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Towards Sustainable Bioinoculants: A Fermentation Strategy for High Cell Density Cultivation of *Paraburkholderia* sp. SOS3, a Plant Growth-Promoting Bacterium Isolated in Queensland, Australia

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Abstract: *Paraburkholderia* sp. SOS3 is a plant growth-promoting bacterium (PGPB) that displays pleiotropic effects and has the potential to be applied at a large scale across several agronomically important crops. The use of SOS3 is a suitable option to reduce the use of chemical fertilisers. While the benefits of SOS3 have been demonstrated in vitro, its potential applications at large scale are limited due to low biomass yield in current batch culture systems. Here, we developed a strategy for high-cell density cultivation of SOS3 in instrumented bioreactors, moving from low-biomass yield in a complex medium to high-biomass yield in a semi-defined medium. We achieved a 40-fold increase in biomass production, achieving cell densities of up to 11 g/L ($OD_{600} = 40$). This result was achieved when SOS3 was cultivated using a fed-batch strategy. Biomass productivity, initially 0.02 g/L/h in batch cultures, was improved 12-fold, reaching 0.24 g/L/h during fed-batch cultures. The biomass yield was also improved 10-fold from 0.07 to 0.71 g_{biomass}/g_{solids}. Analysis of the fermentation profile of SOS3 indicated minimal production of by-products and accumulation of polyhydroxybutyrate (PHB) during the exponential growth phase associated with nitrogen limitation in the medium. By implementing proteomics analysis in fed-batch cultures, we identified the expression of four metabolic pathways associated with growth-promoting effects, which may be used as a qualitative parameter to guarantee the efficacy of SOS3 when used as a bioinoculant. Ultimately, we confirmed that the high-cell density cultures maintained their plant growth-promoting capacity when tested in sorghum and maize under glasshouse conditions.

Keywords: biofertiliser; plant growth-promoting bacteria; fed-batch cultivation; maize; sorghum

1. Introduction

The agriculture sector faces significant new challenges associated with climate change, resistance to biopesticides and soil degradation. An emerging solution towards sustainable agricultural practices is the use of cost-effective, environmentally friendly biofertilisers. Biofertilisers are a suitable alternative to chemical fertilisers and exploit native microorganisms present in the soil, known as plant growth-promoting bacteria (PGPB). Such products are termed 'bioinoculants', as they induce rhizospheric colonisation with the target bacterium rather than merely supplying nutrients to the soil as chemical fertilisers do.

The use of PGPB in sustainable agriculture has gained importance in the past decade due to their beneficial effects on soil and crop productivity. PGPB exhibit different mechanisms of action in their interactions with plants, leading to improved productivity through

various means. Those mechanisms can be direct (production of phytohormones, nitrogen fixation and phosphate solubilisation) or indirect (production of antibiotics, iron-sequestering siderophores, cell wall degrading enzymes and quorum quenching) [1,2]. PGPB can be classified based on their mode of action as biofertilisers, phytostimulators, biopesticides and bio-remediators. Biofertilisers are used to promote growth by supplying or increasing the availability of nutrients. Phytostimulators can regulate the concentration of growth regulators. Biopesticides allow plant growth by killing phytopathogenic agents, usually through the production of antibiotics. Bioremediators exhibit the ability to sequester heavy metals [3]. Most research in biofertilisers focuses on the understanding of the diversity, importance and mechanisms of soil PGPB and their beneficial role in agricultural productivity [3]. There are a wide range of bacterial species with plant growth-promoting effects (PGPE), including *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Serratia*, among others [4]. The mode of action of these PGPB varies according to the host plant. However, it is also influenced by several other biotic factors such as the soil composition, the developmental stage of the plant, the diversity of the associated microbial communities in the soil, and the climatic conditions. Those factors define the specificity of PGPB-plant interactions, providing a wide range of potential products. However, the ideal candidate for large scale production would be a PGPB that exhibits pleiotropic effects, covering more than one mode of action in more than one host plant.

Successful inoculants that have been reported to be effective biofertilisers have typically been cultivated in shake flasks. This production method presents several inconveniences, starting with the lack of tight control of growth parameters [5]. Recently, an increasing number of studies have aimed to improve biomass production of a particular PGPB. In most cases, liquid fermentation was studied, and the key focus was to optimise the medium composition. Interestingly, most studies report the use of complex, rich media, despite the issues that complex media introduces (expensive materials, batch to batch variation and little reproducibility) [6]. Reported media compositions have included different substrates such as glycerol [7], yeast extract [8], sweet potato wastewater [9], chicken manure [10] and whey. While it is clear that the use of these complex mixtures leads to higher biomass yields, their complexity and batch-to-batch variability make it difficult to generate a standardised product. In this regard, several efforts have been implemented to develop an optimised culture medium composition that yields high cell densities [6].

The market for PGPB inoculants commercially available is limited, representing less than 5% of the total chemical fertiliser market [11]. Amongst the available products, liquid formulations are preferred because they are easier to process and imply lower production costs. However, it has been reported that many current commercial products are low-quality bio inoculants. This is associated with low cell density in the product ($<10^7$ CFU/mL of product), the loss of PGPE due to the cultivation strategy employed [11], the short shelf life of the product, and even contamination with other species. Those inconsistencies harm the perception of the products and reduce the confidence of consumers. It is possible to develop a high-quality PGPB bioinoculant by selecting adequate fermentation processes and the smart formulation of the final product [12].

Recently, a PGPB isolate, *Paraburkholderia* sp. SOS3, was identified as a promising bioinoculant. Providing multiple benefits to plants, SOS3 has the potential to be implemented in several agronomically important crops such as rice, sugarcane, and pasture [13–15]. Still, the potential of SOS3 is restricted by limited cultivation strategies in bioreactors and poor growth in instrumented fermenters. These limitations can be overcome through the implementation of an efficient fermentation bioprocess. Here, we present a scale-up strategy for the cultivation of SOS3 at high cell density in a low-cost semi-defined medium. The bioprocess described here was validated through plant trials, demonstrating that the final product maintains PGPE and is an adequate candidate for large scale production.

2. Materials and Methods

2.1. Bacterial Strains

SOS3 bacterium (NCBI ID: 1926494) was provided by Sustainable Organic Solutions Pty. Ltd. (Brisbane, Australia).

2.2. Media Composition

R2A agar solid media (Difco) was used to grow SOS3 from glycerol stock. Five different liquid media were used for production: nutrient broth, or NB (8 g/L NB granules, Difco); minimal salts medium (MMS) (10 g/L mannitol, 1 g/L glucose, 0.5 g/L arabinose, 1 g/L yeast extract, 0.5 g/L K_2HPO_4 , 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.03 g/L Fe(EDTA)); and three MMS variants, namely arabinose-MMS (10 g/L arabinose, 1 g/L yeast extract, 0.5 g/L K_2HPO_4 , 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.03 g/L Fe(EDTA)); glucose-MMS (10 g/L glucose, 1 g/L yeast extract, 0.5 g/L K_2HPO_4 , 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.03 g/L Fe(EDTA)); and mannitol-MMS (10 g/L mannitol, 1 g/L yeast extract, 0.5 g/L K_2HPO_4 , 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L NaCl, 0.03 g/L Fe(EDTA)). Ammonium sulphate was added when indicated. The media for 1 L reactors were autoclaved at 121 °C for 20 min. Sugars and Fe(EDTA) were autoclaved separately and then added to the media. All chemicals were of analytical grade (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Fermentations in 1 L Bioreactor

Batch and fed-batch fermentations for biomass production were performed in Sartorius Biostat A bioreactors (Sartorius Stedim, Biostat B-Plus, Gottingen, Germany) equipped with a 1.3 L vessel and up to 1 L working volume. Complex nutrient broth (NB) and semi-defined media were used as indicated for each experiment. Liquid media and reactors were autoclaved at 121 °C for 20 min. One litre of the autoclaved medium was transferred to each reactor under aseptic conditions. Reactors were inoculated with cultures grown for no more than 16 h. Initial optical density at 600 nm (OD_{600}) was adjusted to be 0.1–0.2 units. The reactors were run at 30 °C. When indicated, pH was maintained by the addition of acid (2 M HCl) and base (2 M NaOH). When indicated, dissolved oxygen was maintained at 50% through a cascade control (stirring speed, 200–500 rpm; air flow rate, 0.5–3.0 vvm). For fed-batch fermentation, the initial volume was 500 mL. Fresh media were supplied at a constant flow rate (1 mL/min) using an external Masterflex peristaltic pump.

2.4. Assays for Plant-Growth Promotion: Seed Germination and Vigour Index

Sorghum (*Sorghum bicolor* L.) and Maize (*Zea mays* L.) Seeds Treatment

Seeds were coated with their specified treatment in a Petri dish and then left to dry (sorghum, 10 µL per seed; maize, 20 µL per seed). A sheet of sterile filter paper was placed into a Petri dish and soaked with sterile water (sorghum, 2 mL; maize, 3 mL). Seeds were evenly spread onto the paper using sterile forceps (sorghum, 20 seeds; maize, 10 seeds). Another piece of filter paper was placed on top and soaked with 2 mL sterile water. This process was repeated for all of the required treatments and their replicates. Each treatment consisted of four replicate Petri dishes. The plates were closed, covered in plastic wrap, and then incubated at 30 °C. Before harvest, the number of seeds that had germinated in each plate was counted. After five days, roots and shoots were manually separated from the seed and dried in an oven at 60 °C for three days. The dry weights were then measured. The effects of the microbial inoculant on germination was measured using the vigour index:

$$\text{Vigour index} = \text{Germination\%} \times \text{Seedling dry weight}$$

2.5. Glasshouse Trials—Full Term Growth

Sterilised zeolite (1–5 mm in size) was used to immobilise SOS3 cells recovered from fermentations [13]. Cells grown in either MMS or glucose-MMS medium were immobilised onto zeolite at a 1 to 3 ratio (mL per g) and dried at 30 °C for 16 h. The number of viable CFU per gram of zeolite was determined by resuspending 1 g of coated zeolite in 10 mL of

sterile water. Serial dilutions were made, and 1 mL of each was plated in R2A agar plates. Plates were grown for 48 h at 30 °C, and the number of CFU was calculated.

Ninety pots (125 mm) were filled with University of California (UC) potting mix C (0.33 m³ sand, 0.17 m³ bale peat, 4.15 kg fertiliser mix) [16]. The fertiliser mix (10 kg dolomite, 6 kg blood and bone, 6 kg superphosphate, 6 kg hydrated lime, 3 kg gypsum, 1.2 kg Micromax, 1 kg potassium nitrate, 0.5 kg potassium sulphate) was prepared separately and then added. All pots received an additional 1.8 g (per pot) of Osmocote fertiliser, a standard application as instructed by the company (Osmocote Exact 3M, Scotts. Evergreen Garden Care Australia, New South Wales, Australia). A small hole was pressed into the pot, and two maize seeds were added along with 1 g of zeolite with or without SOS3 that was previously cultured in MMS or Glucose-MMS. Each treatment consisted of 30 replicate pots. This hole was filled in, and then the pots were watered and left to grow in a glasshouse for 22 days. The pots were watered twice daily. The number of emerged seedlings for each pot was counted at various intervals, and pots with two emerged seedlings had one transplanted into a pot with no emerged seedlings of the same treatment group. Once there were no more empty pots for that treatment group, the additionally emerged seedlings were discarded. Before the harvest, the height measurements were taken from the top of the soil to the growth point of the newest leaf, and the number of leaves was counted. The pots were then upturned, and the soil shaken off the roots. The roots were then washed gently to ensure that as little of the root mass as possible was lost during cleaning.

2.6. Polyhydroxybutyrate (PHB) Extraction

PHB content in the cells may affect the shelf-life of inoculum or their in-field competitive efficacy. We measured PHB content by taking 1 mL of SOS3 culture. The sample was transferred into a clean Eppendorf tube and spun down for 3 min at 20,000 g. The supernatant was removed and the pellet frozen at −20 °C until processing. Pellets were thawed and washed twice with 1 mL of Milli-Q water. Then, 1 mL of 18 M H₂SO₄ was added to each tube before vortexing to dissolve the pellet. The cell suspension was transferred to a 10 mL glass vial and incubated in a 90 °C water bath for 1 h. Every 20 min, the vials were removed and vortexed to mix. The vials were cooled down, and 4 mL of 7 mM H₂SO₄ was added to each vial. The solution was then filtered using a 0.22 µm PES filter. Samples were diluted 1:10 with 7 mM H₂SO₄, and 50 µL of the diluted samples were mixed with 50 µL of 0.2 g/L adipic acid solution. The samples were sent to the Queensland node of Metabolomics Australia for crotonic acid analysis [17].

2.7. Proteomics Analysis

Liquid culture samples of SOS3 were centrifuged for 3 min at 20,000 g. The supernatant was removed, and the pellet was stored at −20 °C for later processing. The frozen pellet was thawed and washed with 1 mL of PBS buffer. The pellet was then resuspended in 1 mL of lysis buffer (5% SDS, 100 mM TEAB buffer, 100 mM DTT; pH 7.55) and transferred to a 2 mL screwcap microcentrifuge tube prefilled with 0.1 mm glass beads (10% of the volume of the tube was filled with glass beads). Cells were disrupted using a Bead Ruptor 24 elite homogeniser (Omni International, Kennesaw, GA, USA) using the following cycle setting: speed, 6 m/s, 30 s on, 45 s off, 20 cycles. Samples were centrifuged at 20,000 g for 10 min, and the supernatant was recovered. Protein digestion was performed in S-trap mini-columns according to the manufacturer's instruction using Promega trypsin. The recovered peptides were eluted from the column and the residual acetonitrile was removed by vacuum centrifugation (Eppendorf, Hamburg, Germany). Peptides were resuspended in 0.1% formic acid for LC-MS/MS analysis. Peptide identification was performed using an UltiMate 3000 RSLCnano LC-MS/MS system (ThermoFisher, Bremen, Germany). The LC () was equipped with a Waters Acquity 1.7 µm CSH C18 130Å, 100 mm × 300 µm column (Waters, MA, USA) operated at 40 °C with a 4–76% acetonitrile gradient in 0.1% formic acid for 60 min at a flow rate of 2.0 µL/min. Eluted peptides were directly analysed on a Q Exactive HF mass spectrometer equipped with a Nanoflex interface. Gas pressures were

set according to the manufacturer recommendation, and the ion spray source was operated at 2100 V. Protein Discoverer 2.4.1.15 software (ThermoFisher) was used to identify the proteins. The mass tolerance values for precursor ions and fragment ions were set to 10 ppm and 0.05 Da, respectively. The genome annotation for SOS3 was taken from NCBI (GenBank assembly accession: GCA_001922345.1)

2.8. HPLC Analysis

Supernatant samples were retrieved from the freezer and left to thaw. A total of 400 µL of each sample was transferred into an HPLC vial. The Queensland node of Metabolomics Australia performed the sample analysis. Organic acids, carbohydrates, and alcohols were quantified by ion-exclusion chromatography using an Agilent 1200 HPLC system and an Agilent Hi-Plex H column (300 × 7.7 mm, PL1170-6830) with a guard column (SecurityGuard Carbo-H, Phenomenex PN: AJO-4490) as previously reported [18].

3. Results

3.1. Growth of SOS3 in Complex Media

Previously, SOS3 was cultivated in the nutrient broth medium [13]. Cultures were grown for 16 h in flasks, reaching cell densities of less than 1.5 OD₆₀₀. Few kinetic data were reported, and the only reported parameters were initial pH (7.0) and temperature (30 °C). An initial characterisation of SOS3 growth in a complex medium (NB) was performed in 1 L Sartorius Biostat A bioreactors (Figure 1). We first scaled-up the growth of SOS3 from flasks to 1 L reactors (Figure 1a), providing only temperature control at 30 °C and maintaining a constant airflow at 0.5 vvm (reference culture). Under these conditions, the growth rate was 0.33 h⁻¹. The culture reached the stationary phase by 20 h post-inoculation, as indicated by the increase in DO. The surge in pH from 6.5 to 8.8 can be attributed to using a complex medium comprised mostly of peptides. As peptides are consumed, ammonia (NH₃⁺) is released, increasing the pH in the culture. Aeration was maintained and pH control was set at 6.5 (Figure 1b) and 7.0 (Figure 1c), and there was a nearly 50% increase in the final OD₆₀₀ at 20 h post-inoculation compared to the reference culture; no significant changes in growth rate were observed (pH 6.5, 0.34 h⁻¹; pH 7.0, 0.33 h⁻¹). No growth was detected when pH was controlled at 7.5, showing that high initial pH led to growth inhibition at low cell densities.

Next, we maintained DO at 50% using a cascade controller. For both pH 6.5 and 7.0, the cultures reached the exponential phase faster, producing up to 2.8 OD₆₀₀ units by 10 h post-inoculation. Growth rates were slightly higher when DO was kept at 50%; however, there was no significant difference across pH (pH 6.5, 0.36 h⁻¹; pH 7.0, 0.35 h⁻¹). For both conditions, cell density decreased slightly after reaching the highest value.

On average, a value of 1 OD₆₀₀ corresponded to 0.29 g/L of g dry cell weight (DCW) for SOS3 cultures. When considering the final biomass yields, it is clear that the process is inefficient. In the reference cultures, we obtained 0.07 gDCW per g of solids (g_{solids}, which refers to the mass of solids in the growth medium and accounts for components such as sugars, yeast extract) while for the cultures where pH and DO were controlled, the final yields were 0.11 and 0.12 gDCW/g_{solids} for pH 6.5 and 7.0, respectively. For further experiments, we kept pH and DO cascade controls at 7.0 and 50%, respectively.

We aimed to improve biomass production by extending the exponential growth phase for 8 h by implementing a fed-batch stage (Figure 2). We set a constant feeding rate (F) of 1 mL/min and evaluated biomass production when a 2× and 4× concentrated NB medium was fed. The feed started when the cultures reached the mid-exponential phase (13 h after inoculation). The constant feed rate allowed the extension of exponential growth for 8 h before reaching a plateau. By the end of the fed-batch stage, biomass remained constant up to 60 h. Compared to the reference batch culture (Figure 1), the cell density reached by 20 h post-inoculation in the fed-batch cultures increased nearly 50% using the 2× concentrated feed and nearly 200% using the 4× concentrated medium. However, we observed no improvement in the biomass yield, obtaining around 0.10 gDCW/g_{solids} in both conditions.

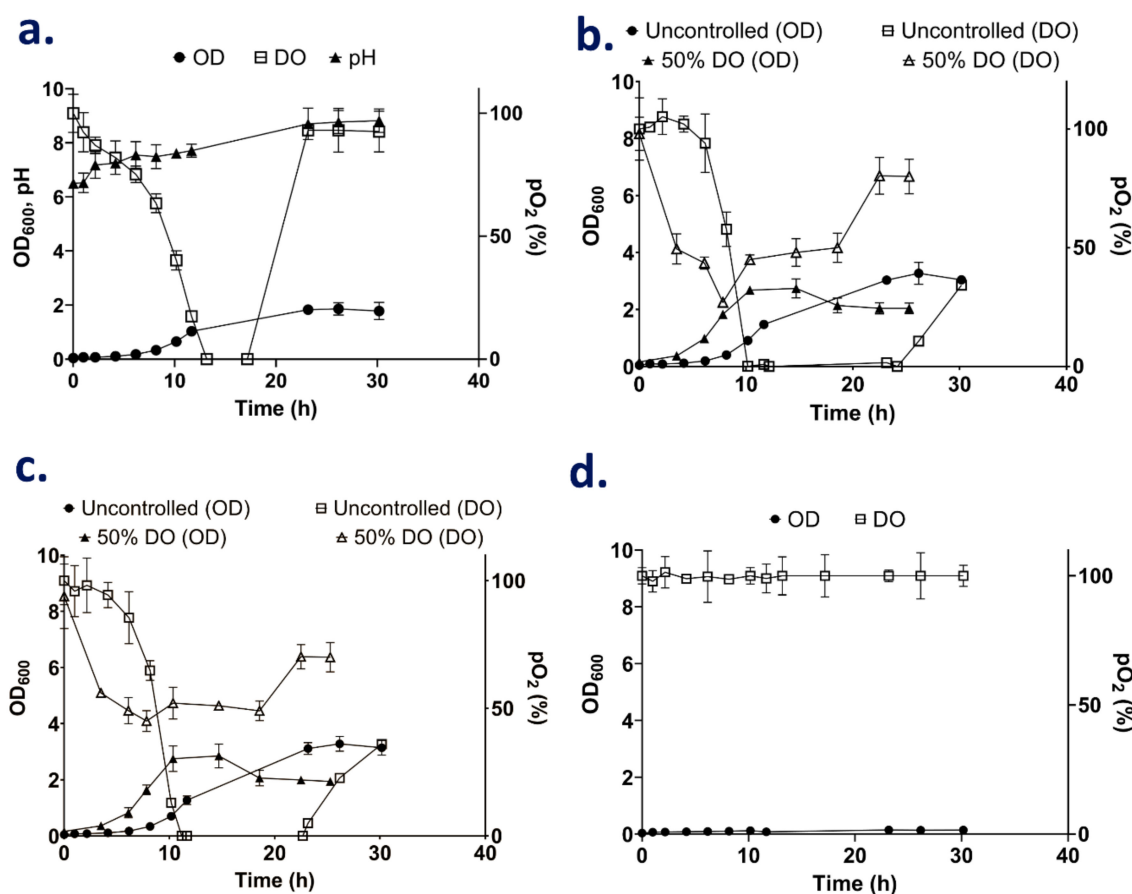


Figure 1. Fermentation profiles of SOS3 batch cultures in Biostat A bioreactors. (a) Reference fermentation; no pH control; no DO control. (b) pH 6.5. (c) pH 7.0. (d) pH 7.5. Data correspond to the average of two replicates.

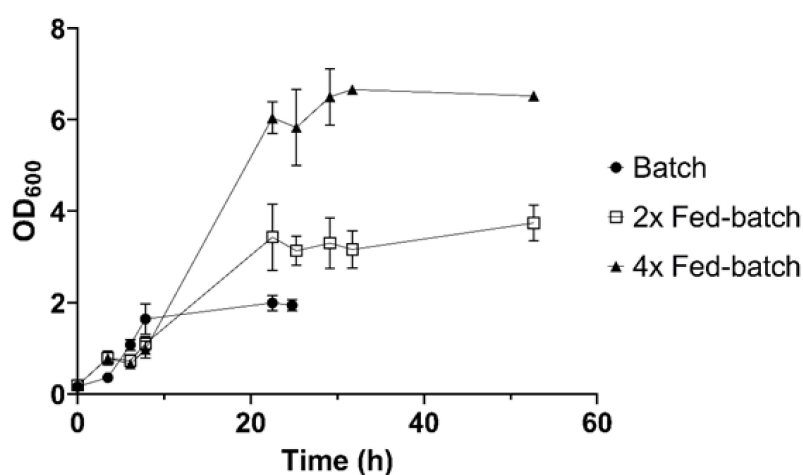


Figure 2. Fed batch cultivation of SOS3 in complex nutrient broth (NB) media. Data correspond to the average of two replicates.

3.2. The Use of Semi-Defined Media Increases Final Biomass Yield in Batch Cultivation

The use of complex media for large-scale production can lead to variations in productivity due to batch-to-batch variability of the raw materials [19,20]. We evaluated the growth of SOS3 in mannitol minimal salts medium (MMS), a semi-defined medium containing 0.1% yeast extract. The potential for SOS3 to grow on MMS medium was initially assessed in shake flasks (Figure 3a). MMS contains mannitol, glucose and arabi-

nose as carbon sources; however, we also evaluated three variations of MMS media, with each utilising only one of these carbon sources. While the growth rates of SOS3 in the four semi-defined media variations were slower (MMS, 0.28 h^{-1} ; glucose-MMS, 0.24 h^{-1} ; mannitol-MMS, 0.24 h^{-1} ; arabinose-MMS, 0.25 h^{-1}) than in NB medium (0.32 h^{-1}), the final biomass titre increased more than three-fold. In NB medium, SOS3 cultures reached the highest OD_{600} by 10 h post-inoculation, whereas cultures grown on MMS exhibited an extended growth phase up to 30 h post-inoculation. In the MMS variants, the cultures reached the stationary phase by 25 h post-inoculation. The biomass concentration obtained with Mannitol-MMS and Glucose-MMS was similar ($\text{OD}_{600} = 5.5$), while cultures grown in Arabinose-MMS only reached $\text{OD}_{600} = 4.8$. Given the complexity of the media, we calculated yields by considering the total amount of soluble solids present in the media. Biomass yields for those cultures increased slightly, obtaining $0.15 \text{ gDCW/g}_{\text{solids}}$ in mannitol-MMS, $0.14 \text{ gDCW/g}_{\text{solids}}$ in glucose-MMS, and $0.13 \text{ gDCW/g}_{\text{solids}}$ in arabinose-MMS. These results suggest that using MMS media leads to higher cell densities and could provide more efficient production, opening up avenues for large-scale production. In this regard, arabinose ($0.625/\text{kg}$) [21] implies a higher cost compared to glucose (USD $0.36/\text{kg}$) [22] when used in combination with mannitol (USD $3\text{--}7/\text{kg}$) [23].

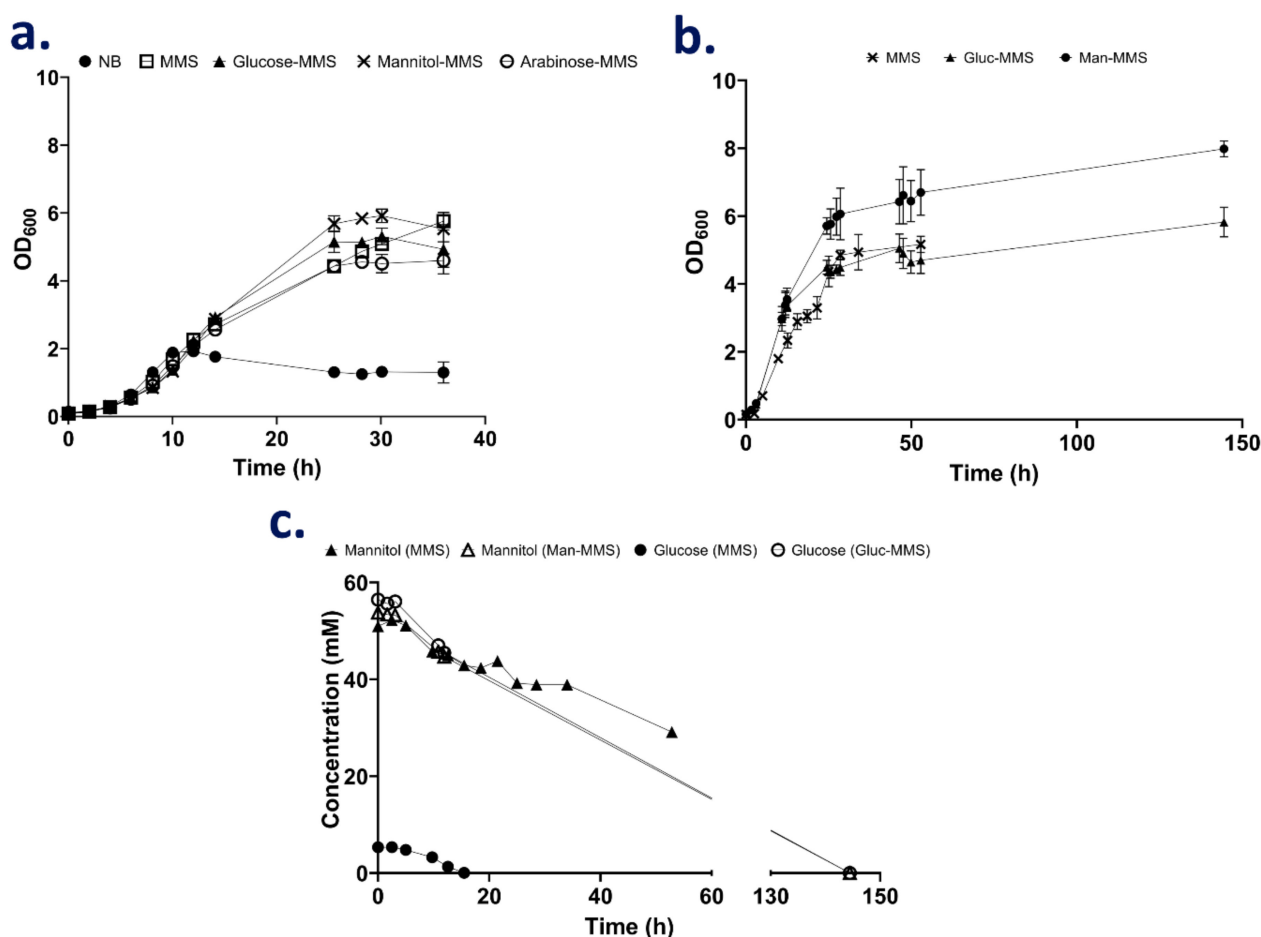


Figure 3. Batch cultivation of SOS3 in semi-defined media. (a) Growth curves in Shake flasks. (b) Growth curves in instrumented bioreactors. (c) Substrate consumption in instrumented bioreactors. pH and dissolved oxygen were controlled at 7.0 and 50%, respectively. Data correspond to the average of two replicates.

We used MMS, mannitol-MMS and glucose-MMS media to evaluate the performance of SOS3 in 1 L bioreactors (Figure 3b). For all three conditions, growth rates were similar (0.31 h^{-1}). Cultures grown on single-carbon source media reached OD_{600} values up to 6 units after 150 h, while cultures grown on MMS media reached more than 8 OD_{600} units. The substrate consumption profile in MMS medium shows how SOS3 assimilates mannitol and glucose simultaneously. Glucose was consumed during the first 20 h after inoculation, while only 12 mM of mannitol was consumed. Cell growth was maintained up to 30 h post-inoculation before reaching the stationary phase. At this point, one third of the initial mannitol was still present. Sixty hours after inoculation, biomass remained constant, while mannitol consumption continued. Mannitol was entirely consumed by 150 h post-inoculation, and there was a slight increase in biomass. Similar behaviour was observed for the glucose-MMS and mannitol-MMS media, suggesting a nutrient limitation was preventing the remaining carbon sources from being utilised for further growth. Overall, the use of semi-defined media leads to higher final cell densities in SOS3 cultures. The best results were obtained in MMS, with a 4-fold increase compared to batch cultures and similar results in fed-batch cultivation. Cultures grown on glucose-MMS and mannitol-MMS had similar biomass yields of 0.14 and $0.13 \text{ gDCW/g}_{\text{solids}}$. The highest yield was obtained using MMS medium, reaching up to $0.15 \text{ gDCW/g}_{\text{solids}}$.

3.3. Fed-Batch Strategy for High Cell Density Cultures

Following the improved yields obtained during fed-batch cultures on complex media, we implemented a similar strategy to increase the biomass production in SOS3 (Figure 4). We compared three different conditions depending on the media used during the batch and fed-batch stages: MMS medium during the batch stage and fresh MMS medium during the fed-batch (MMS + MMS); MMS medium during the batch stage and fresh Glucose-MMS medium during the fed-batch (MMS + G-MMS); and G-MMS medium during the batch stage and fresh G-MMS during the fed-batch. The concentration of the components in the fresh media was doubled for all feeds. All three conditions presented similar growth profiles during the batch stage. Feeding started at 13 h post-inoculation at a constant rate (1 mL/min) and lasted for 8 h. There were no noticeable differences between the three cultures during the fed-batch stage either. There was no difference in the final biomass concentration, reaching 14 OD_{600} units by 40 h after inoculation. Cell density was maintained up to 60 h post-inoculation. Accumulation of glucose and mannitol occurred during the fed-batch stage, reaching up to 87 mM by the end of the feeding stage. The carbon sources were then consumed steadily for up to 130 h post-inoculation, and no changes in biomass were observed. The accumulation of glucose and the little variation in growth profile across the three conditions tested suggest that biomass production could be limited by the availability of nitrogen supplied as yeast extract in these carbon-rich growth environments.

The change to fed-batch operation improved the biomass production yield significantly. At 60 h post-inoculation, MMS + MMS cultures reached a yield of $0.40 \text{ gDCW/g}_{\text{solids}}$. In MMS + G-MMS and G-MMS + G-MMS cultures, the estimated yields were $0.59 \text{ gDCW/g}_{\text{solids}}$ and $0.71 \text{ gDCW/g}_{\text{solids}}$, respectively. However, the variation between replicates might indicate no difference across treatments despite the observed trend. All cultures reached stationary phase by 60 h post-inoculation, and OD_{600} remained unchanged around 15 OD_{600} . The productivity for all three conditions averaged $0.08 \text{ g}_{\text{biomass}}/\text{L/h}$.

For the fed-batch experiments, the total amount of media components was increased 1.5-fold, considering the initial volume (0.5 L) at $1\times$ concentration and the $2\times$ concentration of the feed (0.5 L). From Figure 3, the cultures reached the stationary phase when sugars were still available. Thus, carbon source limitation can be discarded. Similarly, nearly two thirds of the biomass produced in the fed-batch cultures is produced while the fresh medium is supplied.

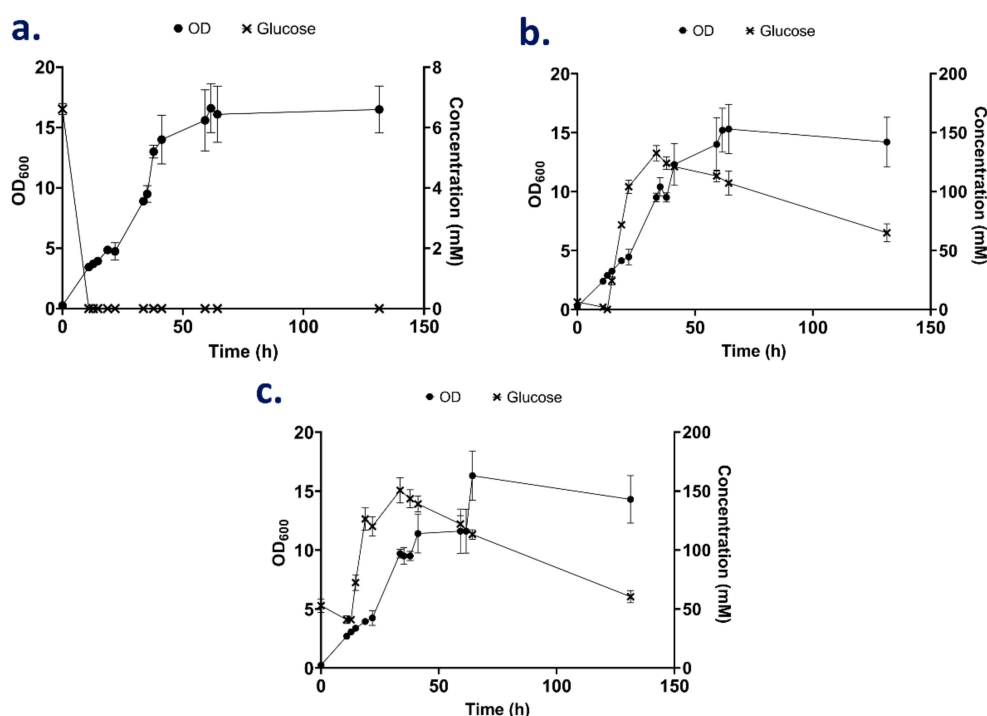


Figure 4. Fed-batch cultivation of SOS3 in semi defined media. (a) MMS + MMS. (b) MM + G-MMS. (c) G-MMS + G-MMS. Data correspond to the average of two replicates.

3.3.1. The Effect of Nitrogen Supplementation during High Cell Cultivation PGPBs Are Complex

We observed the accumulation of glucose and mannitol during fed-batch in the high cell density cultures (Figure 5a,b). We suspected the cultures were subjected to limitation by nitrogen. To avoid this phenomenon during fed-batch cultivation, we supplemented the feed medium (2× G-MMS) with increased ammonium sulphate (AS) concentrations (2, 4, 12, and 20 g/L). The extra nitrogen increased the biomass concentration considerably by the end of the fed-batch stage. Cultures supplemented with 12 and 20 g/L reached almost four times the OD₆₀₀ obtained in the cultures where no extra nitrogen was supplied. While biomass yield averaged about 0.52 gDCW/g_{solids} in the four conditions, productivity nearly doubled from 0.13 (AS, 2 g/L) to 0.24 g_{biomass}/L/h (AS 20 g/L). Cultures with added nitrogen also displayed faster glucose consumption. Interestingly, we observed a decrease in the final biomass after the fed-batch in the cultures supplemented with AS.

3.4. Plant Growth-Promoting Effects from High-Cell Density Cultures

3.4.1. SOS3 Maintains Its Growth-Promoting Effect under Control and Glasshouse Conditions

SOS3 biomass was used as a bioinoculant, and growth promotion was evaluated in sorghum and maize (Figure 6). The effect of SOS3 culture on the germination of sorghum was examined using seeds coated with cells grown in NB, MMS, or glucose-MMS batch cultures (Section 3.2) taken during the exponential phase. The effect of SOS3 cultures on germination was tested in a fresh batch of sorghum seeds, separating the sample into two fractions: supernatant and cell pellet. NB media cultures produced less dry mass per seed than the control, MMS and G-MMS cultures (Figure 6a). Supernatant treatments appeared to show higher yields compared to the control for both MMS and G-MMS. Similar results are observed for the vigour index (Figure 6b). For all treatments, samples were taken during the exponential growth phase. Preliminary data (not shown) suggest that there is a reduction in the vigour index and dry mass for sorghum seeds when SOS3 cells are taken during the late stationary phase.

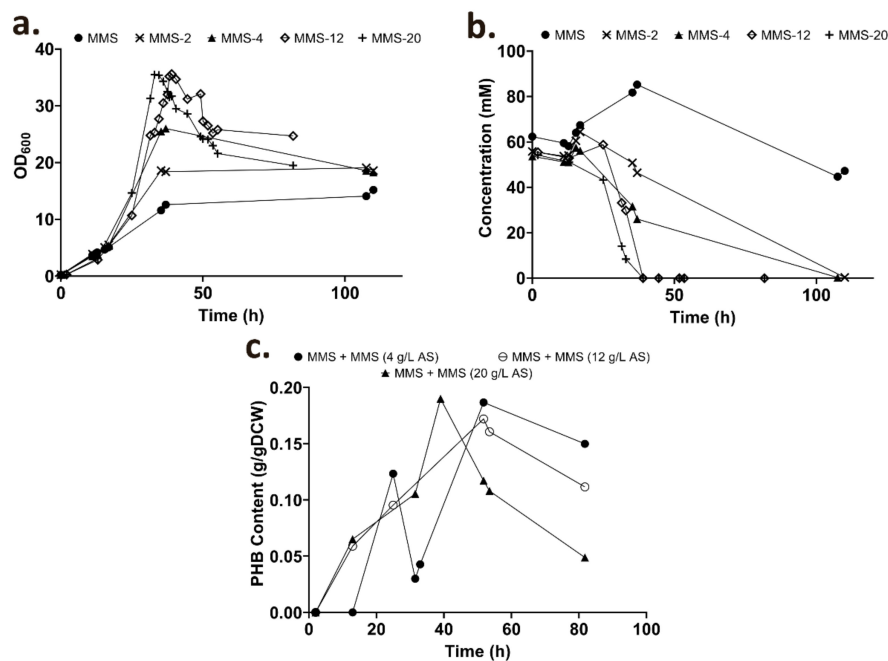


Figure 5. Growth of SOS3 in media supplemented with varying amounts of nitrogen. (a) Growth curves. (b) Sugar concentrations throughout. (c) PHB content over time of SOS3 cultures supplemented with varying amounts of nitrogen.

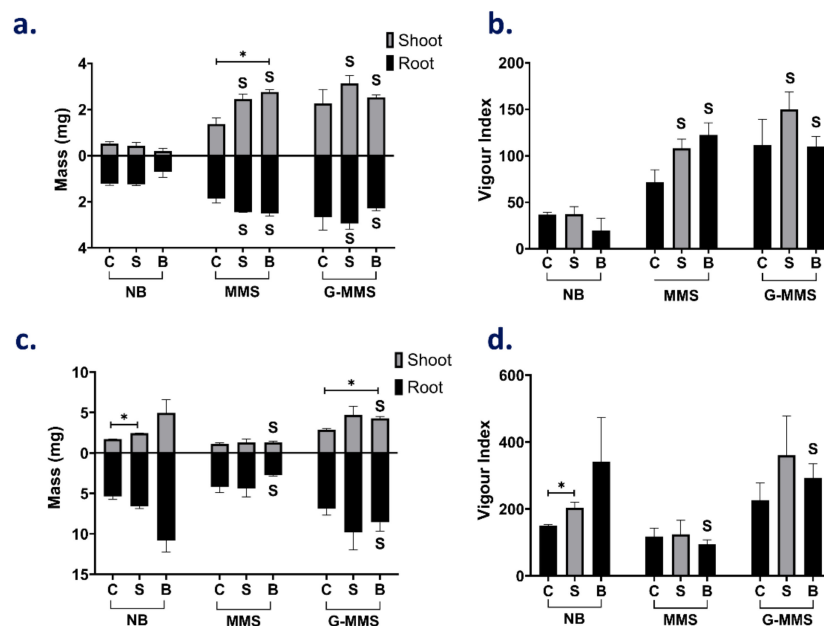


Figure 6. Effect of SOS3 on biomass and vigour of sorghum (*Sorghum bicolor* L.) and maize (*Zea mays* L.). Dry biomass (a) and vigour index (b) of sorghum seeds. Dry biomass (c) and vigour index (d) of maize seeds. Seeds were treated with sterile media control (C), SOS3 supernatant (S), or SOS3 bacterial culture (B). Three media were tested: NB, MMS, and G-MMS. Each treatment had four replicates. Measurements were performed 5 days after germination. Significance was calculated using standard *t*-tests. For simplicity, significance was only tested between relevant groups (i.e., between the three treatments of each medium, the three controls, the three supernatant treatments, and the three bacteria treatments). S represents significance compared to the corresponding treatment of at least one other media type (only shown on MMS and G-MMS). Levels of significance are not noted in either the S or * labels. SOS3 cultures also exhibited growth-promoting effects on maize seeds (Figure 5c,d); however, the results do not follow the same behaviour as in sorghum. For maize seeds, SOS3 cultures grown on MMS medium showed a lower dry mass in roots and shoots compared to NB and G-MMS media. Similarly, MMS conditions displayed a lower vigour index compared to NB and G-MMS.

The performance of SOS3 was simulated in field conditions using immobilised cells grown in MMS and G-MMS cultures. Maize seeds were planted in pots and inoculated with immobilised SOS3 cells (Figure 7). SOS3-treated plants had significantly higher emergence rates than the control 6 days after planting, although, after 13 days, these differences were no longer significant. G-MMS-treated plants had the highest emergence at both time points (Figure 7a). The SOS3-treated pots also showed increased leaf counts and height. The average leaf count was increased by roughly 12% and 7% in MMS and G-MMS, respectively, and the average height was increased by roughly 18% and 19% in MMS and G-MMS, respectively. Similarly to the response observed in sorghum, the inoculated plants produced more dry mass than the controls (Figure 7d), and the vigour index increased by nearly 50%. In Figure 7f, it can be seen that there are also morphological differences amongst the treatments, specifically in root thickness and plant height.

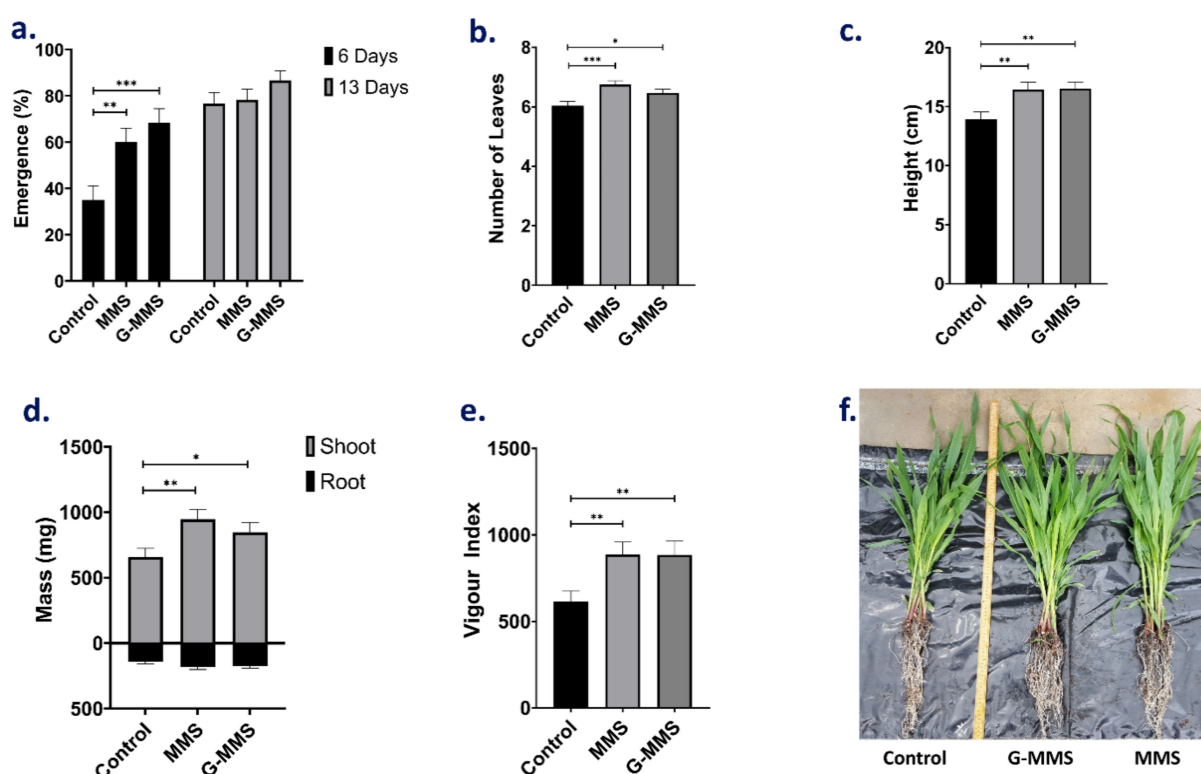


Figure 7. Response of maize (*Zea mays* L.) to SOS3 inoculation. The seeds were grown in glasshouse conditions for 22 days before harvest. Each treatment had 28 replicates: one seedling per pot. (a) Seedling emergence at 6 and 13 days of untreated maize seeds (control) compared with seeds inoculated with SOS3 grown in MMS (MMS) or SOS3 grown in glucose-MMS (G-MMS); (b) analysis of leaf number at harvest; (c) height of seedlings at harvest; (d) dry biomass; (e) vigour index; (f) maize plants at harvest after removal from pots and washing of the roots. Error bars denote standard error from the mean, and asterisks indicate statistically significant differences from the control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.4.2. Proteomics Fingerprint of SOS3 Cultures as a Quality Measure

PGPBs are complex products, and the efficacy of a product is affected by both raw materials and the bioprocess. While functional bioassays (e.g., Figures 6 and 7) are critical, the development of a better understanding of the mode of action would greatly facilitate process development and production quality control [24]. Here, we used proteomics to screen for potential biomarkers as a qualitative measure of the effectiveness of SOS3 as a biostimulant.

A comparative analysis was performed of samples taken from three different growth media (MMS, G-MMS, and M-MMS) by sampling at three time points (the early exponential

phase, t1; late exponential phase, t2; and stationary phase, t3). The PC2 component analysis of the proteomics data indicates that the primary source of variation relative to protein abundance was between time points rather than media (Supplementary Materials File 1). Thus, the simpler G-MMS media should not significantly affect the bacterium's metabolic state or disrupt triggering the observed growth-promoting traits.

Consulting the existing literature on PGPB, key mechanisms associated with pleiotropic effects were identified: nitrogen fixation, phosphate solubilisation, phytohormone production (including indole-3-acetate, cytokinins, and gibberellins), siderophore production, and modulation of stress signalling. Changes in the abundance of the most representative protein involved in the biochemical pathways that lead to the synthesis of those products appear in Figure 8.

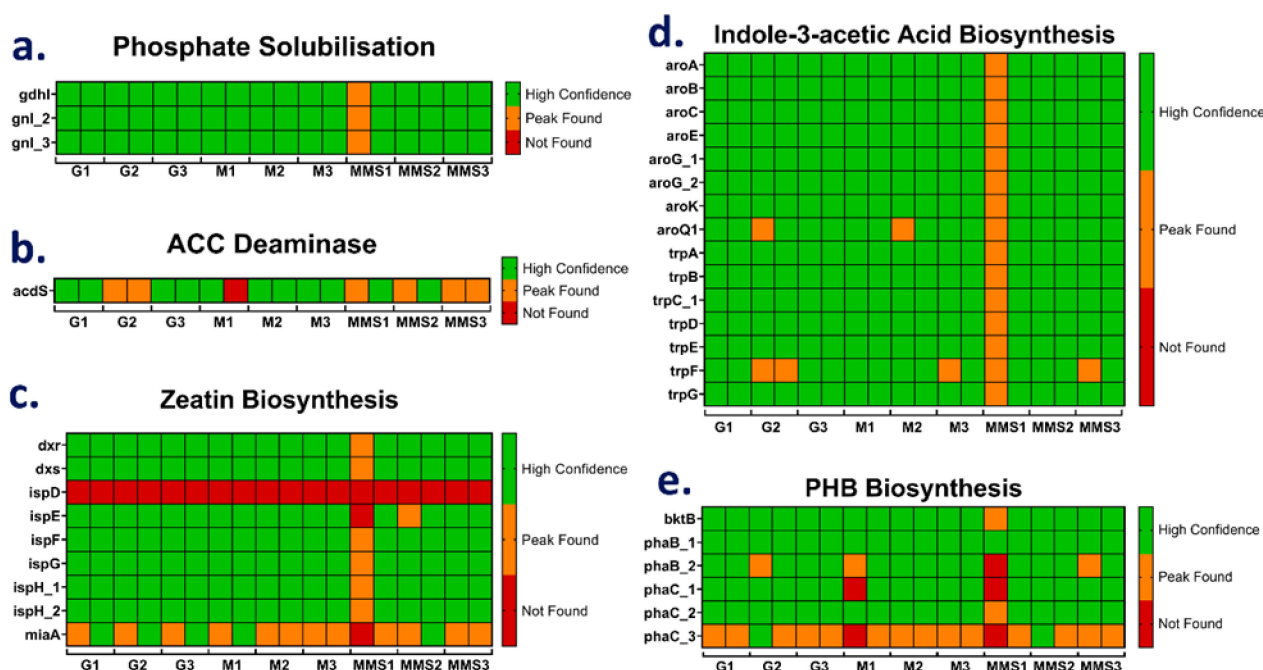


Figure 8. Proteomics identification data of SOS3 cultures grown in various media and at different time-points. (a) Key phosphate solubilisation proteins; (b) ACC deaminase; (c) key zeatin biosynthesis proteins; (d) key IAA biosynthesis proteins; (e) key PHB biosynthesis proteins. Each medium–time combination (e.g., G2 meaning G-MMS at time 2) was sampled in duplicate from separate fermentations.

The proteomics analysis showed that the proteins involved in PHB biosynthesis and accumulation were present during SOS3 cultivation. The measurement of intracellular PHB (Figure 5c) in the cultures supplemented with 4, 12 and 20 g/L of ammonium sulphate reached up to 0.2 g per gDCW by the end of the fed-batch stage. Intracellular PHB then decreased in all conditions. There was an inverse correlation between the final PHB content and the AS concentration in the feed.

4. Discussion

The adoption of semi-defined MMS media to replace the complex NB medium greatly increases biomass production in SOS3 cultures (Figure 3). NB batch cultures showed higher growth rates during the exponential phase than MMS cultures, however, these cultures have a reduced yield due to a much shorter growth phase. This higher growth rate is likely due to the increased presence of essential complex nutrients in NB (peptone and yeast extract) compared to MMS, which only has 1 g/L of yeast extract. In a complex medium, specific growth rates are almost always higher than a defined minimal medium because cells do not need to synthesise many essential compounds from base nutrients [25]. Despite this, NB batch cultures quickly reached the stationary phase at 1.5 OD₆₀₀ compared to

MMS batch cultures, where OD_{600} reached around 5 units with an extended growth phase. NB cultures are likely being limited in growth, as they fully deplete their carbon source. The concentration of peptone and yeast extract makes nitrogen limitation unlikely. While supplementation of NB media with additional carbon sources could lead to improvements in biomass production as observed in MMS derived media, this strategy is not compatible with the ultimate goal, which is the use of chemically defined media [5]. Recently, Lobo et al. (2019) [6] summarised several studies on biomass production of bioinoculants. In most cases, media composition consists of complex mixtures, including corn flour (*Pseudomonas putida* Rs-198) and dairy sludge (*Rhizobium trifolii*). While these strategies aim to be sustainable by using relatively cheap material, still the final formulation can lead to inconsistent results [5].

The apparent diauxic growth seen in SOS3 fermentations in MMS batch cultures suggested that one of the carbon sources is preferred by the bacterium (Figure 3). It is not uncommon for rhizobacteria to exhibit this behaviour when grown in controlled conditions [26]. The carbon source analysis confirmed that glucose is consumed preferentially. Once glucose has been depleted, the culture exhibits a small diauxic shift as the bacterium seemingly adjusts its metabolism to primarily consume mannitol. However, the data suggest that mannitol is still being co-consumed during this first stage of growth. While mannitol and glucose have similar biomass yields, glucose is consumed much more rapidly than mannitol (even when mannitol was the sole carbon source). Despite these differences in substrate preference and yield per biomass during growth, all MMS variants showed similar yields in preliminary fermentations. This can be related to the fact that the cultures were nitrogen-limited.

It was clear that nitrogen supplementation in SOS3 culture promotes higher cell densities during the fed-batch stage, followed by a substantial reduction in the biomass concentration. It has been reported that ammonia (NH_3^+) uptake is coupled to carbon uptake [27]. On the other hand, the non-supplemented cultures showed a rise and a relatively consistent stationary phase whilst consuming the remaining carbon sources. The supplemented cultures likely grew until they had depleted their carbon sources, and then the bacteria began to die off and eventually plateau. This was confirmed in Figure 5, where it can be seen that total depletion of glucose and mannitol was achieved at roughly the same period as the cultures peaked in OD_{600} . Whilst this increase in yield was one of the key goals, it brings a new parameter to consider: harvest time. Whereas non-supplemented cultures remain relatively stable after the fed-batch stage, cultures supplemented with nitrogen must be harvested in a window of a few hours to collect the maximum amount of biomass. Late harvesting could lead to a weaker performance associated not only with the lower cell density but also with a reduced PHB content. Reduced PHB content in the cells may also have a detrimental effect on their potential as shelf-stable biofertilisers.

The PHB production profiles of SOS3 cultures seen in Figure 5 are associated with the availability of nitrogen and carbon sources, a feature observed in several PGPR [28,29]. However, while most PHB producer accumulate the bioplastic when nitrogen is limiting [30], SOS3 accumulates PHB when both carbon and nitrogen sources are available. PHB synthesis during the exponential growth phase can be associated with both nutrient limitation in the culture, such as phosphate and accumulation due to an excess of carbon (storage mechanism) [30]. PHB accumulation is desirable in liquid formulations of biofertilisers where the PGPR does not produce spores. This can also help cells to remain viable after immobilisation into a solid matrix or onto seeds [31].

Similar fed-batch and media supplementation strategies have been implemented in the cultivation of bioinoculants such as *Azospirillum* sp., with growth-promoting properties associated with the production of growth regulators [5,32]. Trujillo et al. (2013) [30] implemented a control strategy where pH, dissolved oxygen, stirring speed, and aeration were kept constant while varying the carbon to nitrogen ratio of the feed. Their process reached up to 3 OD_{600} units, which is ten times lower than the highest OD_{600} reached by the SOS3 cultures tested in this work. While SOS3 reached higher biomass production, it

should be noted that SOS3 does not produce spores, as *Azospirillum* sp. does. The higher cells densities reached in SOS3 are a desirable characteristic for biofertiliser formulation in non-sporulating organisms [33].

Generation of by-products such as organic acids and alcohols seemed to vary with AS supplementation (Supplementary Materials File 2). In particular, non-supplemented cultures produced significant amounts of formate with a small amount of acetate production. On the other hand, the supplemented cultures show no formate production but significant acetate production. It might be possible that the addition of AS triggered multiple metabolic functions other than improving growth and promoting PHB accumulation.

Implementing multi-omics approaches has been instrumental in deciphering the mechanisms that lead to growth promotion [34]. While omics' implementation has focused primarily on the plant-bacterium interaction to build accurate and reliable data [34], there is little information regarding quality controls for bioinoculant formulation. We sought to identify potential biomarkers for the pleiotropic growth-promoting effects observed during trials by identifying key expressed proteins using proteomics. The results obtained provide an initial snapshot of the potential biomarkers that can be used as a quality parameter during the formulation of inoculants. Still, further work is required to demonstrate to what extent these biomarkers contribute to growth promotion as long as they are present at the time of harvesting.

Overall, SOS3 exhibits four main pathways during fed-batch cultivation that can arguably be associated with an immediate response in plant growth post-inoculation. While phosphate solubilisation, synthesis of ACC deaminase, synthesis of zeatin, and synthesis of IAA are active in harvested cells, targeted experiments to assess the translation of such effects into plant growth are needed to confirm the robustness of the biofertiliser [35,36]. Only zeatin biosynthesis seems to be incomplete as *IspD* was not found in any sample. While *nif* genes are present in the SOS3 genome and the proteins were present in the analysed samples, no nitrogenase production was detected in SOS3 during cultivation in bioreactors. Proteomic analysis of SOS3 in the rhizosphere of growing plants may hold evidence for the production and activity of nitrogenases. Still, trial experiments show the positive effect that SOS3 has regarding germination, emergence, and vigour.

High-cell density cultures maintained the growth-promoting effects previously observed. In both sorghum and maize coated with culture supernatant, an increased germination rate is likely associated with metabolites or peptides secreted by SOS3. Although this is an exciting result, the germination experiments are not quality substitutes for real field conditions. Many plant growth-promoting mechanisms involve longer-term interactions, and so only the production of phytohormones would be expected to play a role in germination improvements over only a few days. The identification of the molecules (small molecules, peptides, glycoproteins) in the supernatant that trigger growth-promotion in plants can provide further information to build the next generation of fertiliser.

Using a solid matrix as a delivery method allowed us to test the benefits of SOS3 in model crops. The most valuable experiment for assessing the effects of SOS3 on plant growth was the maize glasshouse trial. Significant increases in all metrics of plant growth were observed in SOS3 treatments. This shows the potential for SOS3 use in farming applications as a drop-in biofertiliser (with zeolite as the carrier).

Preliminary data on the effectiveness of SOS3 cultures grown on MMS medium suggest that cultures harvested during the late stationary phase have a detrimental effect on the germination, vigour index, and dry mass of sorghum seeds. This observation becomes relevant as it influences the shelf life of a liquid formulation. Interestingly, the inhibition of maize seed germination by treatment with SOS3 grown in MMS compared to G-MMS was not seen in the glasshouse trial, where MMS and G-MMS yields were similar. This further highlights the importance of the glasshouse trial, as the germination experiments alone suggest that MMS is not a suitable growth medium for maize treatments. Whether this was due to the use of zeolite as a carrier instead of direct seed coating or some impacts from actually being planted in the soil is not yet known. By planting maize seeds coated with

MMS culture and comparing them with maize seeds treated with MMS culture zeolite, this cause should be identified.

5. Conclusions

Previously, the use of SOS3 cultures as bio-inoculants was limited by the use of small shake flask cultures and low biomass yields. Here, we evaluated different conditions for culturing SOS3 in bioreactors and implemented a fed-batch strategy for reaching high cell densities up to 40 OD₆₀₀ units. Moreover, the cultures grown in semi-defined media displayed higher yields and productivities up to 10 times higher than the initial conditions tested. The cultures maintained their plant growth-promoting traits when tested on seeds and in model crops in glasshouse conditions, opening new avenues for the implementation of SOS3 as a broad-spectrum biofertiliser.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/fermentation7020058/s1>, Supplementary Materials File 1. Supplementary Materials File 2.

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