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Investigation of a Broad-Bean Based Low-Cost Medium Formulation for *Bacillus subtilis* MSCL 897 Spore Production

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Abstract: *Bacillus subtilis* (Bs) is a bacterium that benefits plants and is used in the production of bio-fungicides. The cultivation of Bs is a crucial step in bio-control preparation production, as it greatly impacts the quality and price of the final product. In a series of shake flask experiments, we investigated the economically feasible broth composition for spore production of *Bacillus subtilis* MSCL 897, a Latvian soil isolate. Our study investigated the impact of utilizing legume-based flours (such as broad bean, grey pea, and soybean) as the primary nitrogen source, along with sugar-beet molasses, sucrose, or glucose as the carbon source, and yeast extract, peptone, and corn-steep liquor as growth factor additives. Additionally, we examined the effect of using $(NH_4)_2HPO_4$ or urea as supplementary nitrogen sources, as well as previously established media formulations, on spore yield. Our results showed that a culture medium composed of broad bean flour (10 g/L) and molasses (10 g/L) led to spore productivity of $1.35 \pm 0.47 \times 10^8$ CFU/mL at 48 h. By enriching the culture medium base constituents with a minor (0.5–1.0 g/L) yeast extract or corn-steep liquor additive, a notable increase in spore productivity was observed, with values of $2.00 \pm 0.28 \times 10^8$ and $2.34 \pm 0.18 \times 10^8$ CFU/mL at 48 h, respectively, and sporulation efficiency > 80–90%. As a result, we achieved a high spore yield of the *Bacillus subtilis* MSCL 897 strain, demonstrating the competitiveness of our approach, which relied on a low-cost medium made mainly from locally available and renewable raw materials.

Keywords: *Bacillus subtilis*; low-cost medium/broth; endospores; bio-fungicide

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1. Introduction

Bacillus subtilis (Bs) is a type of gram-positive, spore-forming bacteria that is commonly found in both the soil and gastrointestinal tracts of ruminants and humans. This bacterium has significant biotechnological applications, particularly in the production of microbiological formulations that promote and protect plant growth (PGPFs) [1], enzymes [2], probiotics, and other products such as medicines and industrial chemicals [3]. Bs acts as both a microbial fertilizer, boosting crop productivity, as well as a biocontrol agent, suppressing the growth of plant pathogens. Additionally, the bacterium is able to simultaneously produce antibiotics and endospores, which enhances its ability to survive in unfavorable conditions and eliminate competing organisms. The strain of *B. subtilis* MSCL 897 was isolated from Latvian soil and carefully chosen for its ability to thrive in the local environment, including the climate and soil properties, making it a suitable candidate for use as a PGPF in the region.

In a bioreactor production process, Bs vegetative growth, sporulation, and release of antifungal compounds are critical aspects [4]. To ensure optimal endospore formation, it is essential to achieve a maximum cell count during vegetative growth. An endospore is a dormant form of Bs that possesses remarkable mechanical strength and high resistance to external factors such as drying, ultraviolet radiation, organic solvents, and high temperatures,

making it an ideal choice for preparation, processing, storage, and application in microbial formulations. Additionally, during sporulation, the majority of antimicrobial compounds, including antifungal and antibacterial compounds, are synthesized [5]. On average, 4–5% of the Bs genome is dedicated to antibiotic production, with several antibiotics being produced by various Bs strains (such as subtilosin, surfactin, and bacilysin), while others are produced strain-specifically (such as lantibiotics subtilin, ericin, and mersacidin) [5]. The efficiency of sporulation and the accumulation of antifungal compounds depend on several cultivation process conditions, including temperature, pH, cultivation time, dissolved oxygen level, broth composition, broth element concentration, and more [4,6].

The cultivation medium for Bs typically comprises a combination of (1) a carbon source (e.g., glucose, sucrose), (2) a nitrogen source (e.g., ammonium salts or complex, protein-rich organic sources), (3) mineral salts (e.g., potassium phosphate, sodium chloride, and magnesium sulfate), (4) micro-elements (e.g., iron, zinc, and manganese), and (5) vitamins (e.g., thiamine and riboflavin). The composition and concentration of these components can be adjusted based on specific growth conditions and the desired outcome. In both flask [6–11] and bioreactor [7,9,12,13] experiments, *Bacillus subtilis* has been extensively studied using media of complex composition. Other components, such as amino acids or growth factors, may also be added to the medium to enhance growth [14].

Legumes, such as broad beans and soybeans, are rich in organic and inorganic compounds that are suitable for use as a nutritional base for microbial cultivation. Examples of Bs culture using defatted soybean meal (soybean flour) [6,7] and milled soybean [10] are available, and broad bean flour has also been used for lengthy *Bacillus thuringiensis* cultivation [15]. It is worth noting that broad bean production is preferred in temperate climate regions, making them an attractive renewable bio-processing feedstock in Northern and Eastern Europe. *Vicia faba* type of broad and horse beans is the major legume produced on the European continent, covering around 25% of the world's harvest area with an average yield of 2.9 t/ha (compared to the world's average of 2.1 t/ha) [16]. As a result, broad beans are an excellent choice for sustainable cultivation and bioprocessing applications.

Sugar-beet molasses is a byproduct of the sugar refining process and is rich in sucrose, which can be used by microorganisms as a carbon source for energy and growth. In addition, beet molasses contains other nutrients, such as vitamins, minerals, and organic acids, that can support microbial growth [17]. Overall, beet molasses is a viable and cost-effective carbon source for microbial cultivation and can support the growth of a wide range of microorganisms, including Bs [18,19].

The combination of the components discussed above, along with the strain type and cultivation parameters, can significantly impact the process productivity, such as Bs spore yield and antifungal properties of the product. Various flask-scale investigations have reported spore yields ranging from approximately 1×10^6 to 7×10^9 spores/mL [7–10,20]. These yields were obtained in media that contained a combination of components, such as soybean flour, corn-steep liquor, yeast extract, meat extract, peptone, salts (with Mg, Mn, Ca, Zn, Fe, K ions), glucose, arabinose, food, or food industry waste.

In this study, we investigated the impact of utilizing legume-based flours (broad bean, grey pea, and soybean) as the primary nitrogen source, along with sugar–beet molasses, sucrose, or glucose as the carbon source, and yeast extract, peptone, and corn-steep liquor as growth factor additives. Additionally, we examined the effect of using $(NH_4)_2HPO_4$ or urea as supplementary nitrogen sources, as well as previously established media formulations, on spore yield. Our findings provide novel insights into the competitive spore productivity of this cost-effective medium and will likely enable the efficient scaling up of PGPFs production for the *B. subtilis* MSCL 897 strain.

2. Materials and Methods

2.1. Microorganism and Inoculum Preparation

Bacillus subtilis MSCL 897, obtained from the Microbial Strain Collection of Latvia, was stored in 1.5 mL cryovials containing 50% glycerol and kept at −28 °C until required

for inoculum preparation. A 250 mL Erlenmeyer flask containing 50 mL of LB medium was then inoculated with 0.5 mL of the cryovial stock, and the inoculum was incubated in a rotary shaker/incubator (ES-20, Biosan, Riga, Latvia) at 37 °C and 200 rpm for 24 h, resulting in seed material with an optical density around 8–9. The obtained seed material was subsequently used to inoculate flasks during the 'Flask experiments'.

2.2. Flask Experiments

Cultivation experiments were carried out in 250 mL Erlenmeyer flasks. Flasks containing 50 mL medium of the specific composition (investigated medium composition variants shown in Table 1) were seeded with 1% (*v*/*v*) of inoculum and incubated in a rotary shaker/incubator at 37 °C, 200 rpm for 48 h. All experiments were replicated at least twice with two duplicates unless otherwise specified.

Table 1. Overview of the Bs medium composition investigation steps (1–6) and spore productivity results.

Molasses (M), Sucrose (S) or Glucose (G), g/L	Broad Bean Flour (BF), Grey Pea Flour (PF) or Soy Bean Flour (SF) g/L	Yeast Extract (YE), g/L	Peptone (P), g/L	Corn-Steep Liquor (CSL), g/L	$(NH_4)_2HPO_4$ or Urea, g/L	Endospore Yield (48 h), 10^8 CFU/mL	SD, 10^8 CFU/mL	Sporulation Efficiency ($n_{Sporul,\ \%}$), %
(1) Legume based flour with molasses								
10.0 M	10.0 BF	—	—	—	—	1.35	0.47	79
10.0 M	10.0 PF	—	—	—	—	1.10	0.56	61
10.0 M	10.0 SF	—	—	—	—	1.06	0.27	66
(2) BF with glucose or sucrose								
5.0 S	10.0 BF	—	—	—	—	0.54	0.17	57
10.0 S	10.0 BF	—	—	—	—	0.61	0.23	45
5.0 G	10.0 BF	—	—	—	—	0.18	0.13	36
(3) BF with molasses and YE, P or CSL additive; and SF with YE additive								
10.0 M	10.0 BF	0.5	—	—	—	2.00	0.28	77
10.0 M	10.0 BF	1.0	—	—	—	1.48	0.52	78
10.0 M	10.0 BF	2.0	—	—	—	1.40	0.34	78
10.0 M	10.0 BF	—	0.5	—	—	1.29	0.58	75
10.0 M	10.0 BF	—	1.0	—	—	1.16	0.58	80
10.0 M	10.0 BF	—	2.0	—	—	1.06	0.42	64
10.0 M	10.0 BF	—	—	0.5	—	2.09	0.29	95
10.0 M	10.0 BF	—	—	1.0	—	2.34	0.18	97
10.0 M	10.0 BF	—	—	2.0	—	1.91	0.21	94
10.0 M	10.0 BF	10.0	—	—	—	1.41 *	0.08 *	—
20.0 M	10.0 BF	0.5	—	—	—	1.15 *	0.19 *	—
10.0 M	10.0 SF	0.5	—	—	—	1.07 *	0.15 *	—
(4) BF with 10 g/L of molasses and minor additive of YE/CSL, P/CSL or YE/P/CSL								
10.0 M	10.0 BF	0.5	0.5	—	—	1.58	0.70	79
10.0 M	10.0 BF	0.5	—	0.5	—	2.19	0.51	86
10.0 M	10.0 BF	—	0.5	0.5	—	1.94	0.32	85
10.0 M	10.0 BF	0.5	0.5	0.5	—	1.97	0.34	95
(5) BF with 10 g/L of molasses, minor additive of YE and $(NH_4)_2HPO_4$ or urea								
10.0 M	10.0 BF	0.5	—	—	1.0 $(NH_4)_2HPO_4$	1.00	0.08	88
10.0 M	10.0 BF	0.5	—	—	2.0 urea	0.85	0.25	95
(6) Trials of mediums from literature								
Posada-Uribe et al. [9]						0.87	0.34	72
Monteiro et al. [12]						1.10	0.82	77
Khardziani et al. [10]						0.15 *	0.04 *	79

* Endospore yield and SD obtained from one or two replication.

Bacillus subtilis MSCL 897 cultivation experiments were conducted in 250 mL Erlenmeyer flasks containing 50 mL of the specific medium composition variants (as shown in Table 1). Each flask was seeded with 1% (*v*/*v*) of inoculum. The inoculated flasks were incubated at 37 °C and 200 rpm in a rotary shaker/incubator for 48 h. All experiments were conducted in at least four replications unless otherwise specified.

2.3. Culture Media Composition

The experimental design aimed to evaluate and compare several medium components as sources of carbon/energy, nitrogen, minerals, and vitamins for Bs cultivation. Broad bean flour (BF) and sugar-beet molasses were chosen as the main constituents of the medium, while additional legume-based flours (grey pea flour (PF) and soybean flour (SF)) were examined for comparison purposes. Various culture media additives, including yeast extract (YE), peptone (P), corn-steep liquor (CSL), $(NH_4)_2HPO_4$, and urea, were

also investigated. Additionally, some culture media compositions from the literature were tested.

The experiments were designed to investigate the effects of varying components or their concentrations in the cultivation medium on spore yield and were organized into six series:

1. Evaluation of the best-suited flour as a nitrogen source, with analysis of BF, PF, and SF.
2. Comparison of sugar beet molasses, glucose, and sucrose as carbon sources.
3. Investigation of various additives, including YE (Biolife), P (from beef, VWR International), and CSL (CC MOORE).
4. Analysis of combinations of additives provided in minor amounts.
5. Addition of $(NH_4)_2HPO_4$ or urea as additional nitrogen sources.
6. Comparison of spore yields with previously reported media formulations for the particular strain.

Initial medium component concentrations were estimated based on available literature and preliminary experimental results. YE (Biolife), P (from beef, VWR International), and beef extract powder (Biolife) were also utilized in experiments to evaluate reference media compositions from the literature.

The reported literature medium compositions are as follows:

1. Posada-Uribe et al. [9]: glucose 1.04 g/L, yeast extract 5.00 g/L, peptone 3.00 g/L, KH_2PO_4 6.00 g/L, $MgSO_4 \cdot 7H_2O$ 0.59 g/L, $MnCl_2 \cdot 4H_2O$ 0.50 g/L, NaCl 0.01 g/L, trace salts: $FeSO_4$ 0.1 M 1.14 mL/L, $ZnSO_4$ 0.1 M 0.30 mL/L, $CaCl_2$ 1 M 1.00 mL/L, $MnCl_2$ 1 M 3.00 mL/L.
2. Monteiro et al. [12]: glucose 5.00 g/L, beef extract 3.00 g/L, peptone 5.00 g/L, $MgSO_4 \cdot 7H_2O$ 0.50 g/L, KCl 1.00 g/L, trace salts: $MnCl_2$ 10 mM 1 mL/L, $Ca(NO_3)_2$ 1 M 1 mL/L, $FeSO_4$ 1 mM 1 mL/L.
3. Khardziani et al. [10]: glucose 2.00 g/L, yeast extract 3.00 g/L, peptone 3.00 g/L, KH_2PO_4 1.00 g/L, $MgSO_4 \cdot 7H_2O$ 1.02 g/L.

2.4. Optical Density Measurements

The culture's optical density (OD) was monitored at 540 nm using a spectrophotometer (BK-V1000, BIOBASE, Jinan, China) at 0, 6, 24, 30, and 48 h. At each time point, 1 mL of sample was taken for analysis.

2.5. Determination of Total Cell and Spore Colony Forming Units

At 24 and 48 h, 1 mL samples were collected for determination of colony forming units (CFU). Decimal dilution series were prepared, and the standard serial dilution method was used to determine the total CFU by plating appropriate dilutions right after their preparation. The endospore concentrations were determined by plating appropriate dilutions after heating them at 80 °C for 20 min. To ensure uniform and consistent heating conditions, an automatic control autoclave (NC 90M, Nuve, Zurich, Switzerland) with a heating program was used. 100 µL of each appropriate dilution of the sample was spread on a 90 × 14 mm Petri dish containing LB medium with 12 g/L agar and incubated at room temperature for 48 h. Each appropriate dilution was plated in triplicate. The CFU count of each sample was determined by calculating the average colony count (in the range of 30 to 300 colonies) on three plates of one of the dilutions and expressed as average CFU/mL for both total viable cells and spores.

2.6. Statistical Methods

For each plated CFU and spore sample (at least 3 measurements for each sample), the standard deviation (SD) was calculated according to the following equation:

$$\sigma = \sqrt{\frac{\sum (x_i - \mu)^2}{N}} \quad (1)$$

where σ—standard deviation, N—number of plates per sample analyzed, x_i—colony count for each sample, μ—the mean colony count for all samples.

Sporulation efficiency ($n_{Sporul,\ \%}$) was estimated by the following equation:

$$n_{Sporul,\ \%} = \frac{Spores}{Total\ cells} * 100\% \quad (2)$$

where *Spores*—concentration of spores (CFU/mL) and *Total cells*—concentration of spores and vegetative cells (CFU/mL).

3. Results

Table 1 shows the results obtained from six investigation steps conducted in chronological order. Although the experiments yielded similar results overall, some conclusions about the growth and sporulation behavior of the particular Bs strain under the investigated conditions can be drawn.

3.1. Legume Flour

The first investigation step (as indicated in Table 1) was carried out to identify the most suitable nitrogen (broad bean flour (BF), grey pea flour (PF) or soybean flour (SF)) sources for Bs endospore production.

Cultivation of Bs in media containing either BF, PF, or SF (10 g/L) and supplemented with 10 g/L molasses resulted in comparable total cell and spore concentrations at 24 and 48 h (Figure 1B). A slight increase in the total cell number was observed for all three cases within 24–48 h. This trend was also reflected in the OD data (see Figure 1A), where a growth decline during the 30–48 h period can be observed. It is worth noting that there was less variability between parallel measurements in less complex culture media cases, where sucrose or glucose was used instead of molasses. In the case of the PF medium, a sporulation efficiency of approximately 80% at 48 h was observed.

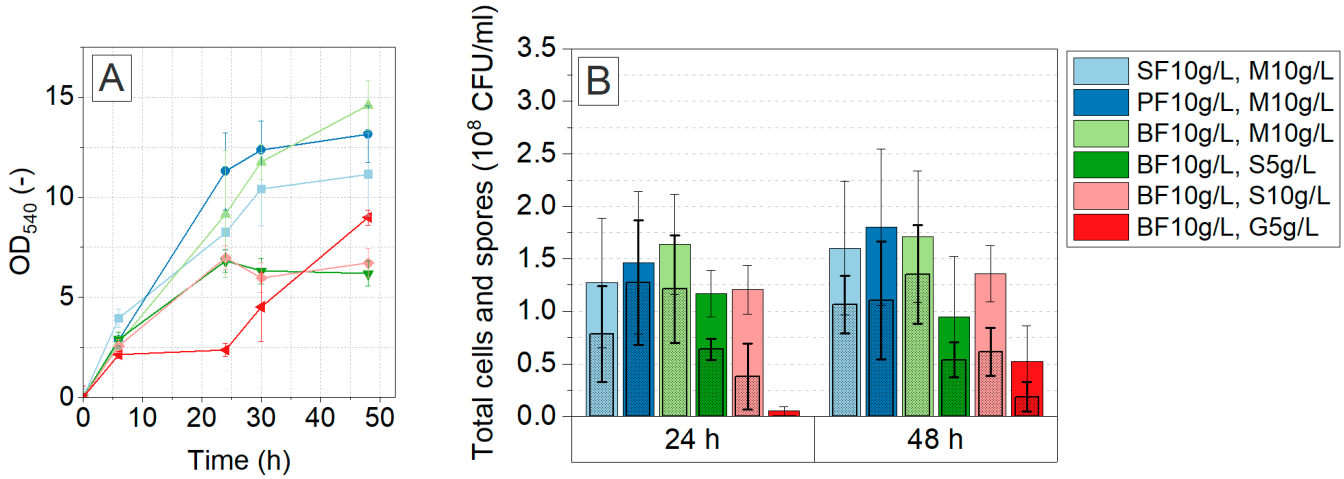

Figure 1. *B. subtilis* MSCL 897 cultivation in medium containing combinations of soybean (SF), broad bean (BF) and gray pea (PF) flour, sucrose (S), glucose (G) and molasses (M): (**A**) the optical density (OD) dynamics over time; (**B**) total cell (outer bars) and spore (inner bars) counts at 24 and 48 h. Legend colors correspond to both plots.

The highest endospore yield ($1.35 \pm 0.47 \times 10^8$ CFU/mL) and sporulation rate (79%) were achieved using BF as the main nitrogen source (Table 1). Therefore, this component was selected as the base for the next investigation steps.

3.2. Main Carbon and Energy Source

In the second experimental step, the effects of different carbon and energy sources on growth and sporulation were evaluated by comparing a medium containing BF and 10 g/L molasses (from the first investigation step) to the medium containing BF and sucrose (5 or 10 g/L) or glucose (5 g/L). Medium containing sucrose, whether 5 or 10 g/L, resulted in a 2- to 3-fold increase in spore yield compared to medium containing 5 g/L glucose, suggesting that sucrose is a more favorable carbon and energy source for biomass growth and spore formation in this particular strain. However, the use of molasses, which contains 60% sucrose along with other components such as proteins, organic acids, microelements, and inorganic acid anions utilized in cell biochemistry, led to a 2-fold increase in spore yield compared to BF media supplemented with pure sucrose (5 or 10 g/L).

Evidently, molasses is the preferred carbon source for Bs endospore formation, as it is not only cheaper than glucose and sucrose but also ensures a higher endospore yield.

3.3. YE, P, CSL, $(NH_4)_2HPO_4$ and Urea Additives to BF and Molasses Medium

To further enhance the BF and molasses medium, we devised an experimental plan that involves enriching the medium with small amounts of YE, P, CSL, $(NH_4)_2HPO_4$, or urea (experimental steps 3–5 in Table 1). Our study aims to examine the impact of the additive components, either individually or in combination (as illustrated in Figure 2), on the medium's overall performance. This approach will allow us to identify the most effective combination of additives for improving the medium's efficiency.

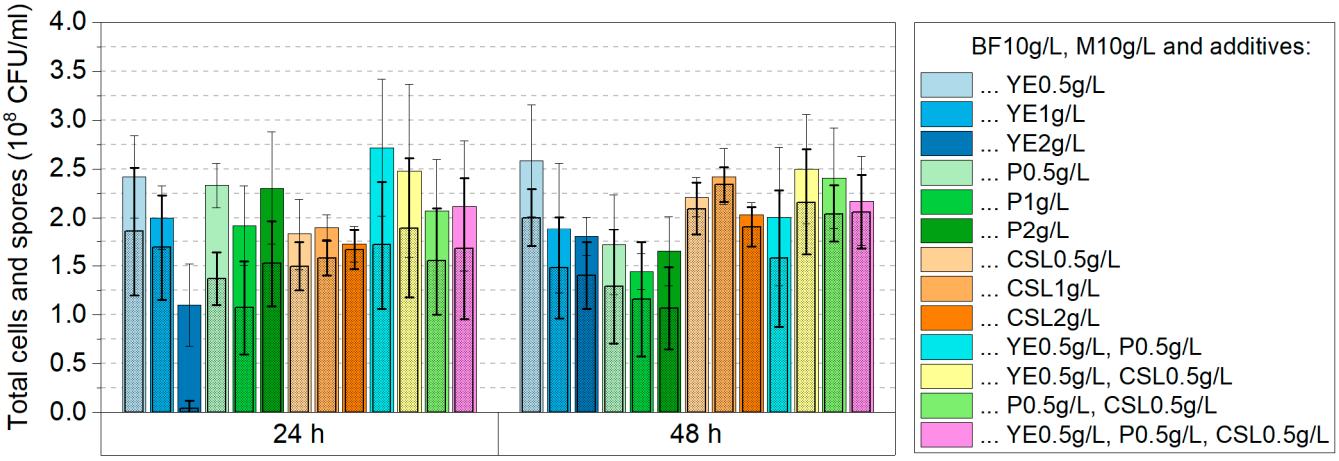

Figure 2. *B. subtilis* MSCL 897 cultivation in medium containing broad bean flour (BF) 10 g/L, molasses (M) 10 g/L and combinations of yeast extract (YE), peptone (P) and corn-steep liquor (CSL). Total cell (outer bars) and spore (inner bars) counts at 24 and 48 h.

3.3.1. YE, P, and CSL as Individual Additives

To determine the impact of different concentrations of YE, P, and CSL on spore concentration, we evaluated three concentrations (0.5 g/L, 1.0 g/L, and 2.0 g/L) of each additive individually. Our findings revealed that only YE and CSL additions had a significant effect on spore concentration, with CSL showing a greater impact compared to YE. Among the tested concentrations, the addition of 1.0 g/L CSL resulted in the highest spore concentration of $2.34 \pm 0.18 \times 10^8$ CFU/mL at 48 h, with a sporulation efficiency of over 90% and a 70% increase in spores compared to the BF and molasses medium. Moreover, the CSL addition showed a tendency of spore concentration increase with increasing CSL concentration from 0.5 to 1.0 g/L. However, this could also be due to liquid CSL having an average solids concentration of 50%.

In contrast, the effect of YE and P additives was similar, with a slightly higher spore concentration of $2.00 \pm 0.28 \times 10^8$ CFU/mL at 48 h observed for the 0.5 g/L YE addition compared to the 0.5 g/L P addition ($1.29 \pm 0.58 \times 10^8$ CFU/mL, 48 h). However, increasing

the concentration of YE or P did not result in higher spore concentration, except for a delayed sporulation in the YE 2.0 g/L additive case at 24 h.

We also conducted additional experiments to investigate the effect of increased molasses concentration (20 g/L molasses, 10 g/L BF, 0.5 g/L YE) and SF addition (10 g/L SF, 10 g/L molasses, 0.5 g/L YE) on spore concentration. Interestingly, both experiments showed similar spore concentrations of $1.15 \pm 0.19 \times 10^8$ and $1.07 \pm 0.15 \times 10^8$ CFU/mL at 48 h, respectively. However, the results were significantly lower than in the case for YE- or CSL- enriched 10 g/L molasses and 10 g/L BF medium, thus confirming that 10 g/L is the optimal molasses concentration and BF is the most suitable nitrogen source.

3.3.2. YE, P, and CSL Combined in Minor Amounts

To explore the impact of additive combinations, we tested several variants including YE and P (both 0.5 g/L), YE and CSL (both 0.5 g/L), P and CSL (both 0.5 g/L), and YE, P, and CSL (each 0.5 g/L). However, none of these combinations showed a positive correlation with spore yield increase. Interestingly, in the group of experiments where both YE and P were added at a concentration of 0.5 g/L, we observed a lower spore yield compared to the control group with no P or CSL additives.

Based on our previous observations, we speculate that the addition of P may prevent sporulation in such flask experiments, while CSL may minimize this effect. Nevertheless, further studies are necessary to confirm these findings and investigate the underlying mechanisms.

3.3.3. $(NH_4)_2HPO_4$ and Urea Additives

To investigate the potential impact of inorganic nitrogen sources such as $(NH_4)_2HPO_4$ and urea on spore yield, we supplemented a medium containing BF (10 g/L), molasses (10 g/L), and YE (0.5 g/L) with either one of these components. This approach was inspired by a recent study by Abuhena and co-authors [6]. We chose to use YE instead of CSL in this experiment due to the availability of YE for our planned pilot-scale bioreactor experiments, which would enable us to compare the results.

Our experimental results indicate that the addition of either $(NH_4)_2HPO_4$ or urea did not improve spore yield (Figure 3). Interestingly, we observed that the sporulation ratio of sporulated cells was higher in the presence of urea than in its absence. However, despite this difference, there was no significant increase in spore yield.

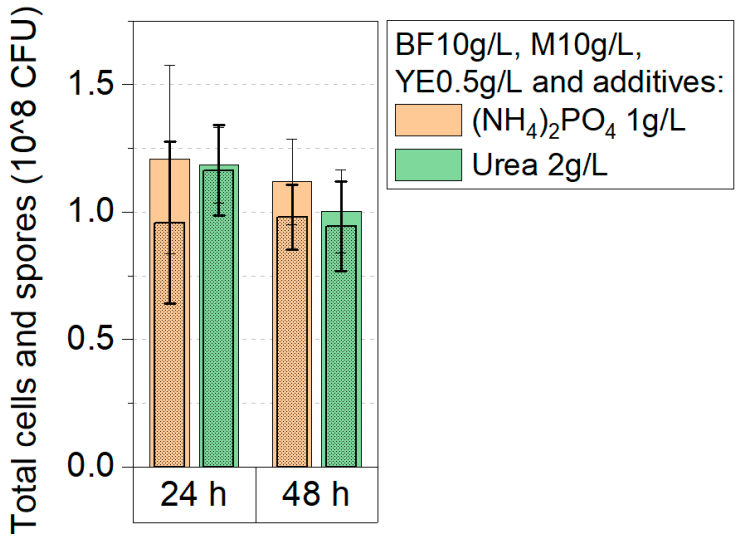

Figure 3. *B. subtilis* MSCL 897 cultivation in medium containing broad bean flour (BF) 10 g/L, molasses (M) 10 g/L, yeast extract (YE) 0.5 g/L and combinations of $(NH_4)_2HPO_4$ and urea. Total cell (outer bars) and spore (inner bars) counts at 24 and 48 h.

It is worth noting that our findings differ from those of a recent study by Abuhena and co-authors [6], where the addition of $(NH_4)_2HPO_4$ and urea was found to increase spore yield. However, we did not include the results from $MgSO_4 \cdot 7H_2O$, K_2HPO_4, or KH_2PO_4 addition experiments, as their addition, contrary to their study, did not result in a notable improvement in spore yield.

3.4. Reported Cultivation Mediums

Finally (in step 6), we evaluated the efficacy of complex and rich culture media compositions used for Bs cultivation in flasks by Posada-Uribe et al. [9], Monteiro et al. [12], and Khardziani et al. [10]. Although the recipes shared similarities, such as the use of glucose and being rich in yeast extract, peptone, or meat extract, different salts and their concentrations were utilized. The spore concentrations achieved under specific conditions ranging from low $0.15 \pm 0.04 \times 10^8$ CFU/mL (48 h) (media composition from Khardziani et al.) to average 0.87 ± 0.34–$1.10 \pm 0.82 \times 10^8$ CFU/mL (48 h) (media compositions from Posada-Uribe et al. and Monteiro et al., respectively). According to Monteiro et al., the media showed highly variable spore concentrations (high SD). The differences and similarities among the investigated broth compositions suggest that the lowered productivity in Khardziani's et al. medium may be due to the absence of some inorganic components, including $MnCl_2$, NaCl, KCl, $FeSO_4$, and $Ca(NO_3)_2$.

4. Discussion

A low-cost medium based on broad bean flour (BF) and molasses, supplemented with minor amounts of corn-steep liquor (CSL) or yeast extract (YE), has been identified as the optimal medium for achieving high *Bacillus subtilis* MSCL 897 spore concentrations and high sporulation efficiency (>90%) in 48-h flask cultivations.

To add more context to our reported results, we analyzed the approximate costs of both the reported mediums from the literature and compared them with the estimated costs of our suggested medium. For comparison purposes, the price of components was obtained from the Sigma–Aldrich catalog (if listed) at the highest available quantity. We estimate that the costs of reported mediums are approx. 1.50 EUR/L for Posada-Uribe et al. [9] medium, 2.17 EUR/L for Monteiro et al. [12] medium, and 0.88 EUR/L for Khardziani et al. [10] medium. In contrast, the costs of our presented medium are around 0.05 EUR/L (molasses and BF prices obtained from local suppliers), indicating, on average, a 17 to 43-fold decrease in price compared to the reported mediums. Since medium costs usually constitute roughly 30–60% of total production costs [21], the potential savings present a significant benefit for industrial production.

Interestingly, the endospore yields we achieved in the previously reported mediums were also significantly lower than the authors reported. The most likely cause for these low endospore yields is the fact that different Bs strains were used in each cultivation. In fact, Khardziani et al. [10] used a strain isolated from dairy and the strain from Posada-Uribe et al. [9] was isolated from the rhizosphere of banana plants in Colombia. Therefore, the difference in reported endospore yields may arise from the genetic differences characteristic of the origin of each strain.

The obtained results provide several important and novel conclusions. Firstly, our study reveals that the investigated Bs strain performs better in a BF medium compared to a soybean flour-based (SF) medium, despite the latter being more commonly used in available *Bacillus* spp. PGPF production examples. Secondly, our findings suggest that the addition of YE, peptone (P), or CSL in a concentration range of 0.5 to 1.0 g/L may significantly enhance spore productivity. However, beyond this range, increasing the additive concentration did not lead to any positive effect on spore concentration. It is important to note that, apart from the uncontrolled and low-aerated flask cultivation setup, other factors may limit the growth of Bs and spore formation under the investigated conditions.

The C:N (carbon:nitrogen) ratio plays a crucial role in the sporulation of *Bacillus* bacteria [22]. Sporulation is triggered by the depletion of nutrients in the environment,

and a balanced C:N ratio (where both C and N are depleted simultaneously) is necessary for efficient sporulation [13,18]. This could explain why increasing the concentrations of YE, P, or CSL beyond a certain point (>0.5–1.0 g/L) did not increase spore productivity and may have even decreased it. In addition, in the experiments where the medium was supplemented with $(NH_4)_2HPO_4$ or urea, we hypothesize that a lower spore yield was achieved due to an elevated N content in the medium, which prevented both C and N from being depleted simultaneously, thus hindering sporulation.

Several studies have demonstrated that the method of inoculum preparation can have a significant impact on the experimental outcomes in Bs cultivations [23,24]. This is because using sporulating or sporulated cells as inoculum can result in prolonged and sluggish Bs growth. To achieve optimal results, inoculum cells should be in the exponential growth phase. Different inoculum preparation methodologies have been reported in the literature for Bs cultivation, including the use of frozen cell stocks [7,12,22] or agar-plated cells [9,10,13] for seed material inoculation. In our study, we used a methodology that allowed the cells to remain in the exponential growth phase after 24 h of seed material cultivation (data not shown).

5. Conclusions

Our findings indicate that a culture medium containing broad bean flour (BF) (10 g/L) and molasses (10 g/L) resulted in a spore productivity of $1.35 \pm 0.47 \times 10^8$ CFU/mL after 48 h. Further enhancement of the culture medium by adding minor amounts (0.5–1.0 g/L) of yeast extract (YE) or corn-steep liquor (CSL) led to a significant increase in spore productivity, with yields of $2.00 \pm 0.28 \times 10^8$ and $2.34 \pm 0.18 \times 10^8$ CFU/mL at 48 h, respectively. In addition, the sporulation efficiency was >80–90%. Our approach, which utilized a low-cost medium primarily made from locally available and renewable raw materials, resulted in a high spore yield of the *Bacillus subtilis* MSCL 897 strain, highlighting the competitiveness of this medium.

Further investigation is needed to evaluate the economic potential of the identified medium composition variant for *Bacillus subtilis* MSCL 897 pilot and production-scale bioreactor processes. However, the promising results obtained in shake flasks suggest that this medium could be a potential candidate for further investigation in larger-scale bioreactor systems.

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References

1. Blake, C.; Christensen, M.N.; Kovács, Á.T. Molecular Aspects of Plant Growth Promotion and Protection by *Bacillus subtilis*. *Mol. Plant-Microbe Interact.* **2021**, *34*, 15–25. [CrossRef] [PubMed]
2. Yoshida, K.-i.; van Dijl, J.M. Engineering *Bacillus subtilis* Cells as Factories: Enzyme Secretion and Value-Added Chemical Production. *Biotechnol. Bioprocess Eng.* **2020**, *25*, 872–885. [CrossRef]

3. Su, Y.; Liu, C.; Fang, H.; Zhang, D. *Bacillus subtilis*: A Universal Cell Factory for Industry, Agriculture, Biomaterials and Medicine. *Microb. Cell Fact.* **2020**, *19*, 173. [CrossRef] [PubMed]
4. Stojanović, S.S.; Karabegović, I.; Beškoski, V.; Nikolić, N.; Lazić, M. Bacillus Based Microbial Formulations: Optimization of the Production Process. *Hem. Ind.* **2019**, *73*, 169–182. [CrossRef]
5. Stein, T. *Bacillus subtilis* Antibiotics: Structures, Syntheses and Specific Functions. *Mol. Microbiol.* **2005**, *56*, 845–857. [CrossRef] [PubMed]
6. Abuhena, M.; Al-Rashid, J.; Azim, M.F.; Khan, M.N.M.; Kabir, M.G.; Barman, N.C.; Rasul, N.M.; Akter, S.; Huq, M.A. Optimization of Industrial (3000 L) Production of *Bacillus subtilis* CW-S and Its Novel Application for Minituber and Industrial-Grade Potato Cultivation. *Sci. Rep.* **2022**, *12*, 11153. [CrossRef] [PubMed]
7. Chen, Z.M.; Li, Q.; Liu, H.M.; Yu, N.; Xie, T.J.; Yang, M.Y.; Shen, P.; Chen, X.D. Greater Enhancement of *Bacillus subtilis* Spore Yields in Submerged Cultures by Optimization of Medium Composition through Statistical Experimental Designs. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1353–1360. [CrossRef] [PubMed]
8. Tavares, M.B.; Souza, R.D.; Luiz, W.B.; Cavalcante, R.C.M.; Casaroli, C.; Martins, E.G.; Ferreira, R.C.C.; Ferreira, L.C.S. *Bacillus subtilis* Endospores at High Purity and Recovery Yields: Optimization of Growth Conditions and Purification Method. *Curr. Microbiol.* **2013**, *66*, 279–285. [CrossRef] [PubMed]
9. Posada-Uribe, L.F.; Romero-Tabarez, M.; Villegas-Escobar, V. Effect of Medium Components and Culture Conditions in *Bacillus subtilis* EA-CB0575 Spore Production. *Bioprocess Biosyst. Eng.* **2015**, *38*, 1879–1888. [CrossRef] [PubMed]
10. Khardziani, T.; Kachlishvili, E.; Sokhadze, K.; Elisashvili, V.; Weeks, R.; Chikindas, M.L.; Chistyakov, V. Elucidation of *Bacillus subtilis* KATMIRA 1933 Potential for Spore Production in Submerged Fermentation of Plant Raw Materials. *Probiotics Antimicrob. Proteins* **2017**, *9*, 435–443. [CrossRef] [PubMed]
11. Umar, A.; Zafar, A.; Wali, H.; Siddique, M.P.; Qazi, M.A.; Naeem, A.H.; Malik, Z.A.; Ahmed, S. Low-Cost Production and Application of Lipopeptide for Bioremediation and Plant Growth by *Bacillus subtilis* SNW3. *AMB Express* **2021**, *11*, 165. [CrossRef] [PubMed]
12. Monteiro, S.M.; Clemente, J.J.; Henriques, A.O.; Gomes, R.J.; Carrondo, M.J.; Cunha, A.E. A Procedure for High-Yield Spore Production by *Bacillus subtilis*. *Biotechnol. Prog.* **2005**, *21*, 1026–1031. [CrossRef] [PubMed]
13. de Carvalho, A.L.U.; de Oliveira, F.H.P.C.; Mariano, R.d.L.R.; Gouveia, E.R.; Souto-Maior, A.M. Growth, Sporulation and Production of Bioactive Compounds by *Bacillus subtilis* R14. *Brazilian Arch. Biol. Technol.* **2010**, *53*, 643–652. [CrossRef]
14. Harwood, C.R.; Cutting, S.M. *Modern Microbial Methods for Bacillus*; John Wiley & Sons, Inc.: New York, NY, USA, 1990.
15. Gounina-Allouane, R.; Acheuk, F.; Sahir-Halouane, F. Efficient and Cost-Effective Production of Bacillus Thuringiensis Subsp. Aizawai Spores and Delta-Endotoxins Using Agricultural Raw Materials and Agro-Industrial Wastes under Submerged Fermentation. *Bioresour. Technol. Rep.* **2022**, *18*, 101001. [CrossRef]
16. Food and Agriculture Organization of the United Nations (FAO). FAOSTAT: Corporal Statistical Database. Crops; Regions/Eurpe & World; Elements/Area Harvested & Yield; Items/Broad Beans and Horse Beans, Dry; Years/2020. Retrieved: November 2022. 2020. Available online: https://www.fao.org/faostat/ (accessed on 22 March 2023).
17. Clarke, M.A. SYRUPS. In *Encyclopedia of Food Sciences and Nutrition*; Elsevier: Amsterdam, The Netherlands, 2003; pp. 5711–5717, ISBN 9781439876732.
18. Luna, C.L.; Mariano, R.L.R.; Souto-Maior, A.M. Production of a Biocontrol Agent for Crucifers Black Rot Disease. *Braz. J. Chem. Eng.* **2002**, *19*, 133–140. [CrossRef]
19. Dehghan Shasaltaneh, M.; Moosavi-Nejad, Z.; Gharavi, S.; Fooladi, J. Cane Molasses as a Source of Precursors in the Bioproduction of Tryptophan by *Bacillus subtilis*. *Iran. J. Microbiol.* **2013**, *5*, 285–292.
20. Warriner, K.; Waites, W.M. Enhanced Sporulation in *Bacillus subtilis* Grown on Medium Containing Glucose: Ribose. *Lett. Appl. Microbiol.* **2005**, *168*, 97–102.
21. Stanbury, P.F.; Whitaker, A.; Hall, S.J. *Principles of Fermentation Technology*; Elsevier: Amsterdam, The Netherlands, 2017; ISBN 9780080999531.
22. Monteiro, S.M.S.; Clemente, J.J.; Carrondo, M.J.T.; Cunha, A.E. Enhanced Spore Production of *Bacillus subtilis* Grown in a Chemically Defined Medium. *Adv. Microbiol.* **2014**, *04*, 444–454. [CrossRef]
23. Mirnawati; Ciptaan, G.; Ferawati. Ferawati the Effect of *Bacillus subtilis* Inoculum Doses and Fermentation Time on Enzyme Activity of Fermented Palm Kernel Cake. *J. World's Poult. Res.* **2019**, *9*, 211–216. [CrossRef]
24. Gomaa, E.Z. Production of Polyhydroxyalkanoates (PHAs) By *Bacillus subtilis* and Escherichia Coli Grown on Cane Molasses Fortified with Ethanol. *Braz. Arch. Biol. Technol.* **2014**, *57157*, 145–154. [CrossRef]