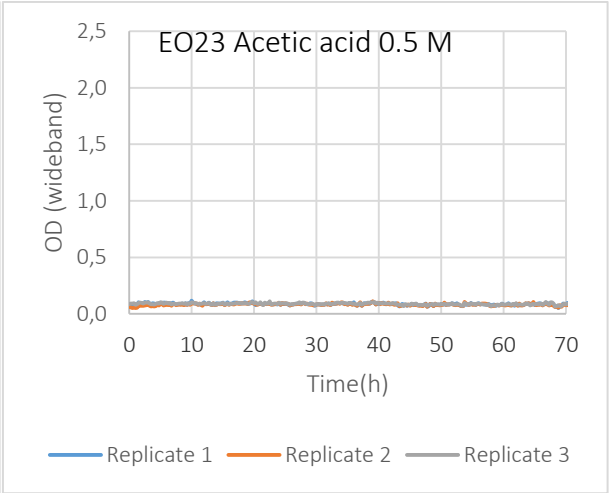
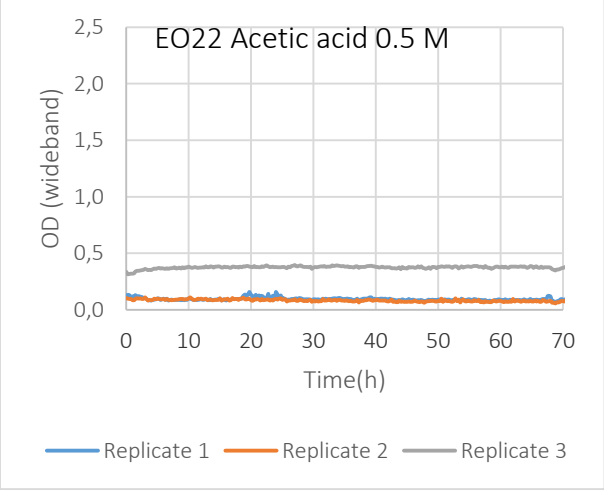
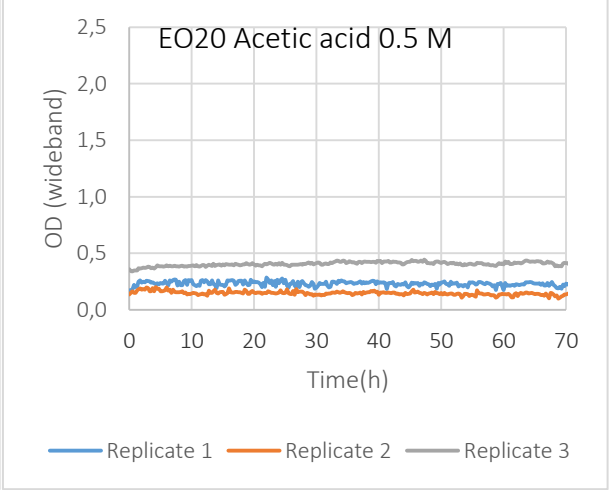
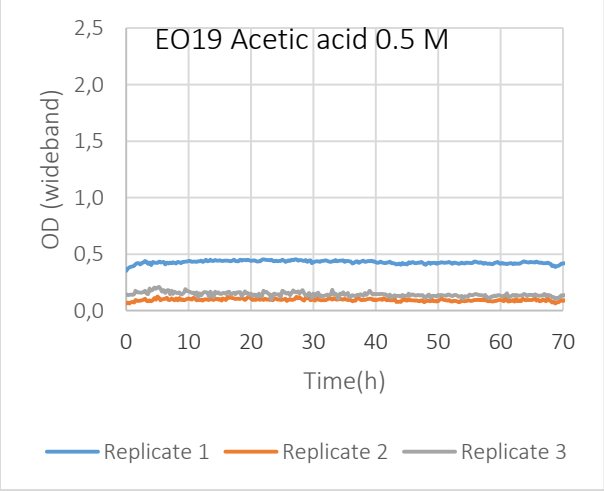
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

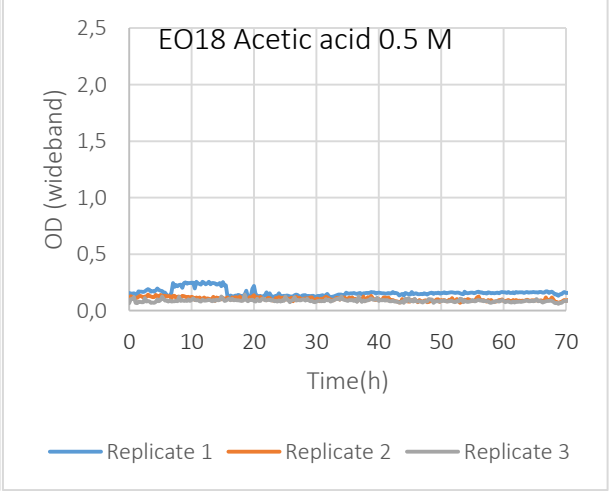

EO24 Acetic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

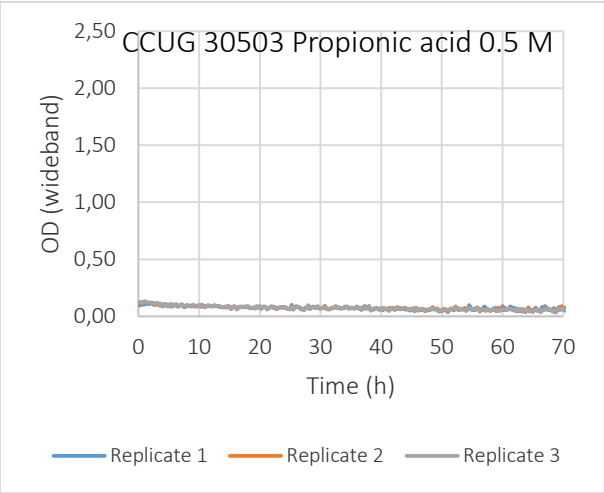

EO23 Acetic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

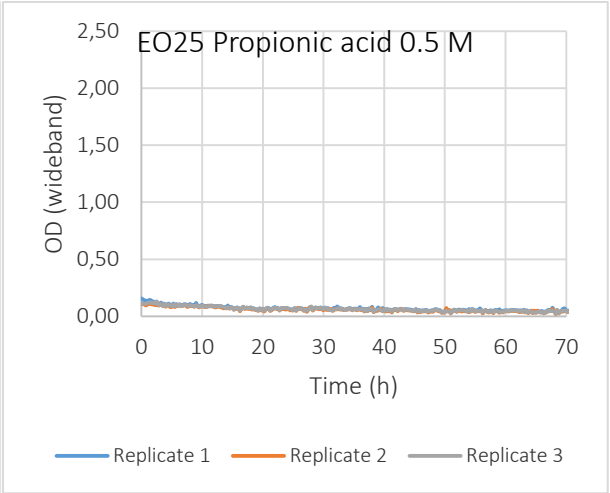

EO22 Acetic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3


EO20 Acetic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3


EO19 Acetic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3


EO18 Acetic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3


CCUG 30503 Propionic acid 0.5 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO25 Propionic acid 0.5 M
OD (wideband)
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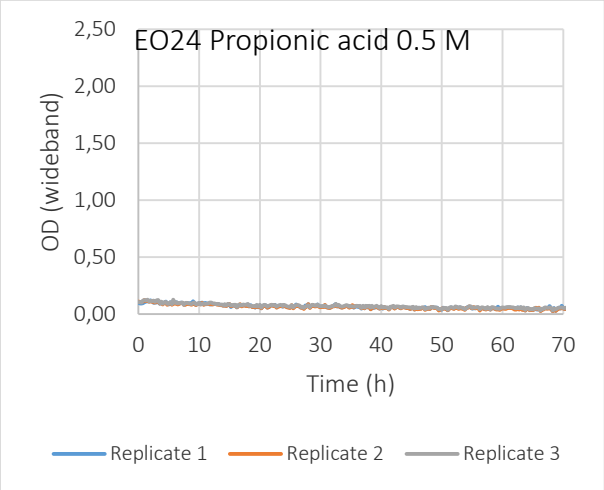
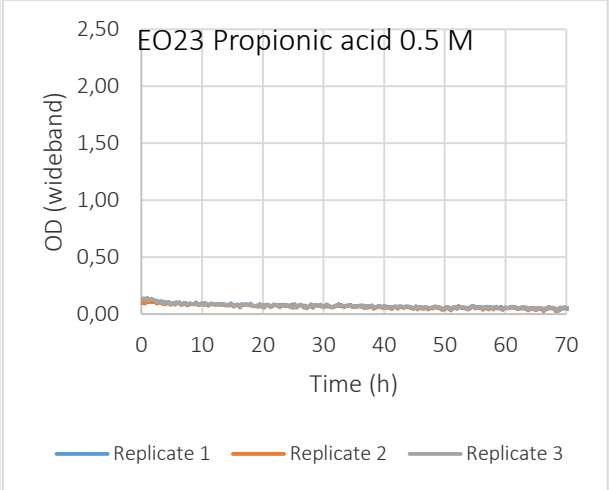
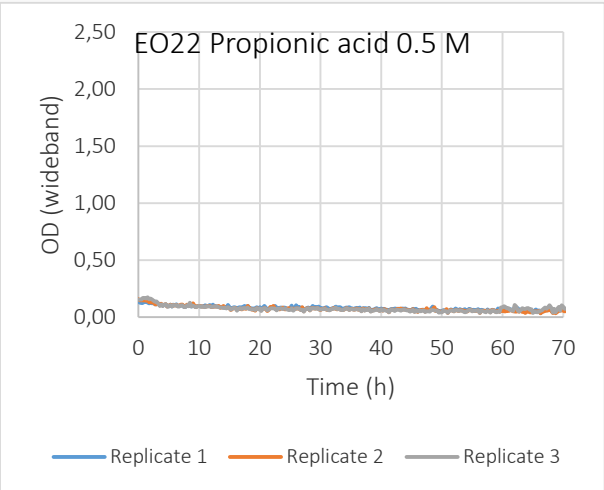
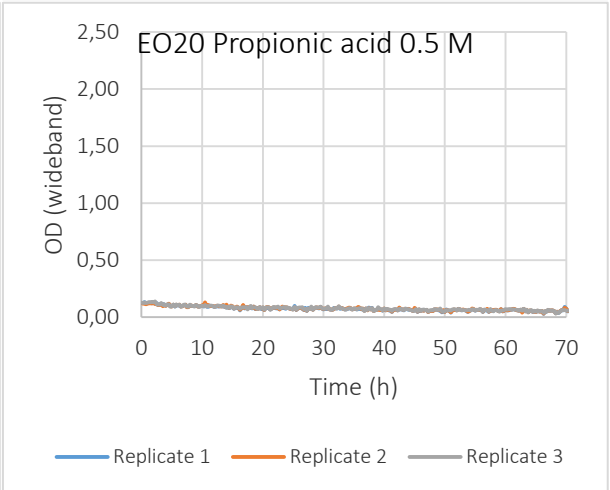
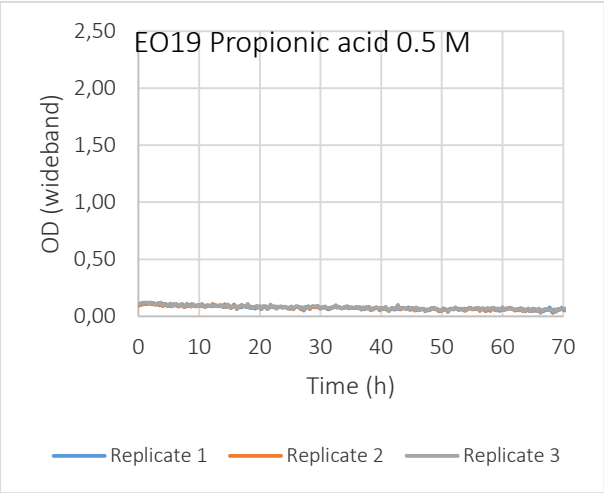
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

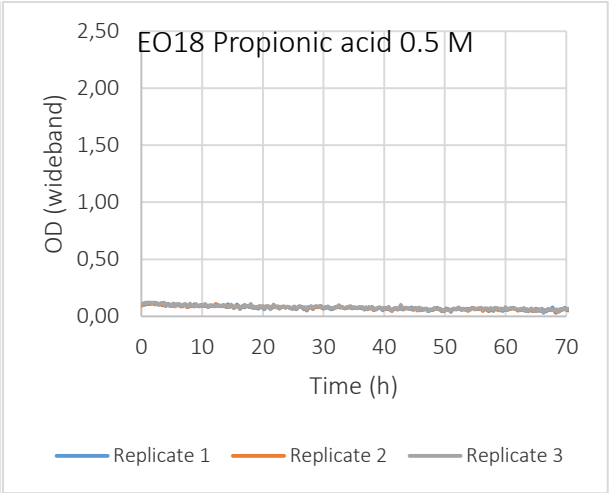

EO24 Propionic acid 0.5 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

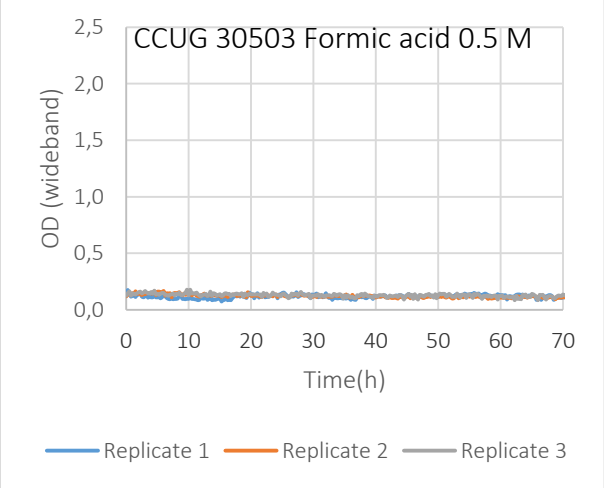

EO23 Propionic acid 0.5 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

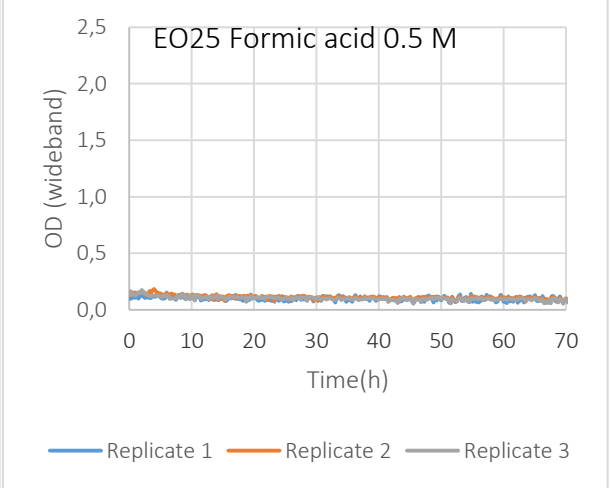

EO22 Propionic acid 0.5 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

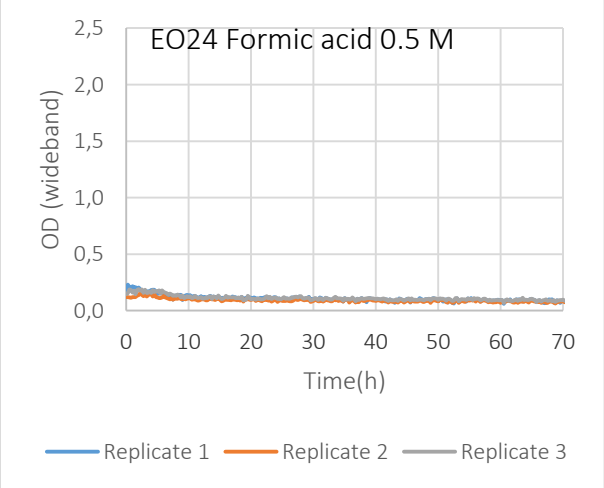

EO20 Propionic acid 0.5 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

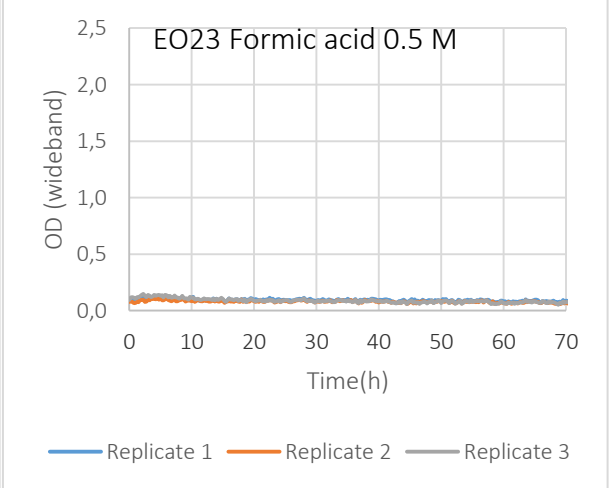

EO19 Propionic acid 0.5 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

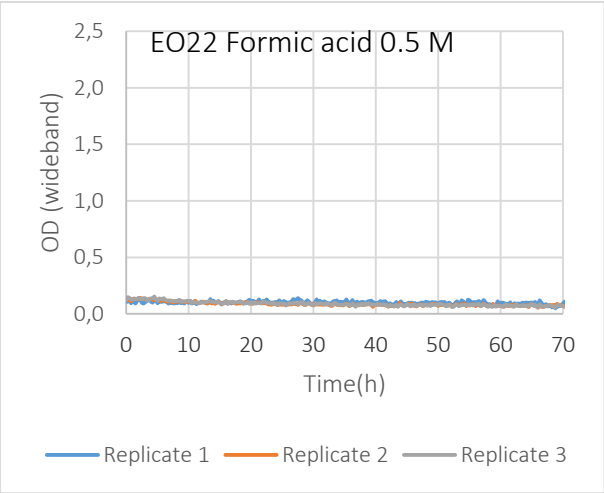

EO18 Propionic acid 0.5 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

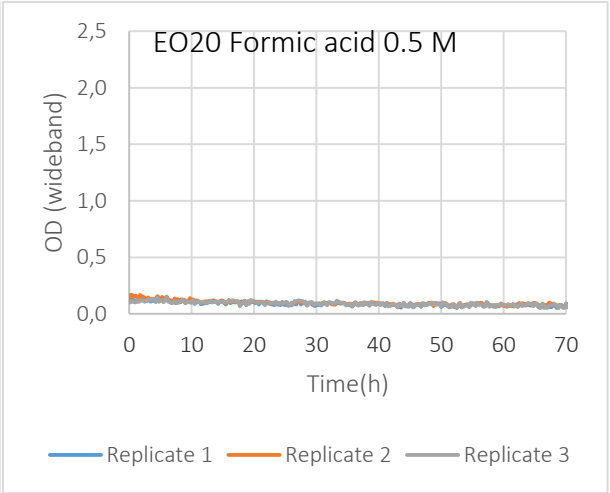

CCUG 30503 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

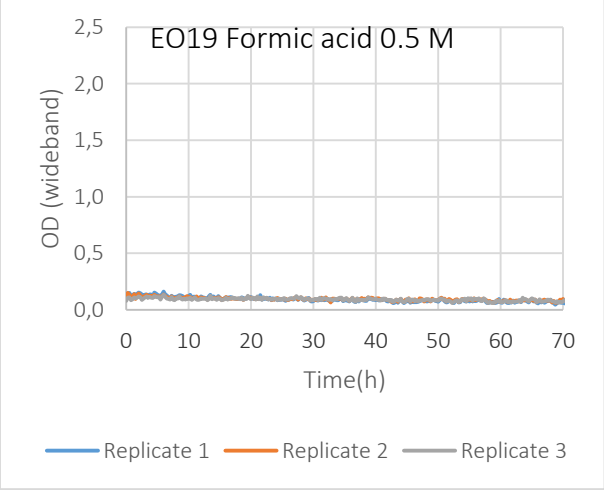

EO25 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

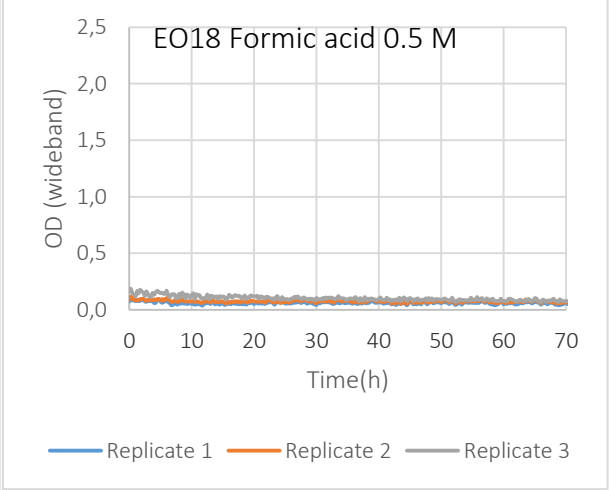

EO24 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

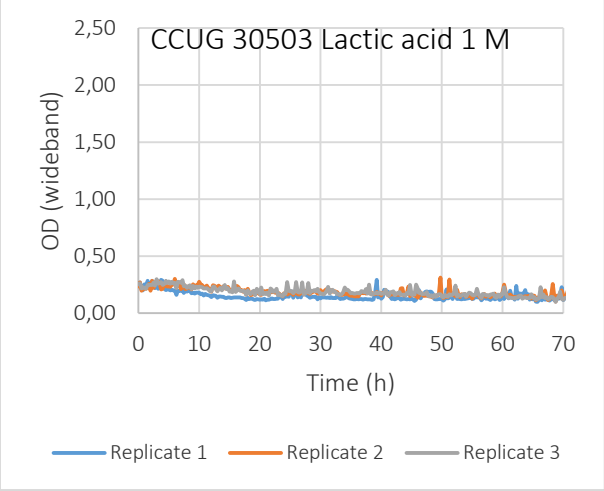

EO23 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

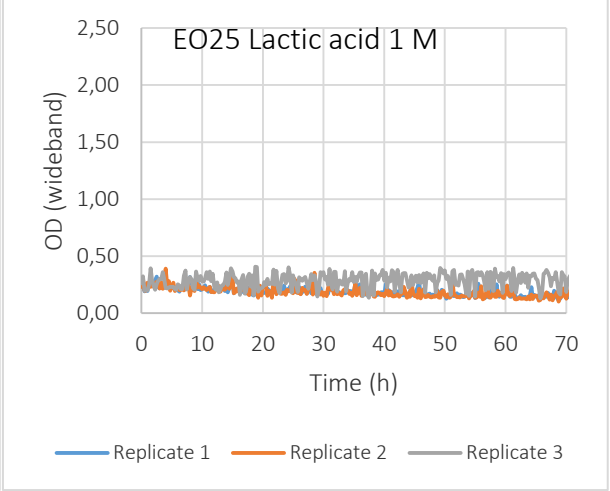

EO22 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

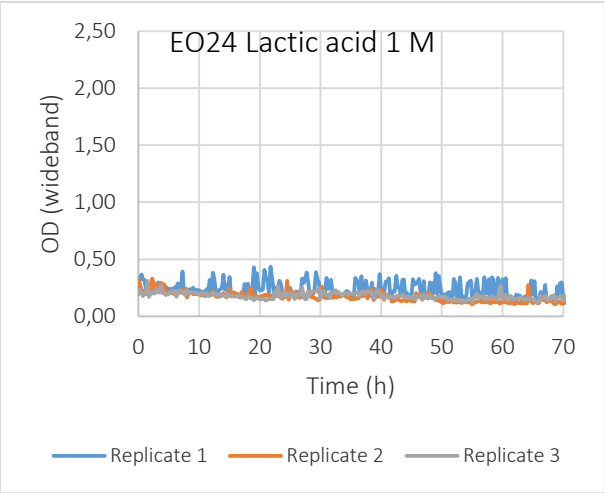

EO20 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

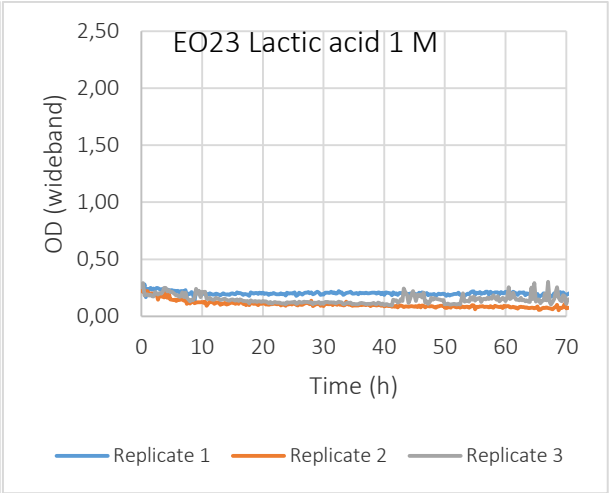

EO19 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

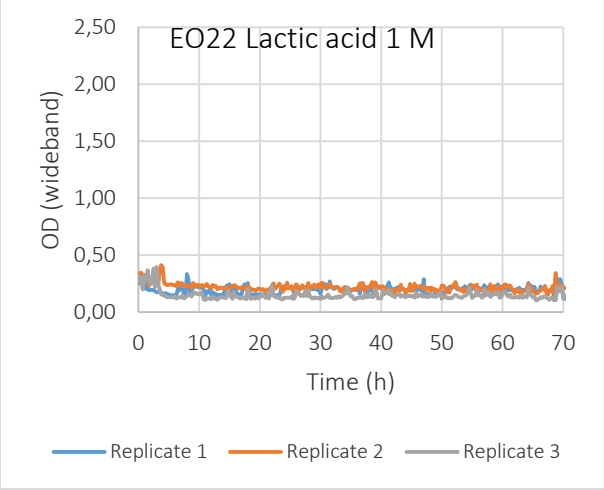

EO18 Formic acid 0.5 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

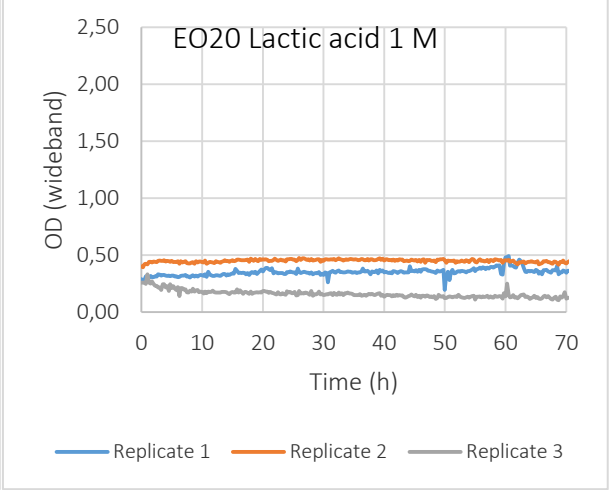

CCUG 30503 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

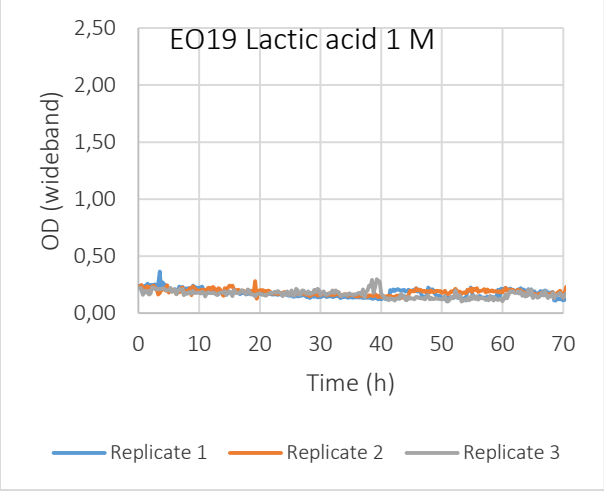

EO25 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

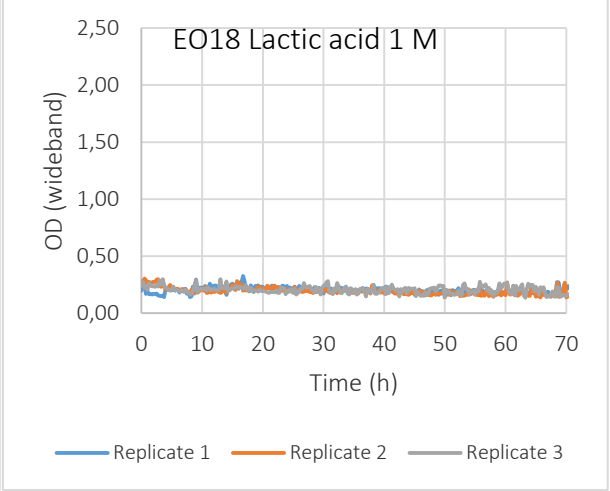

EO24 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

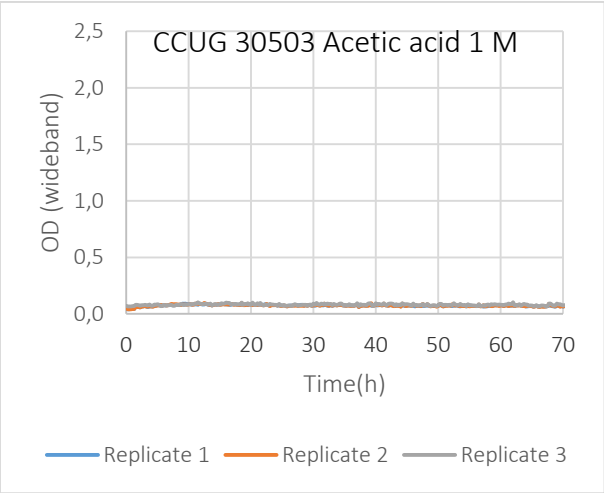

EO23 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

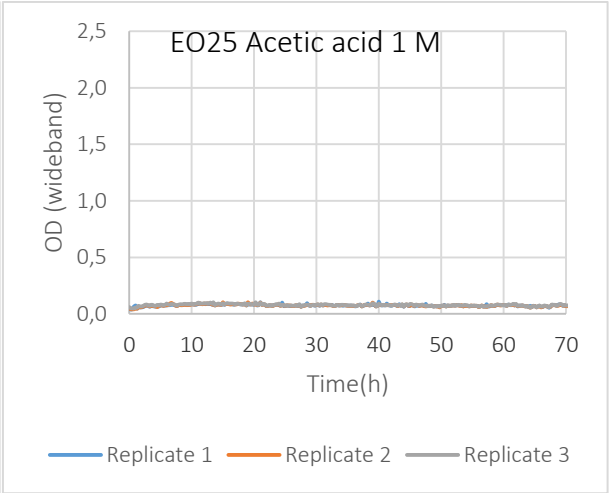

EO22 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

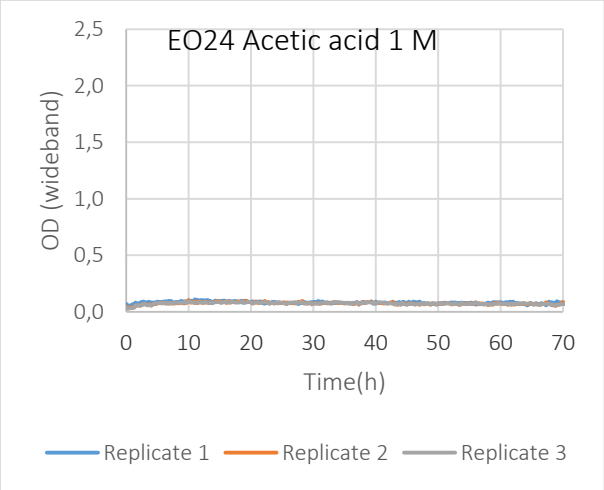

EO20 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

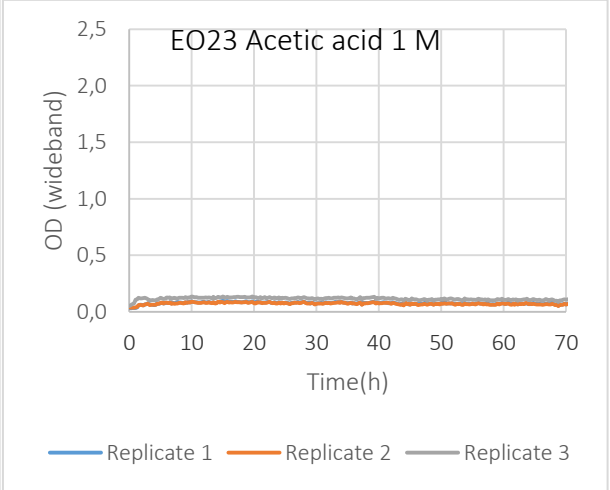

EO19 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

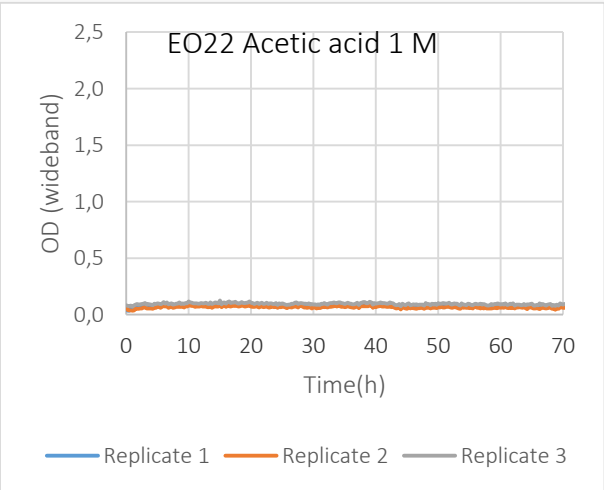

EO18 Lactic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

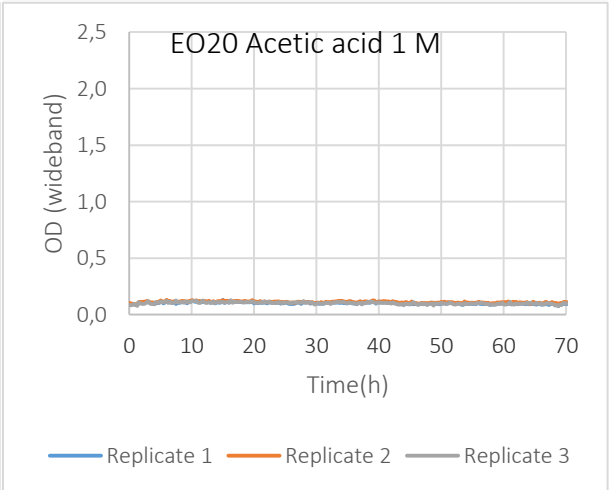

CCUG 30503 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

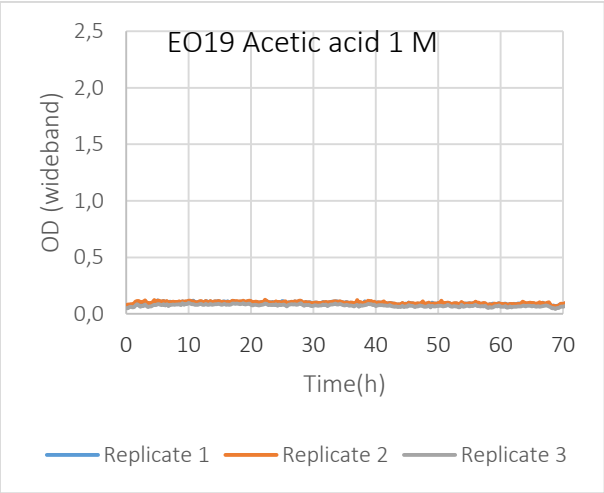

EO25 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

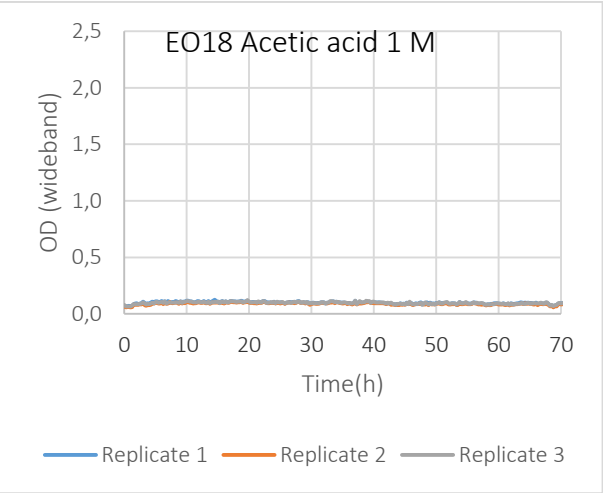

EO24 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

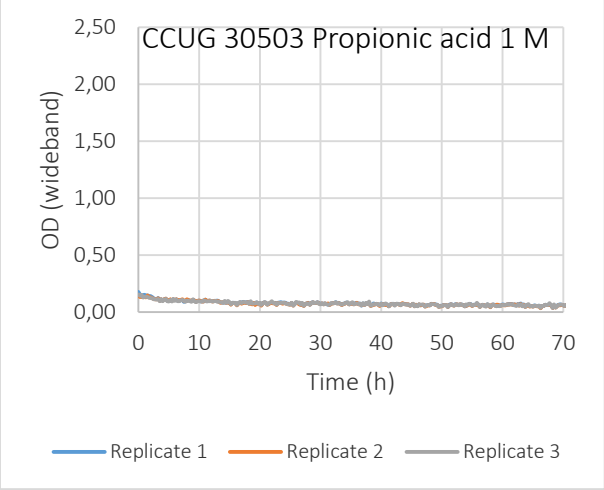

EO23 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

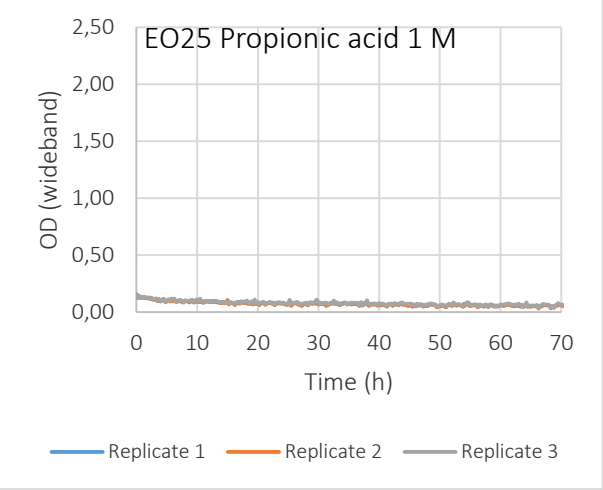

EO22 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

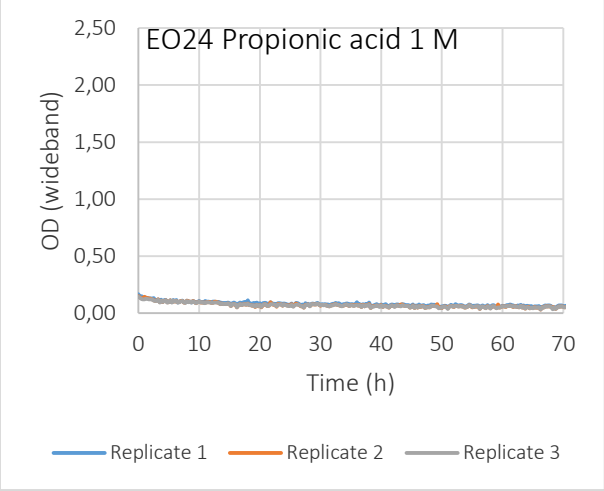

EO20 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

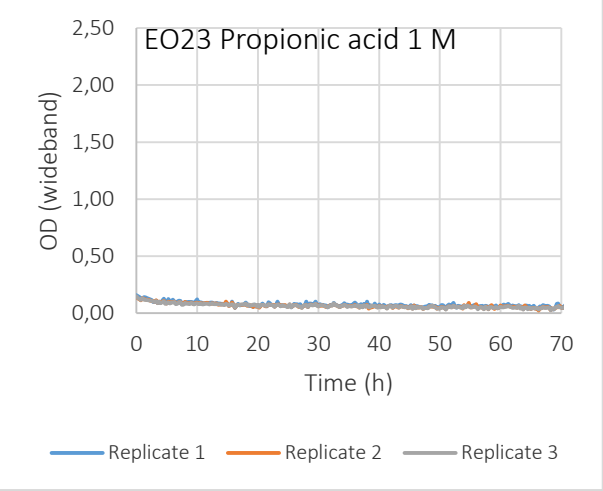

EO19 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

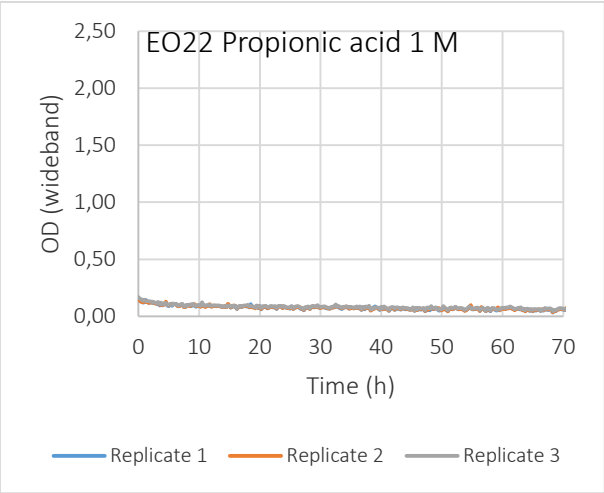

EO18 Acetic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

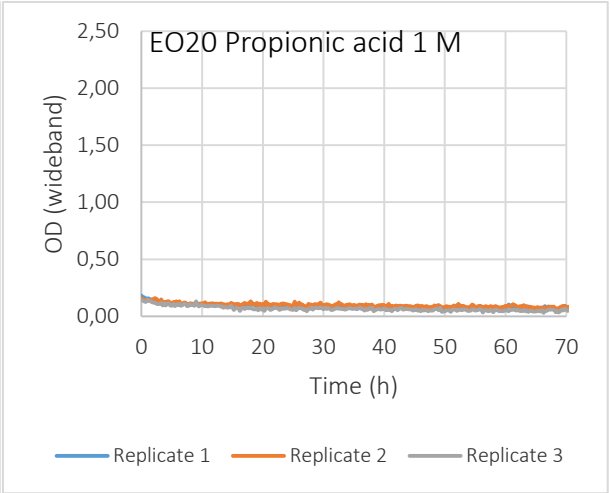

CCUG 30503 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

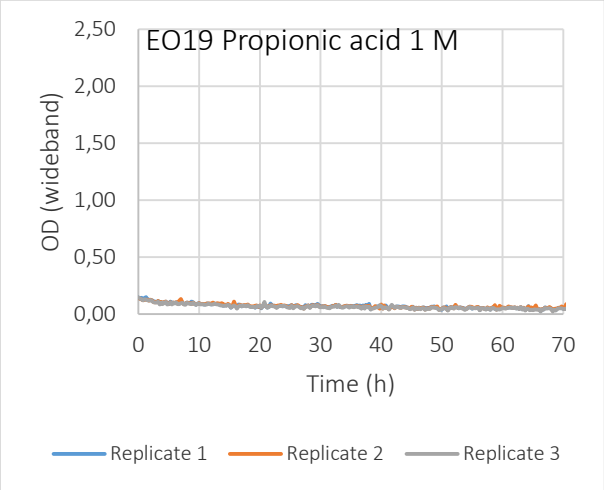

EO25 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

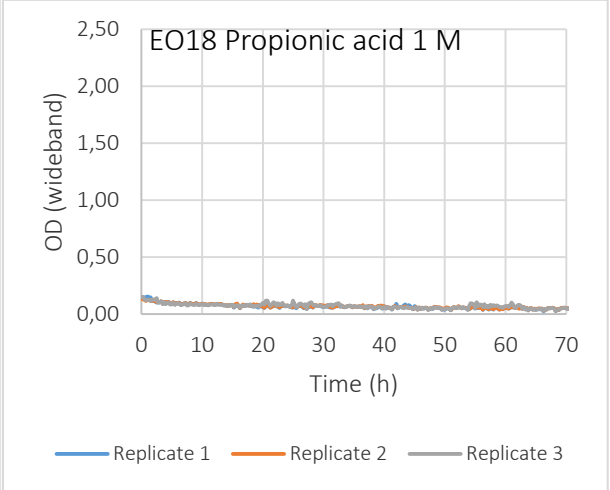

EO24 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

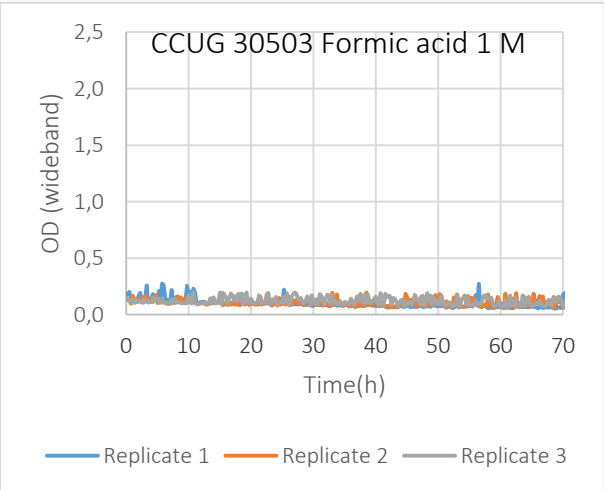

EO23 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

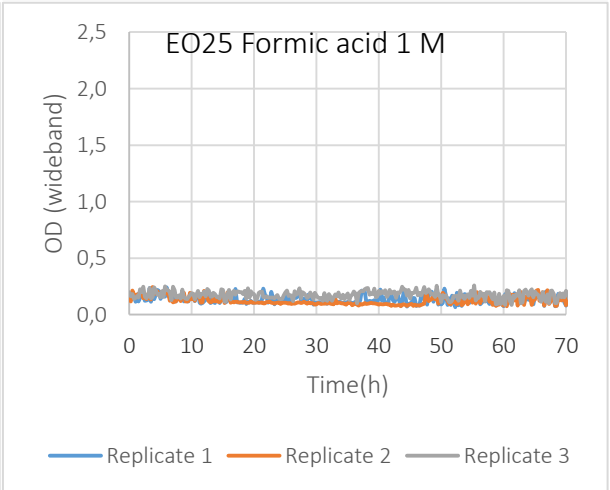

EO22 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

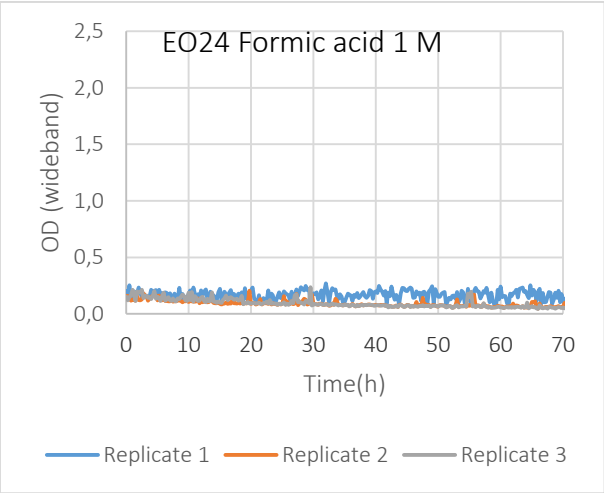

EO20 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

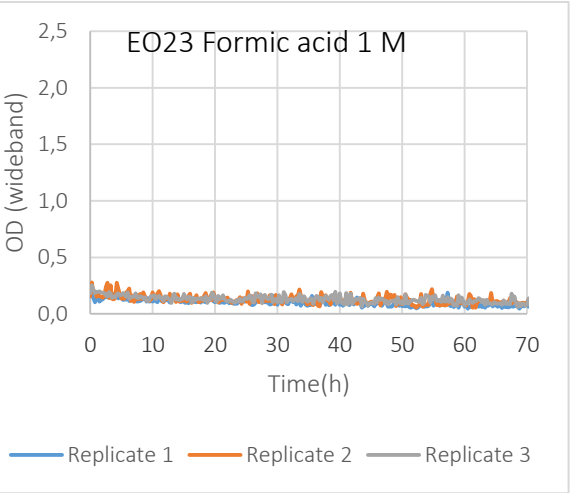

EO19 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

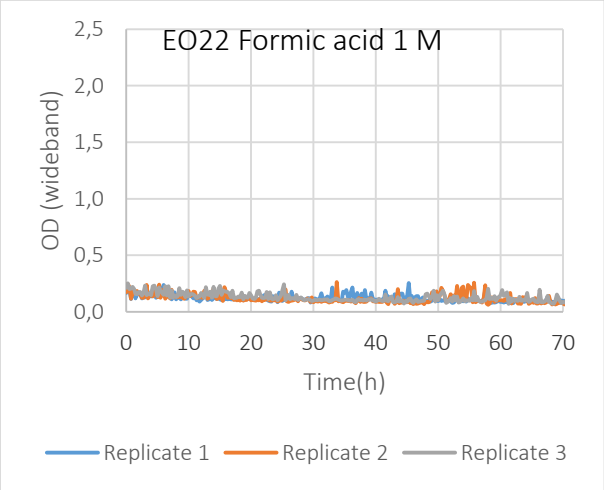

EO18 Propionic acid 1 M
OD (wideband)
2,50
2,00
1,50
1,00
0,50
0,00
0 10 20 30 40 50 60 70
Time (h)
Replicate 1
Replicate 2
Replicate 3

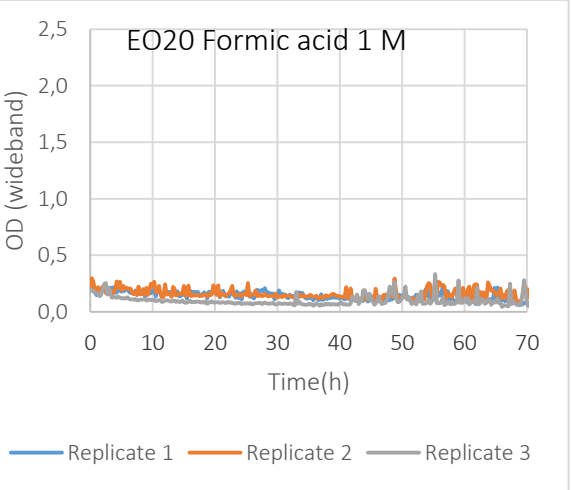

CCUG 30503 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

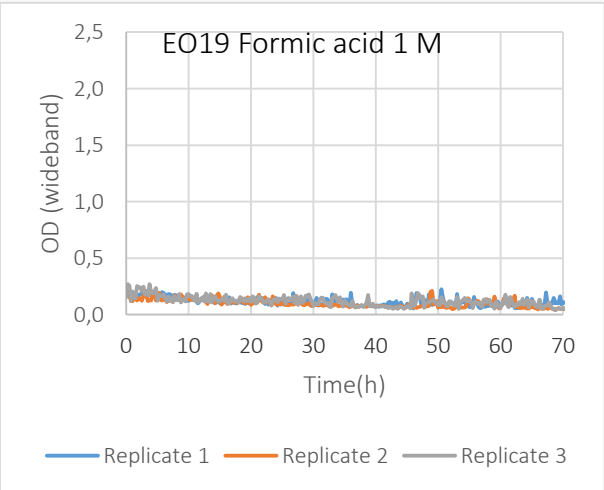

EO25 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

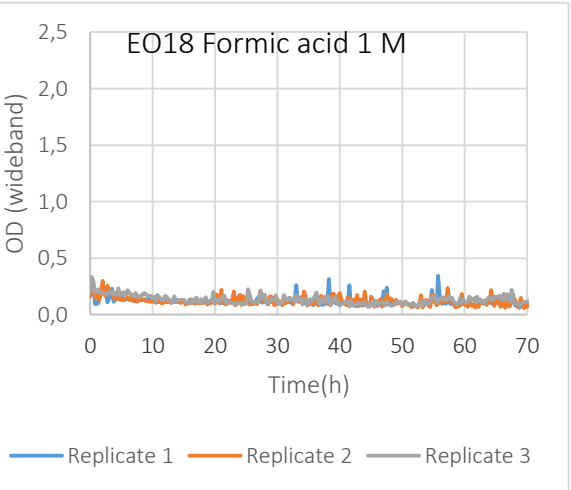

EO24 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3


EO23 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3


EO22 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

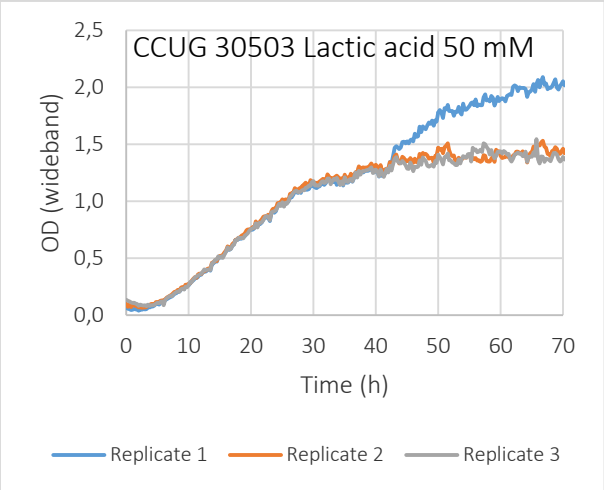

EO20 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

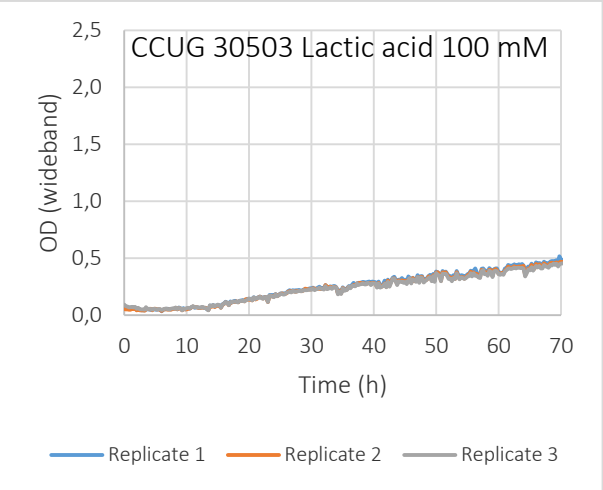

EO19 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

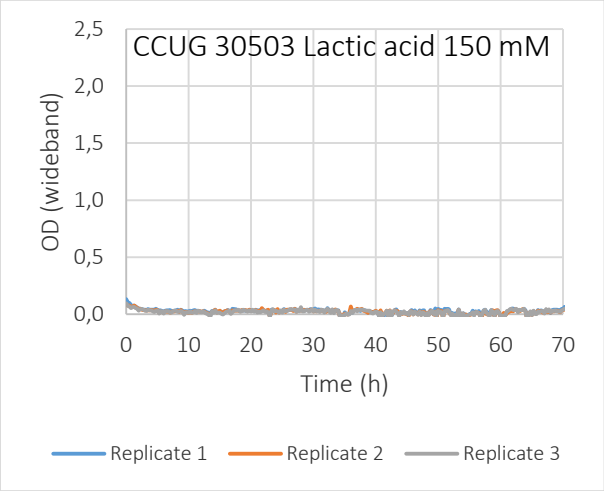

EO18 Formic acid 1 M
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0 10 20 30 40 50 60 70
Time(h)
Replicate 1
Replicate 2
Replicate 3

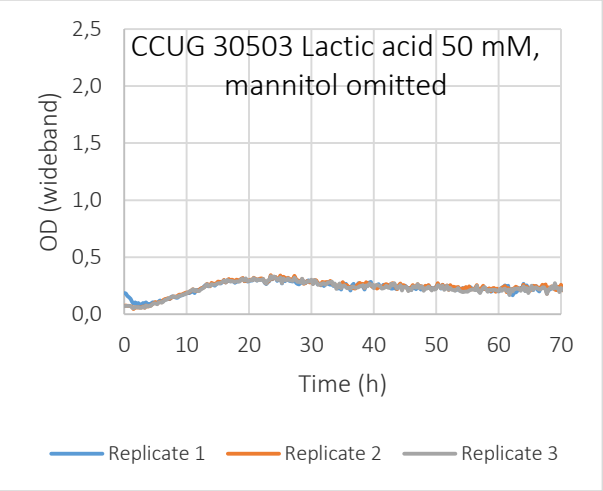
Appendix 4 – Growth curves obtained during the second acid toxicity screening in Bioscreen cultivation

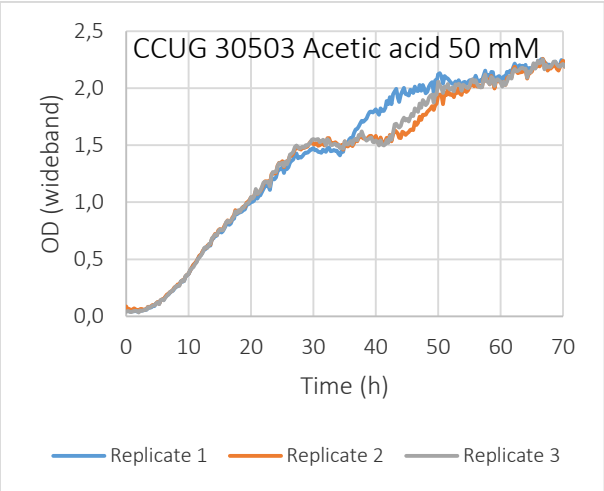
The corresponding growth curves to the values in Table 4 and **Error! Reference source not found.**Table 2, section 4.5. The seven isolates was screened for growth on mannitol (20 g l^{-1}), in presence of lactic, acetic, propionic or formic acid at a concentration of 50, 100 or 150 mM.

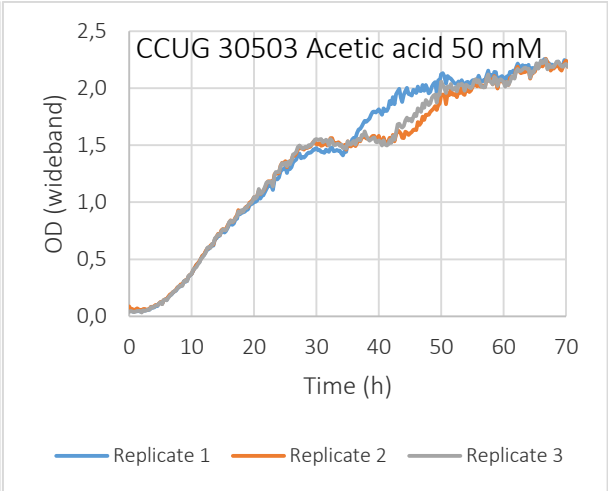


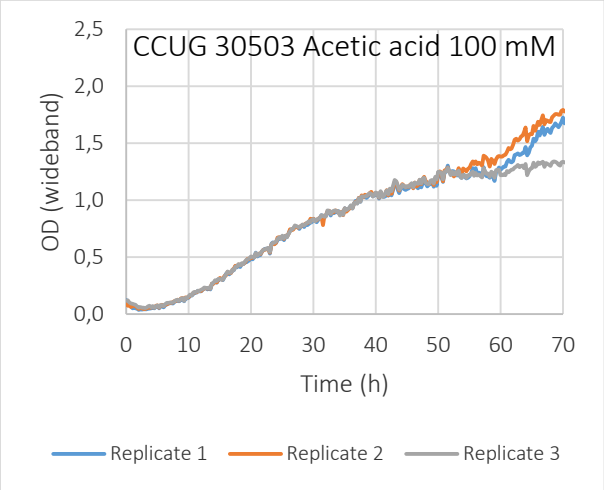


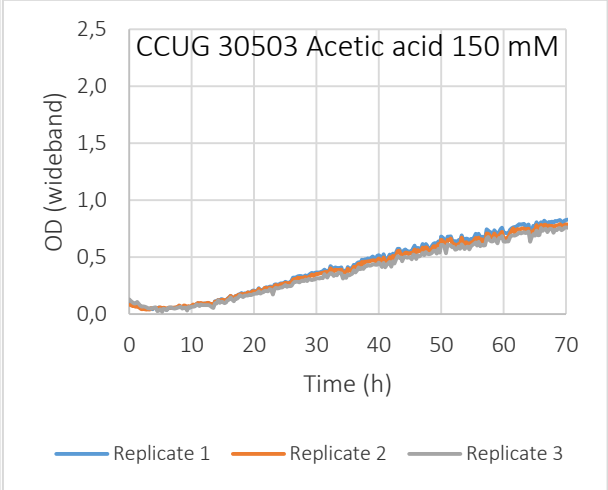


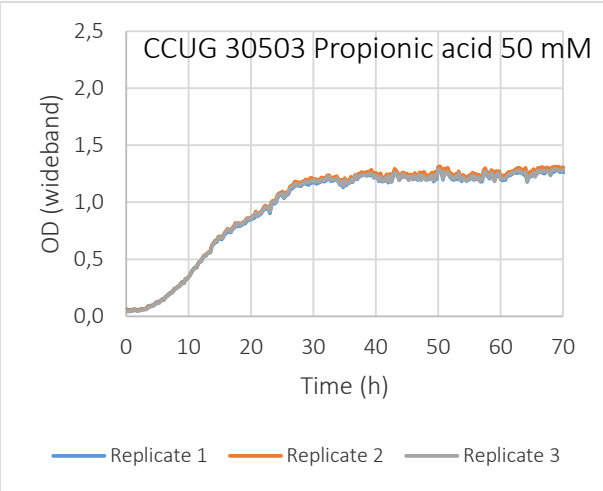


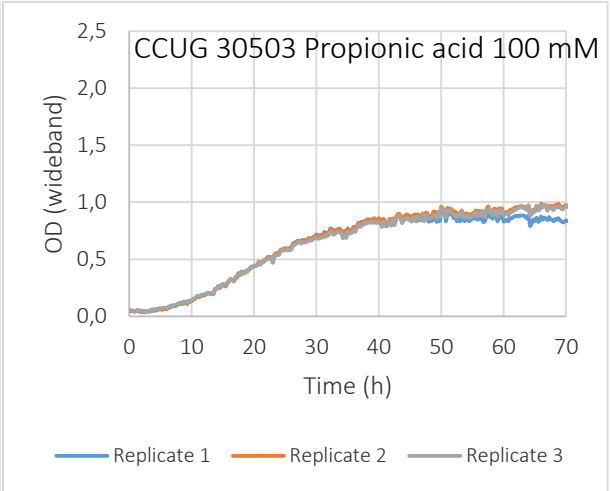

CCUG 30503 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

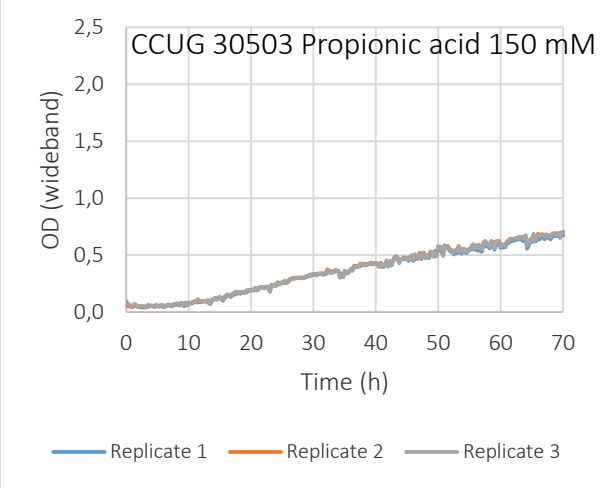

CCUG 30503 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

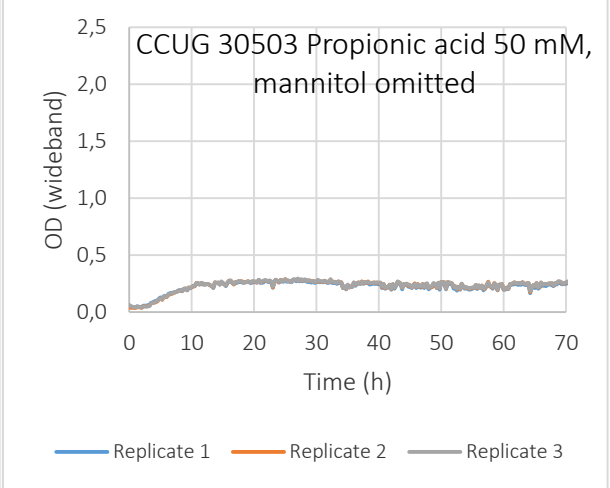

CCUG 30503 Acetic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

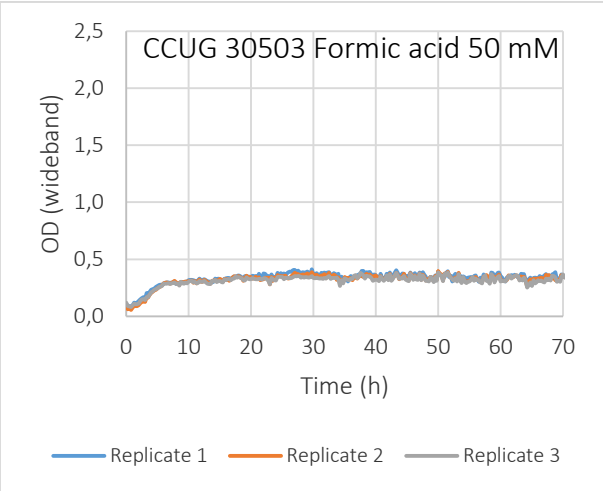

CCUG 30503 Acetic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

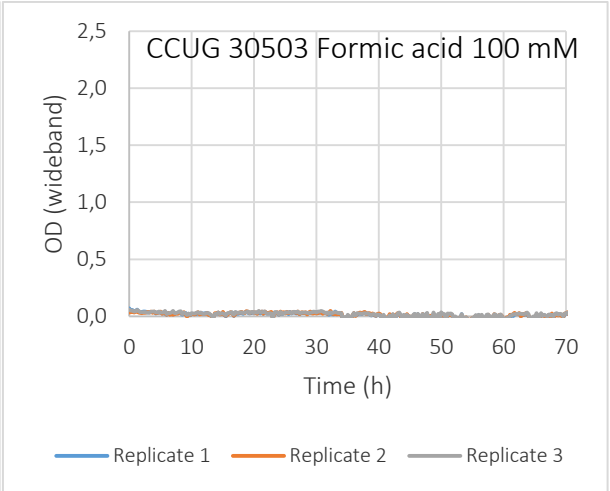

CCUG 30503 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

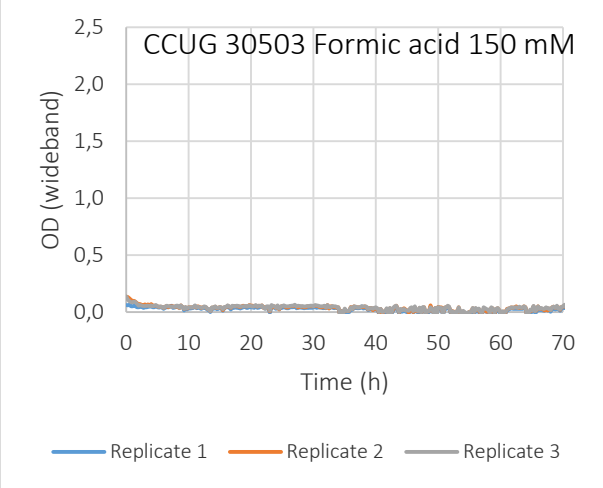

CCUG 30503 Propionic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

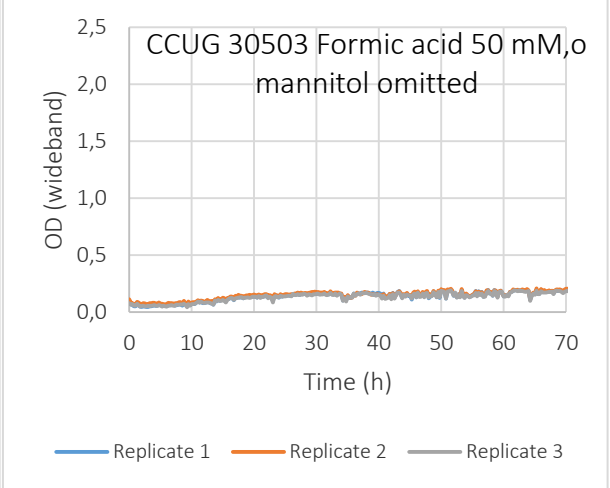

CCUG 30503 Propionic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

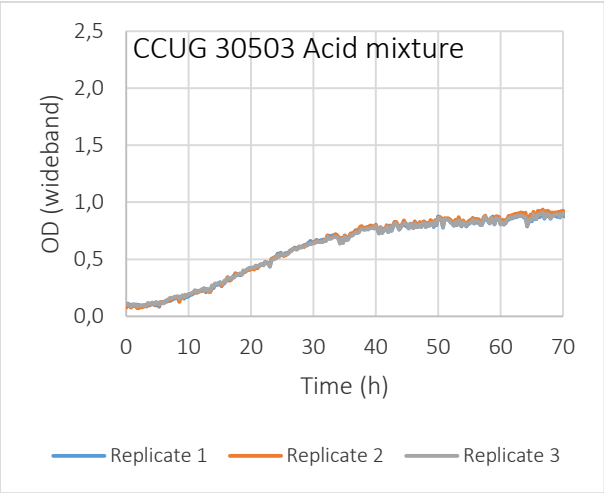

CCUG 30503 Propionic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

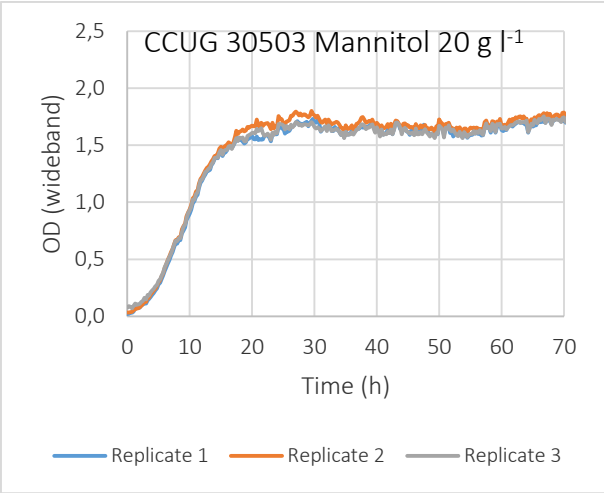

CCUG 30503 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

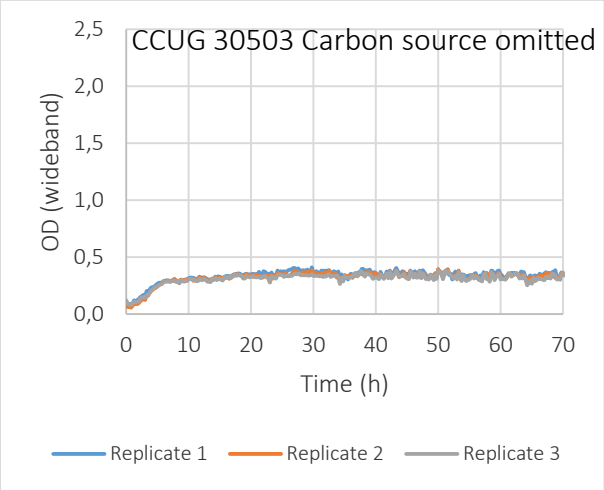

CCUG 30503 Formic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

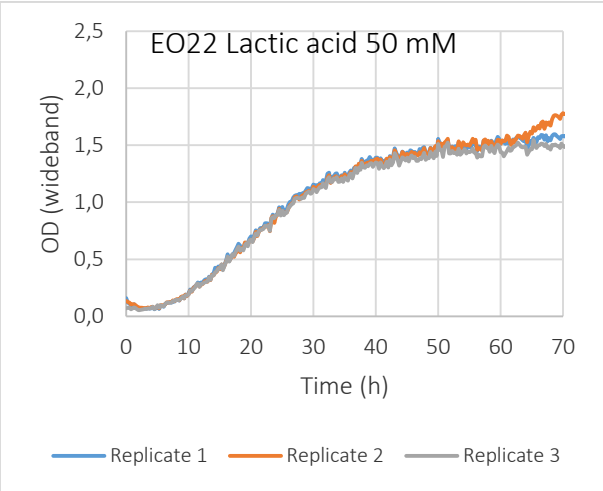

CCUG 30503 Formic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

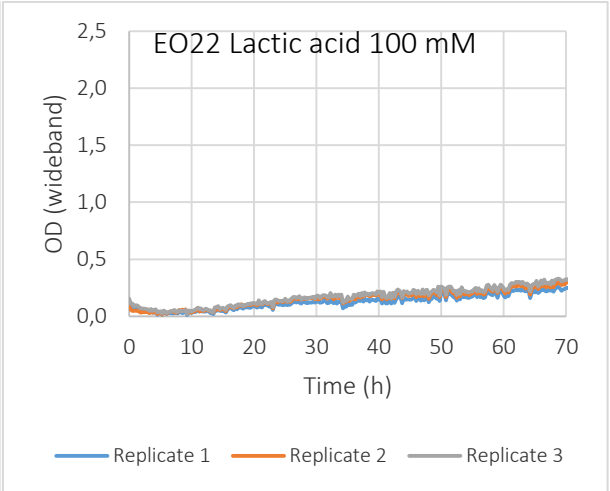

CCUG 30503 Formic acid 50 mM,o
mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

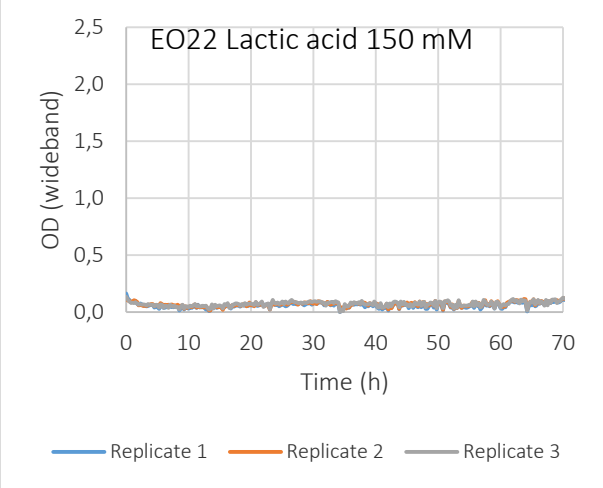

CCUG 30503 Acid mixture
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

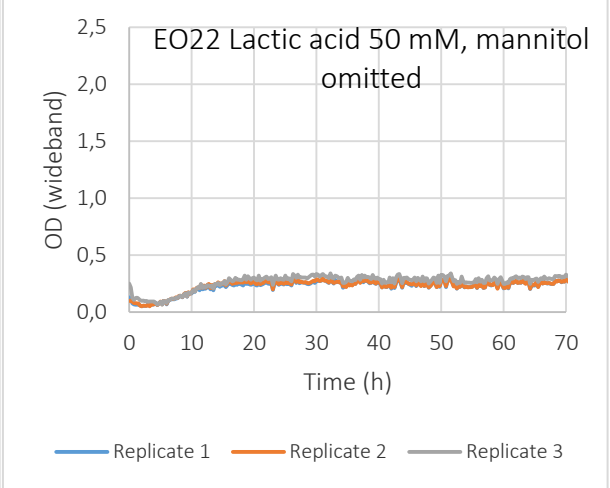

CCUG 30503 Mannitol 20 g l-1
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

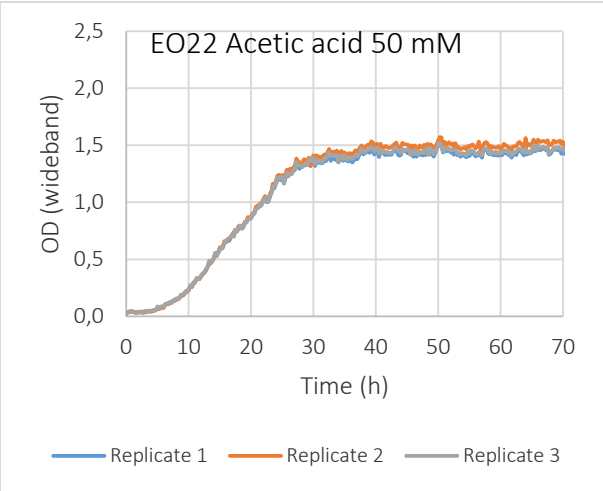

CCUG 30503 Carbon source omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

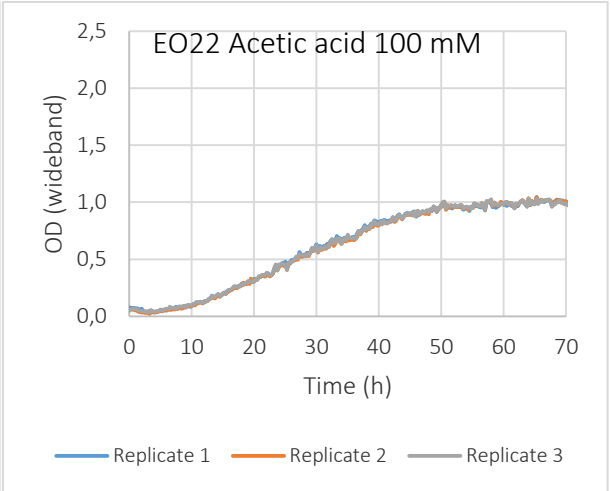

EO22 Lactic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

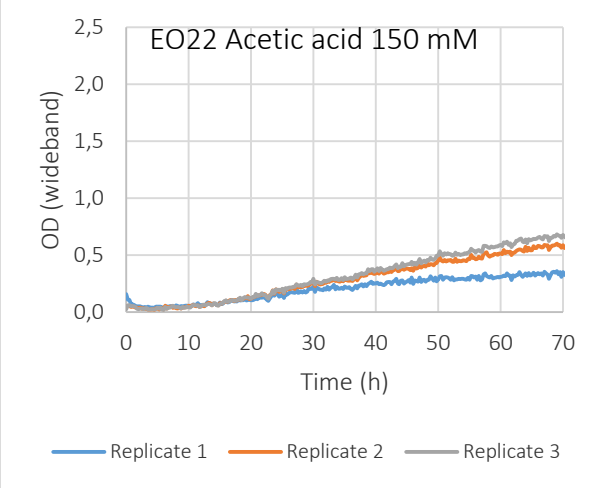

EO22 Lactic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

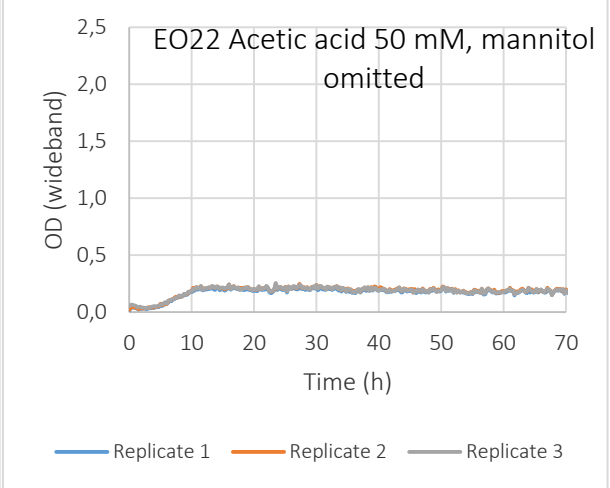

EO22 Lactic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

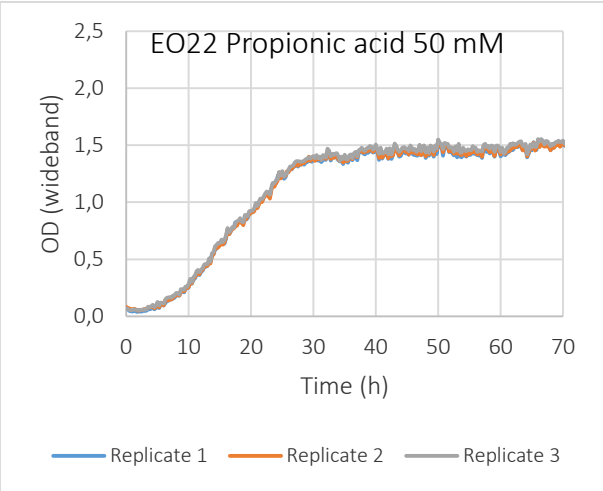

EO22 Lactic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

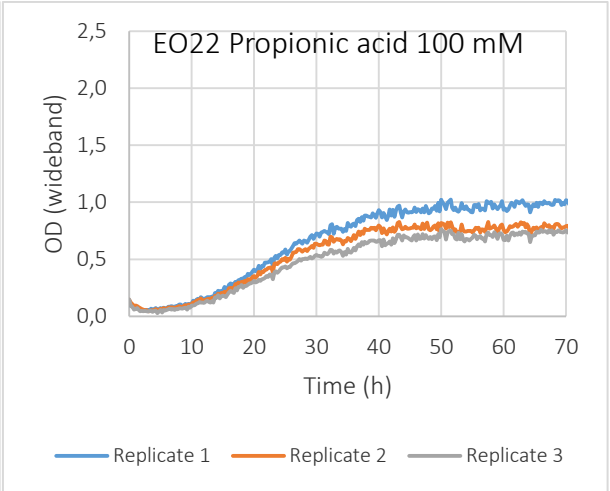

EO22 Acetic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

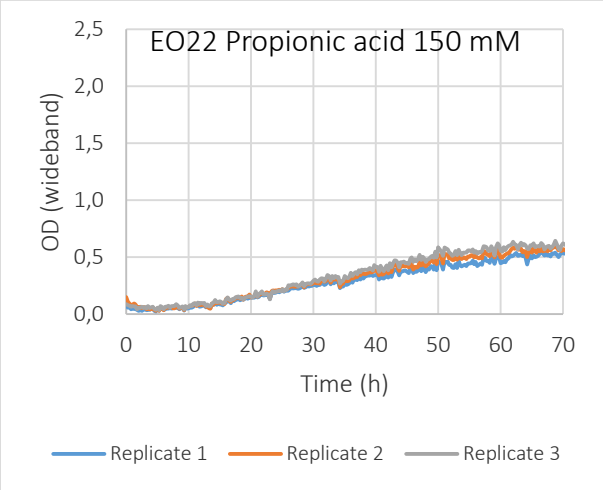

EO22 Acetic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

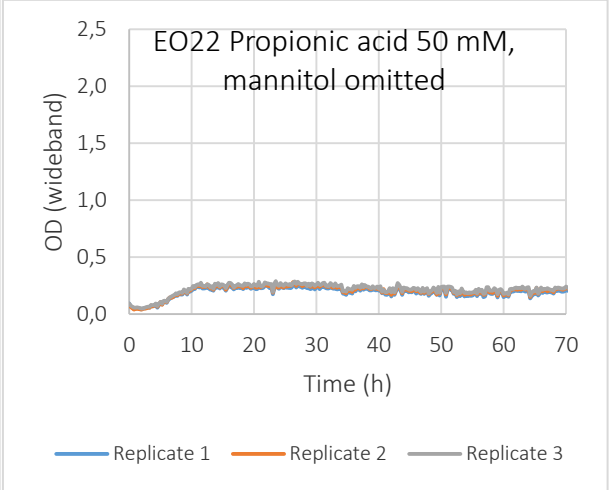

EO22 Acetic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

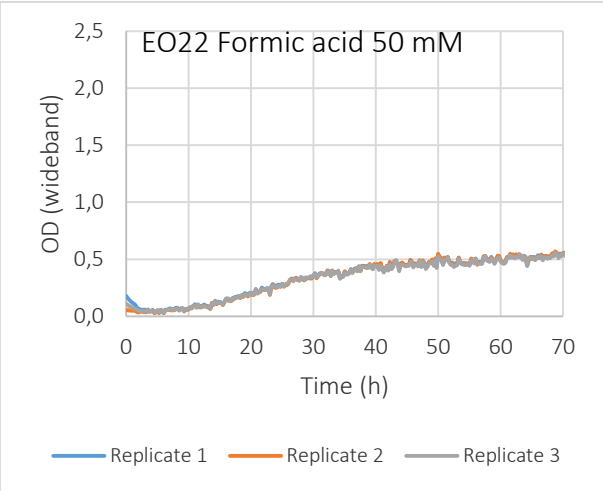

EO22 Acetic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

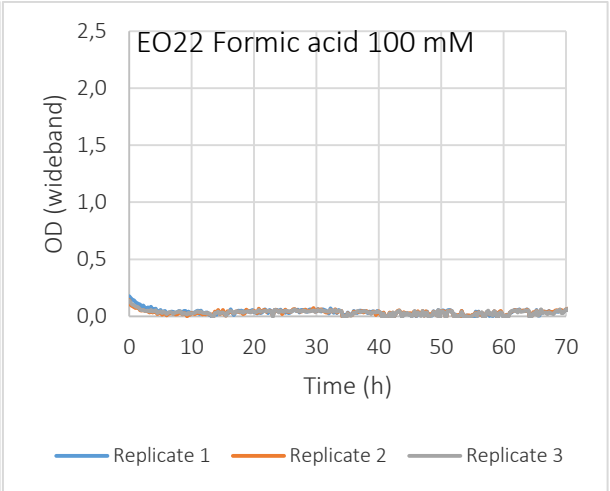

EO22 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

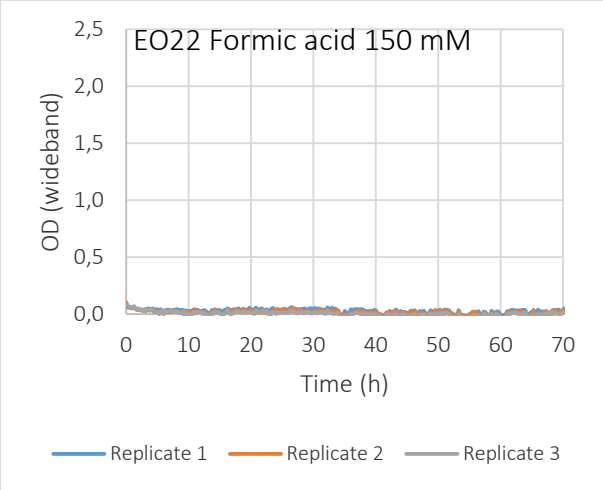

EO22 Propionic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

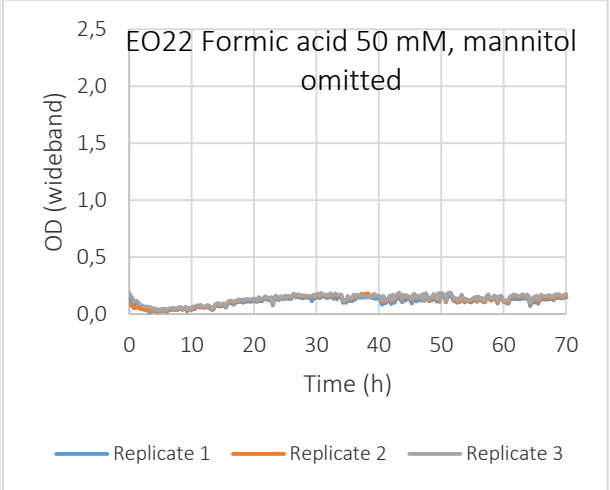

EO22 Propionic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

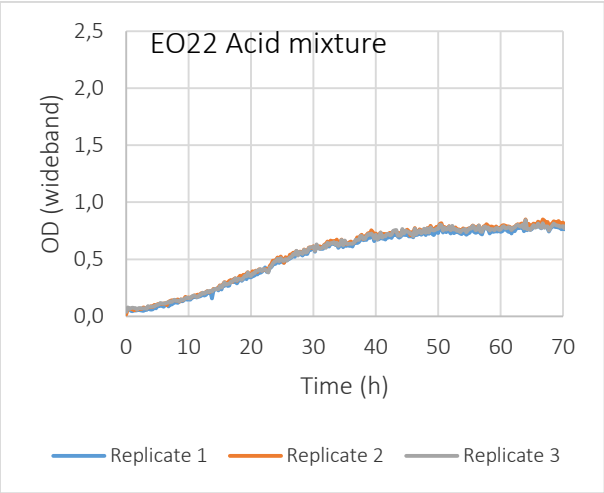

EO22 Propionic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

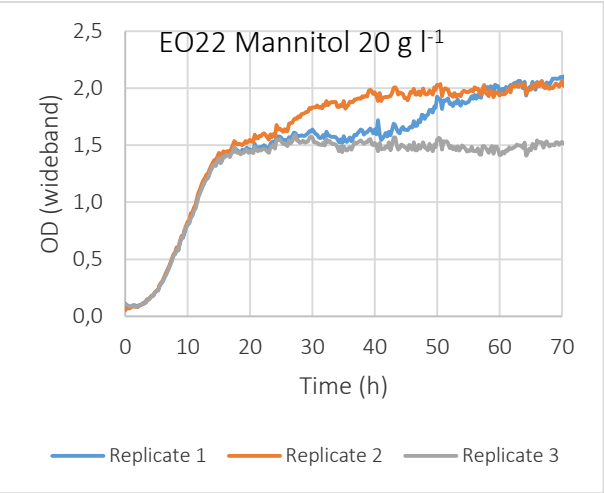

EO22 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

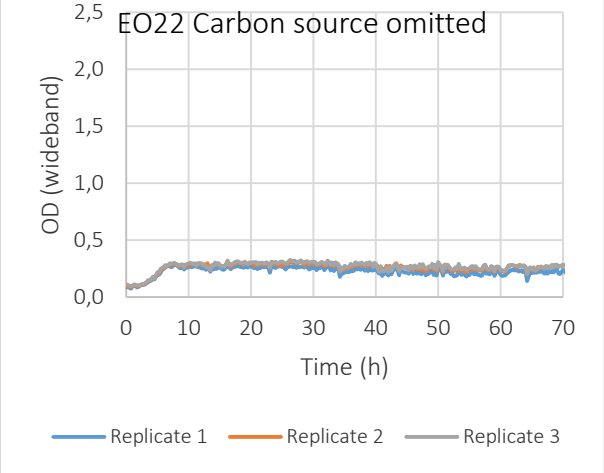

EO22 Formic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

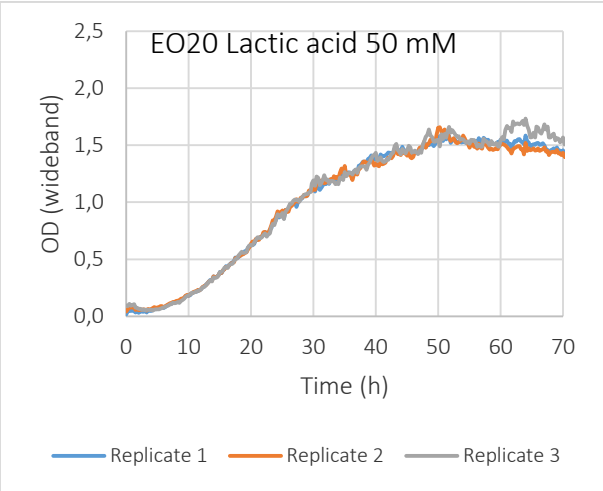

EO22 Formic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

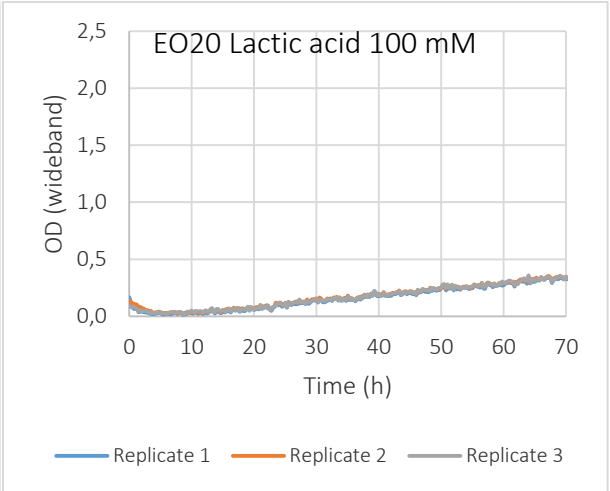

EO22 Formic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

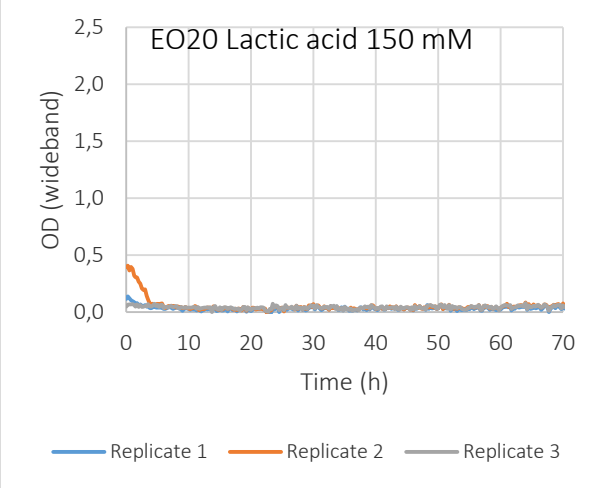

EO22 Acid mixture
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

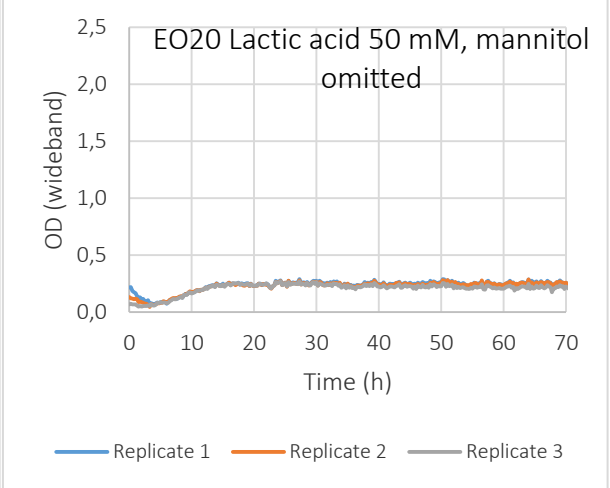

EO22 Mannitol 20 g l-1
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

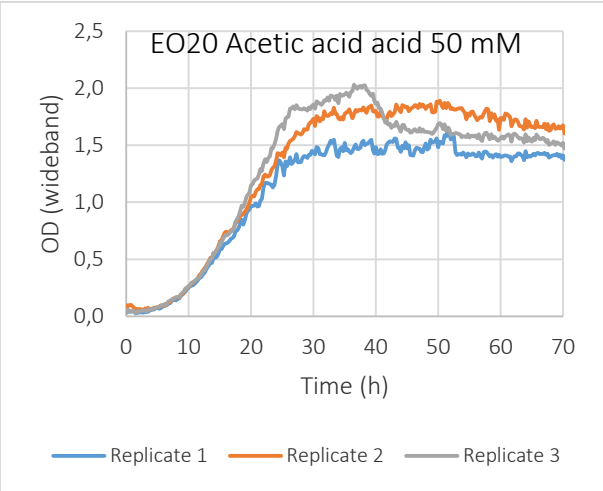

EO22 Carbon source omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

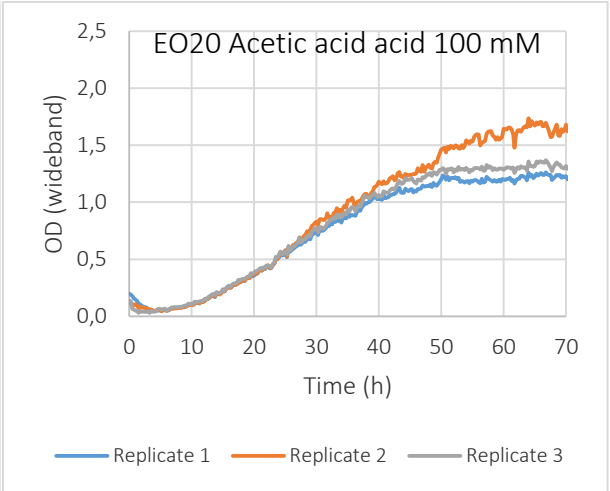

EO20 Lactic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

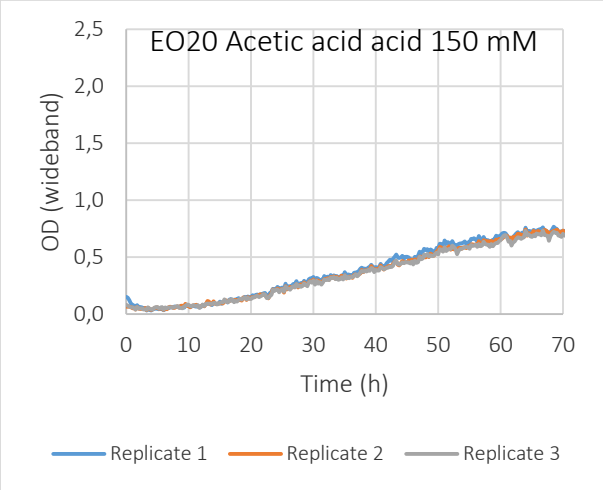

EO20 Lactic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

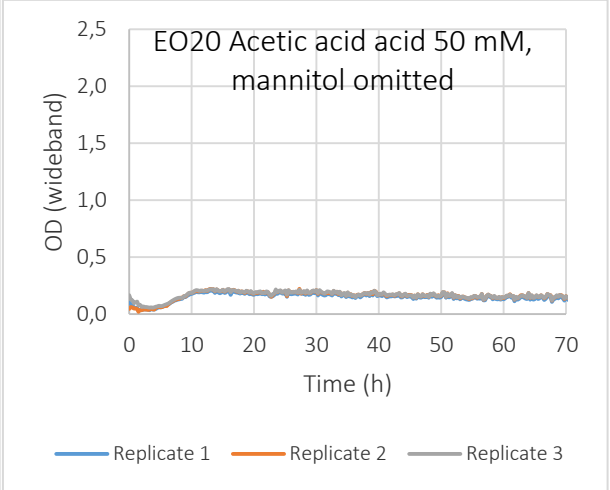

EO20 Lactic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

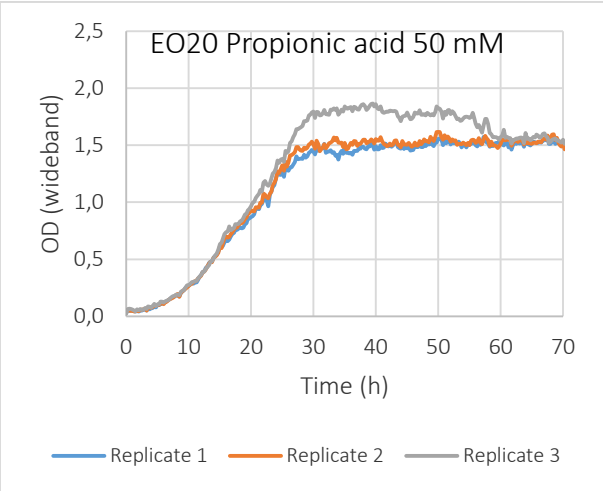

EO20 Lactic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

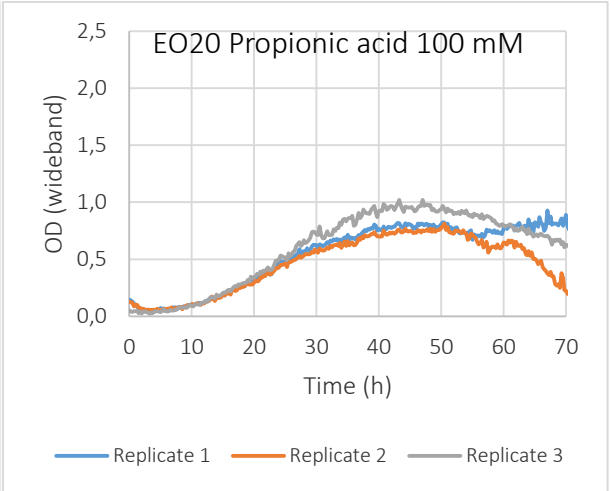

EO20 Acetic acid acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

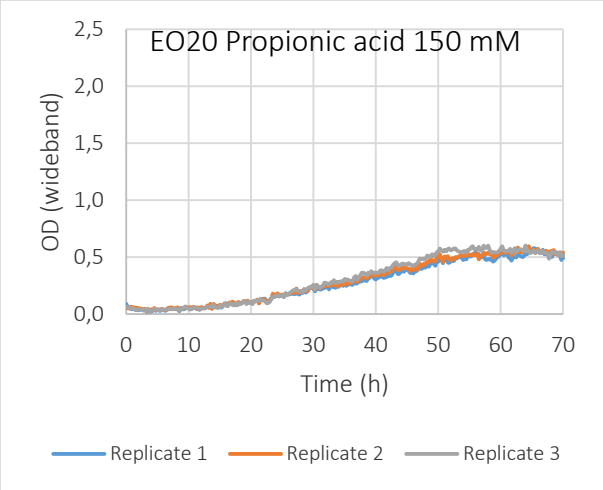

EO20 Acetic acid acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

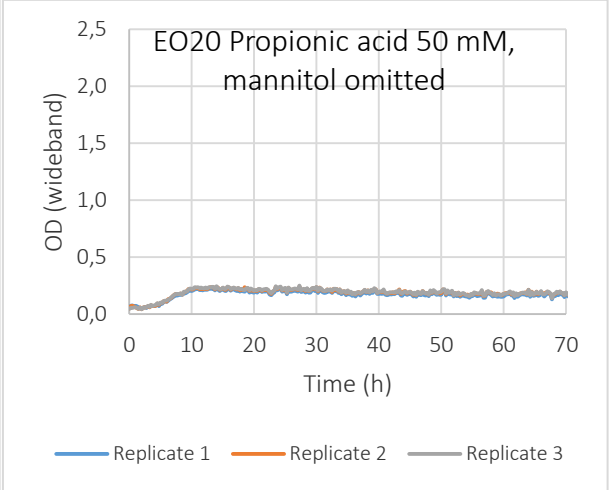

EO20 Acetic acid acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

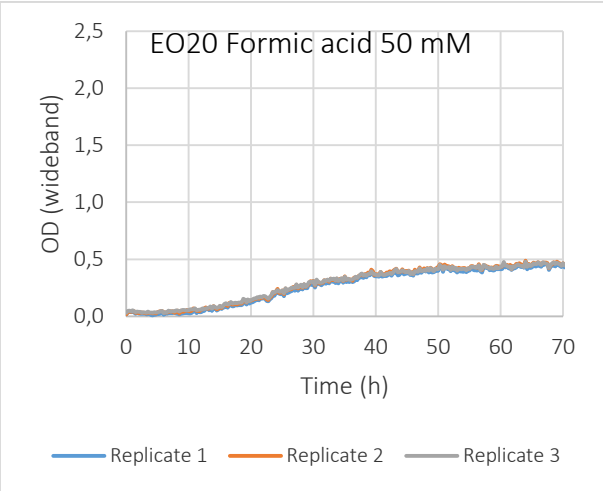

EO20 Acetic acid acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

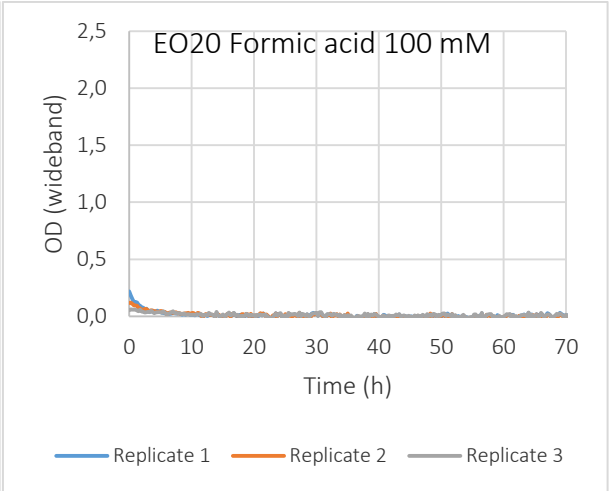

EO20 Propionic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

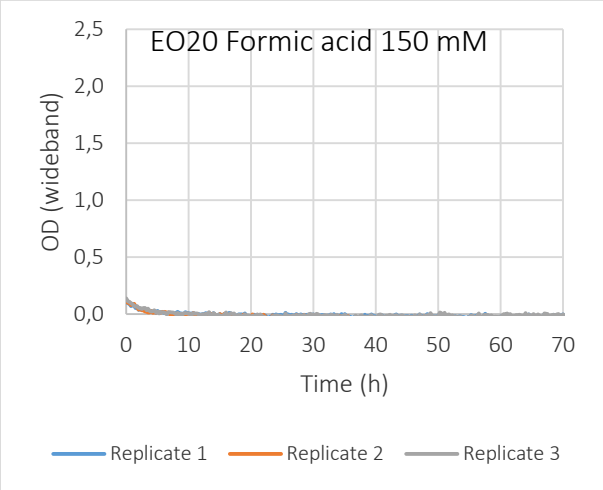

EO20 Propionic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

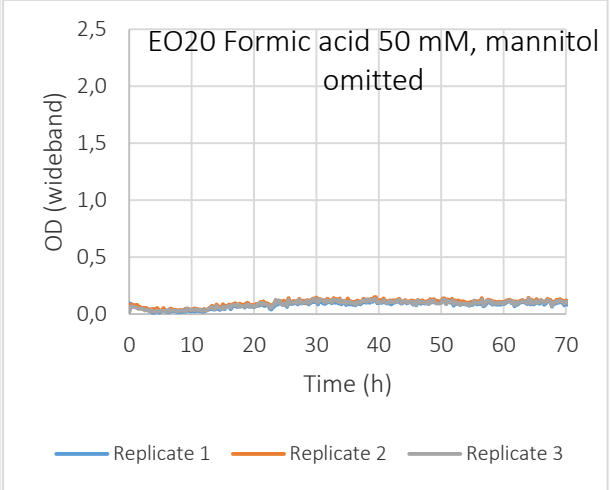

EO20 Propionic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

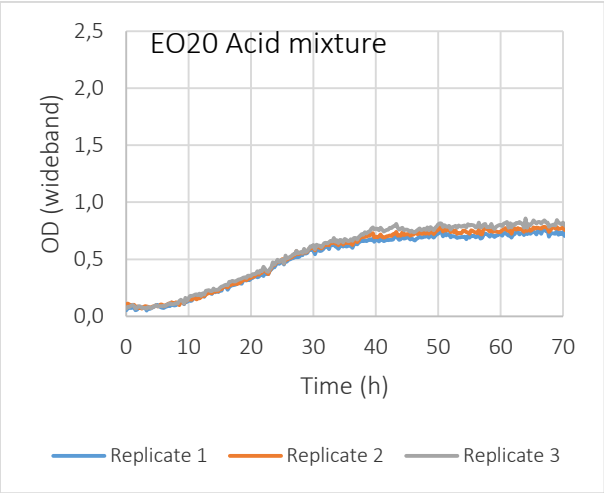

EO20 Propionic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

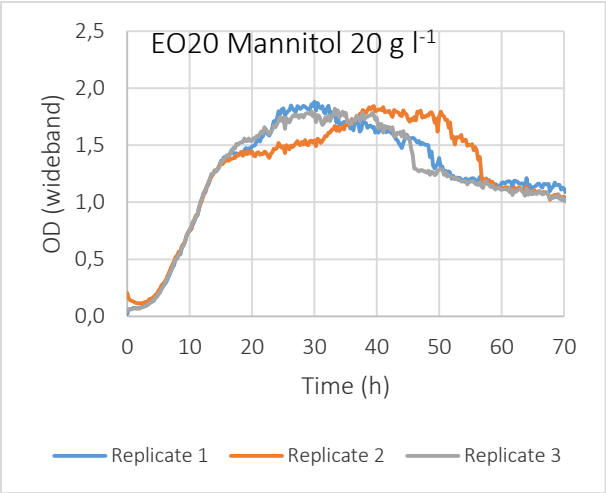

EO20 Formic acid 50 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3

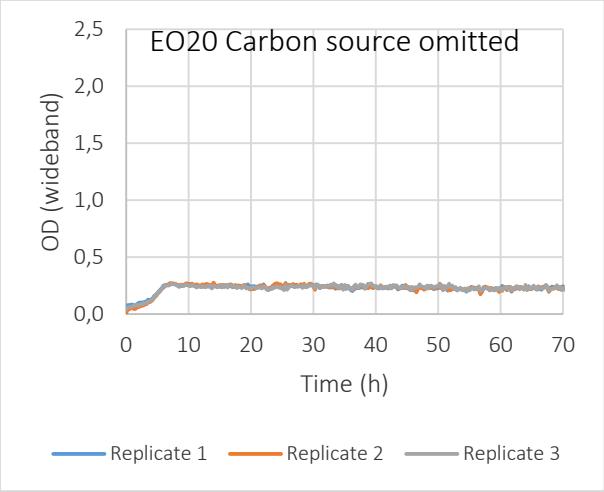

EO20 Formic acid 100 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO20 Formic acid 150 mM
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO20 Formic acid 50 mM, mannitol omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO20 Acid mixture
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO20 Mannitol 20 g l-1
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3


EO20 Carbon source omitted
OD (wideband)
2,5
2,0
1,5
1,0
0,5
0,0
0
10
20
30
40
50
60
70
Time (h)
Replicate 1
Replicate 2
Replicate 3