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# In vitro evaluation of synbiotics combinations of different inulin concentrations and multi-strains probiotics based on microbial growth and digestive enzymes production

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**Abstract.** *Mohammed AA, Aslamyah S, Zainuddin, Djawad MI. 2024. In vitro evaluation of synbiotics combinations of different inulin concentrations and multi-strains probiotics based on microbial growth and digestive enzymes production. Biodiversitas 25: 3693-3702.* This study aimed to isolate and characterize inulin from sweet potatoes, and to determine the best synbiotics combination between inulin and multi-strains probiotics (MSP) (*Bacillus* spp., *Rhizopus* sp., and *Saccharomyces* sp.) using an in vitro methods. In the first phase, inulin was extracted using two methods, namely rotary evaporator and freeze-dryer, and subsequently characterized through fourier transform infrared spectrometry (FT-IR). The results showed that freeze-dryer was more effective compared to evaporator method. In the second phase, an in vitro method was used to evaluate the effect of inulin concentrations (0, 1, 3, and 5 mL), type of medium (nutrient agar (NA), potato dextrose agar (PDA)), and incubation time (24 and 48 hours) on MSP growth by measuring microbial growth and digestive enzymes production. The results showed significant differences ($P<0.05$) in microbial growth based on inulin concentration and incubation time. Higher inulin concentrations led to increased growth of all probiotics strains compared to lower concentrations in both media. In addition, the 48-hour incubation time generally led to a higher microbial growth compared to 24 hours. The findings of this study also showed distinct patterns in the production of protease and amylase enzymes, with higher doses of inulin fostering protease production and lower doses being more conducive to amylase production.

**Keywords:** Digestive enzymes production, extraction methods, inulin concentration, microbial growth, multi-strains probiotics, sweet potato, synbiotics

**Abbreviations**: MSP: Multi-Strains Probiotics, SSP: Single Strain Probiotics, GIT: Gastrointestinal Tract, FT-IR: Fourier Transform Infrared Spectrometer, AOAC: Association of Official Analytical Chemists, NFE: Nitrogen Free Extract, DP: Degree of Polymerization, NB: Nutrient Broth, PDA: Potato Dextrose Broth, CRD: Completely Randomized Design, TPC: Total plate count, CFU: Colony Forming Units, ANOVA: Analysis of Variance, SPSS: Statistical Package for Social Sciences

## INTRODUCTION

Probiotics and prebiotics are two types of feed additives that have gained significant attention due to their potential to promote beneficial effects in the host's gastrointestinal tract (GIT). Furthermore, probiotics are live microorganisms that offer health advantages to the host when provided in suitable proportions, while prebiotics are non-digestible substrates that specifically boost the growth and activity of beneficial microorganisms in the gut (Arumugam et al. 2023). The combined use of probiotics and prebiotics, namely synbiotics, can improve survival rates and modulation of intestinal microbiota (Alvanou et al. 2023). In general, the positive effect of using two or more feed additives results in three patterns, namely additivity, synergism, or potentiation (Cavalcante et al. 2020). The action of probiotics bacteria can be increased by prebiotics such as inulin due to the contribution to growth metabolism and activation (Akhter et al. 2015).

Prebiotics are beneficial compounds that provide food or energy for beneficial microorganisms (Tran and Li 2022). To improve the health of host, prebiotics are used to promote the growth of microorganisms that live in gut (Sanders et al. 2019; Rohani et al. 2021). Inulin is a type of prebiotics present in a variety of plants and foods, such as garlic, onion, agave, artichoke, sweet potatoes, among others (Cruz-Marín et al. 2023). Meanwhile, sweet potato (*Ipomoea batatas* L.) has become a research focus in recent decades because of its nutritional and functional properties as well as ease of availability and economic importance compared to other natural materials. Its leaves, stems and roots are also a valuable source of bioactive carbohydrates, lipids, proteins, carotenoids, anthocyanins, phenolic acids, and flavonoids. These bioactive metabolites have many biological activities, such as antioxidant, antidiabetic, anticancer, hepatoprotective, antimicrobial, antiulcer, and immunostimulant activities (Alam 2021). Inulin is one such bioactive compound found in sweet potatoes (Hiel et al.

2019). Dietary inulin has been shown to promote the growth of beneficial microbiota populations, including bifidobacteria and lactic acid-producing bacteria, such as *Lactobacillus* sp. (Zhu et al. 2020; Gupta et al. 2023). The administration of inulin, associated with certain microbiota fermentations, can influence host health through several physiological and metabolic processes (Régnier et al. 2023). It also provides nutritional benefits due to fermentation transforming bacteria, increasing the absorption of magnesium and calcium. According to Akram et al. (2019), the chemical structure of fermentable inulin is composed of mono-, di-, oligo-, and polyols, which are referred to as small carbohydrates. In this context, small carbohydrates are a group that are digested in the intestine and attract water into the large intestine to manage gastrointestinal diseases.

Multi-strains probiotics (MSP) provide more benefits than single-strain probiotics (SSP) and have synergistic effect (Puvanasundram et al. 2021). The effectiveness of MSP is attributed to the synergistic interactions among strains, fostering symbiosis. Meanwhile, compatibility between strains is crucial in preventing antagonism, which could have detrimental effects on the host. A significant advantage of MSP is the ability to improve the survival rate of the host (Puvanasundram et al. 2022). In aquaculture, the concept of MSP plays a crucial role in improving the non-specific immunity of aquatic animals, including both innate and adaptive immune responses. This enhancement enables the host to better defend against various pathogens and diseases, improving general well-being (Sumon et al. 2022).

The primary role of prebiotics is to support the efficient colonization and proliferation of beneficial probiotics microbes in GIT. In vitro studies are crucial for determining the specificity of prebiotics for selective stimulation of particular probiotics strains and identifying prebiotics that can improve the growth of beneficial bacteria while minimizing the growth of pathogenic microbes (Fehlbaum et al. 2018; Nogacka et al. 2020; Holmes et al. 2022). However, in vitro prebiotics selection for intestinal indigenous microbiota has received less attention in previous studies. Therefore, it remains unclear which prebiotics are the most suitable substrates for the selective proliferation of specific beneficial bacterial strains (Mugwanya et al. 2022).

There is insufficient information regarding in vitro assessment of synbiotics combinations, which is a crucial preliminary study before initiating in vivo experiments. Therefore, it is important to conduct in vitro efficacy test to elucidate the specificity of prebiotics for selectively stimulation the selected probiotics microbes. Inulin acts as prebiotics, selectively stimulating the growth of beneficial microorganisms like probiotics. This selective stimulation can be influenced by the concentration of inulin, leading to different growth patterns and metabolic activities among the probiotics strains (Darilmaz et al. 2019). Currently, there have been no studies of synbiotics combinations involving inulin as prebiotics and various probiotics sources, such as bacteria, yeast, and mold. The aim of this study was to isolate and characterize inulin from sweet potatoes and to determine the optimal synbiotics combination between different inulin concentrations and MSP (*Bacillus* spp., *Rhizopus* sp., and *Saccharomyces* sp.) through in vitro evaluations based on microbial growth and digestive enzymes production.

## MATERIALS AND METHODS

### Study area

Sweet potatoes used in this study were obtained from local markets in Makassar City, South Sulawesi, Indonesia. Furthermore, isolation of inulin was conducted at the Laboratory of Fish Parasites and Diseases, Faculty of Marine Sciences and Fisheries, Hasanuddin University, Makassar, Indonesia. Commercial inulin was obtained from Yasma Natura, Sidoarjo, East Java, Indonesia. In vitro evaluation of synbiotics was conducted at the Laboratory of Microbiology and Animal Health, Faculty of Animal Science, Hasanuddin University, from May 2023 to January 2024.

### Extraction of inulin

The process of sample preparation started with washing, peeling, and chopping of sweet potato with a medium size (1-2 mm), followed by drying in an oven at a temperature of 50°C for 48 hours. The dried sweet potatoes were then ground into flour and sieved using an 80 mesh sieve. The process of sample preparation was based on the method proposed by Kosasih et al. (2015), with some modifications.

Moreover, the extraction process included mixing of flour with distilled water in a ratio of 1:5, followed by soaking for 1 hour. The mixture was heated in a water bath at 80°C, and stirred for 30 minutes. The solution was filtered twice using double filter paper to ensure complete extraction of inulin (Kosasih et al. 2015). The solvents were evaporated through two methods. Initially, a vacuum rotary evaporator was used at 73°C with a speed of 60 rpm for 30 minutes, followed by the use of a freeze-dryer.

Inulin was precipitated by adding 95% ethanol in a ratio of 1:2, followed by homogenization and soaking for 12 hours at room temperature. Subsequently, the mixture was separated by centrifugation for 15 minutes at 5000 rpm and dried in an oven at 60°C for 6 hours (Yudhistira et al. 2020).

#### *Analysis of total sugar, sugar reduction, and degree of polymerization (DP) of isolated inulin*

The determination of total sugar content in 1% inulin was conducted using the phenol-sulfuric acid method as described by Yanti et al. (2019). Fructose solutions with concentrations ranging from 200 to 800 µg/mL were used as the standard. 1 mL of the sample was transferred into a test tube and add 0.5 mL of 5% phenol. The mixture was shaken, and 2.5 mL of $H_2SO_4$ was perpendicularly poured into the test tube. After standing for 10 minutes, solution was shaken and placed in a water bath for 15 minutes before cooling to room temperature. Subsequently, the solution was diluted with 8 mL distilled water and thoroughly homogenized. The absorption of orange-yellowish color was measured at a wavelength of 350 nm. The DNS method was used for the determination of sugar reduction (Miller 1959). Fructose solutions with concentrations ranging

from 200 to 600 µg/mL were used as the standard. 75 µL of 1% inulin and 75 µL of dinitro salicylic acid (DNS) reagent was transferred into a microtube. Further, the mixture was placed in a boiling water bath for 10 min and cooled to room temperature. The mixture was diluted with distilled water and homogenized. Average inulin degree of polymerization as calculated based on the total sugar content per reducing sugar content using the formula of Saengkanuk et al. (2011).

*Proximate analysis of isolated inulin*

The proximate analysis of isolated inulin was conducted using the standard methods by Association of Official Analytical Chemists (AOAC 2016). The analysis included the determination of moisture, crude protein (CP), crude fat (CF), crude fiber, ash, and nitrogen-free extract (NFE). Furthermore, the Kjeldahl method was used to determine the protein content by converting the nitrogen content into a protein percentage using a conversion factor of 6.25. The fat content was measured using the soxhlet method, while ash content was obtained by heating samples in a muffle furnace until a constant weight was achieved. In addition, NFE was calculated by subtracting the sum of ether extract, crude protein, crude fiber, and ash percentages from 100% of the dry matter.

*Characterization of isolated inulin*

This study used fourier transform infrared spectrometer (FT-IR) to compare and characterize the structural features of functional groups in both commercial and isolated inulin (Figure 1). 3 mg of each inulin sample was mixed with 200 mg of potassium bromide anhydrous (KBr) and ground into a fine powder using a pestle and mortar. FT-IR spectra were subsequently analyzed to identify and compare the existing functional groups, as reported by (Fares and Salem 2015; Nurdila et al. 2019). The FT-IR spectra of the samples were obtained using FT-IR spectrophotometer (FT-IR 8400S, Shimadzu, Japan) with a resolution of 4 $cm^{-1}$ and a test speed of 1 cm/s, in the range of 400-4000 $cm^{-1}$.

**In vitro evaluation of synbiotics**

The type of synbiotics used was inulin isolated from sweet potatoes as a prebiotic and MSP (commercial *Bacillus* spp., tempe yeast as a source of *Rhizopus* sp., and baker's yeast as a source of *Saccharomyces* sp.).

*Preparation of the medium*

The nutrient broth (NB) and potato dextrose broth (PDB) were made to observe the growth of MSP. According to the instructions on the product labels, 2.6 g of NB and 4.8 g of PDB were mixed and suspended in 400 mL of distilled water. The mixture was heated on a hotplate and stirred until the medium was completely dissolved. Subsequently, the prepared medium was sterilized in an autoclave at 121°C for 15 minutes (Zainuddin et al. 2008).

**Experimental design and procedure**

The experiment was conducted in a completely randomized design (CRD) with four treatments and two replicates. Each culture bottle was filled to a volume of 50 mL (Figure 2). The synbiotic combinations consisted of different concentrations of inulin (0%, 1%, 3%, and 5%) combined with MSP as follows: (i) A: 0% inulin: 0 mL inulin + 49 mL medium + 1 mL *Bacillus* spp. + 1 g *Saccharomyces* sp. + 1 g *Rhizopus* sp. (ii) B: 1% inulin: 1 mL inulin + 48 mL medium + 1 mL *Bacillus* spp. + 1 g *Saccharomyces* sp. + 1 g *Rhizopus* sp. (iii) C: 3% inulin: 3 mL inulin + 46 mL medium + 1 mL *Bacillus* spp. + 1 g *Saccharomyces* sp. + 1 g Rhizopus sp. (iv) D: 5% inulin: 5 mL inulin + 44 mL medium + 1 mL *Bacillus* spp. + 1 g *Saccharomyces* sp. + 1 g *Rhizopus* sp.

The solutions were incubated at 37°C for 48 hours (Hoseinifar et al. 2017), and the method of combination of MSP (CFU/mL) refers to Aslamyah et al. (2017).

**Figure 1.** Isolated inulin from sweet potatoes


**Figure 2.** Fermentation of synbiotics combination between isolated inulin and MSP

**Assessment of microorganism population**

The populations of probiotics were counted using the plate count method to identify the role of inulin in supporting the growth of MSP. The media used were nutrient agar (NA) and potato dextrose agar (PDA), prepared according to the instructions on the product label (PDA; 39 g/1000 mL and NA. 20 g/1000 mL). Furthermore, 1 mL sample of each concentration, was diluted in the range of $10^{-1}$ to $10^{-6}$. Plate counts were performed using the method described by Nieuwenhof and Hoolwerf (1987). Total Plate Count (TPC) was subsequently carried out with a series dilutions ($10^{-6}$) from each replication and concentration. The petri dishes were tightly sealed with parafilm and incubated for 24 and 48 hours at 37°C. In addition, the count of MSP colonies that grew was determined and expressed in colony-forming units/mL (Log CFU/mL) using a colony counter.

**Determination of digestive enzymes production**

Culture was inoculated into media and the zone was observed for clearance to detect protease and amylase activities. For amylase activity, the bacterial strains were inoculated into modified MRS media (0.5% peptone, 0.7% yeast extract, 0.2% NaCl, 2% starch, and 1.5% agar) supplemented with 0.25% of starch. After incubation, the zone of clearance was observed by adding Gram's iodine as a detecting agent. For the detection of protease activity, 50 mL of cell-free extract was inoculated into skim milk (1%) agar medium and incubated for 48 hours. The zone of clearance was subsequently observed and measured. The analysis of digestive enzymes production followed the method proposed by Tallapragada et al. (2018).

**Data analysis**

The analysis of isolated inulin included measurements of total sugar, sugar reduction, degree of polymerization, and proximate analysis, all of which were analyzed descriptively. The results were presented as means with standard deviations (SD). Furthermore, the effects of inulin concentrations, medium types, and incubation times on the growth of MSP were analyzed using a three-way analysis of variance (ANOVA). Significant differences were identified at a probability level of $P<0.05$, and the Tuckey post-hoc test was applied to compare means. All statistical analysis were performed using SPSS (Statistical Package for Social Sciences, Version 27, IBM Corporation, New York, USA).

## RESULTS AND DISCUSSION

**Isolation of inulin**

*Analysis of total sugar, sugar reduction, and degree of polymerization*

The results of total sugar, sugar reduction, and degree of polymerization of isolated inulin are presented in Table 1. Freeze-dryer method resulted in higher values for both total sugar and sugar reduction compared to evaporator method. Total sugar content in evaporator and freeze-dryer method were (23.55±1.63) and (28.78±0.31) µg/mL, respectively. Similarly, sugar reduction in evaporator method was (10.85±0.49) µg/mL, and (16.55±1.77) µg/mL in freeze-dryer method. The degree of polymerization was lower in freeze-dryer method (1.73±0.42) compared to evaporator method (2.17±0.09).

*Proximate analysis of isolated inulin*

The proximate analysis of isolated inulin is presented in Table 2. The results showed that freeze-dryer method led to lower water, crude fat, and crude protein content compared to evaporator method. On the other hand, freeze-dryer method produced a higher nitrogen-free extract (NFE) content. The ash content was relatively similar for both methods.

*Characterization of inulin*

The results of FT-IR analysis showed a strong asymmetric stretching of -OH bonds at a band around 3.000-3.500 $cm^{-1}$, with commercial inulin exhibiting a peak at 3.381 $cm^{-1}$, evaporator at 3.258 cm-1, and freeze-dryer at 3.271.95 $cm^{-1}$ (Figure 3). This characteristic absorption peaks were a clear indication of the presence of inulin in both samples. Moreover, the bands in the range of 2.800-2950 $cm^{-1}$ had medium intensity and corresponded to the asymmetric and symmetric stretching of C-H bonds in CH2 and CH3 groups.

**Table 1.** Total sugar, sugar reduction, and degree of polymerization of isolated inulin (Mean ± SD)

| Methods | Parameters | | |
|---|---|---|---|
| | Total sugar (µg/mL) | Sugar reduction (µg/mL) | Degree of polymerization |
| Evaporator | 23.55 ± 1.63 | 10.85 ± 0.49 | 2.17 ± 0.09 |
| Freeze-dryer | 28.78 ± 0.31 | 16.55 ± 1.77 | 1.73 ± 0.42 |

**Table 2.** Proximate analysis of isolated inulin (Mean ± SD)

| Methods | Proximate composition (%) | | | | | |
|---|---|---|---|---|---|---|
| | Water | Crude protein | Crude fat | Crude fiber | NFE | Ash |
| Evaporator | 47.01 ± 1.40 | 8.01 ± 1.27 | 3.71 ± 0.85 | 0.00 ± 0.00 | 35.53 ± 2.02 | 5.73 ± 1.67 |
| Freeze-dryer | 42.64 ± 0.62 | 7.21 ± 0.87 | 1.33 ± 0.31 | 0.00 ± 0.00 | 43.16 ± 1.78 | 5.67 ± 0.49 |

Note: NFE: Nitrogen-free extract

The isolated inulin showed an increased low-intensity band in the range of 1.590-1650 $cm^{-1}$, attributed to the presence of C=C bonds. Two bands at 1.000 and 1.100 $cm^{-1}$ were present in both spectra, which were associated with in-plane bending vibrations and internal deformations of CH, CH2, and OH groups from the fructose ring. Also, the peak observed in the range of 900-950 $cm^{-1}$ was more prominent in the isolated inulin spectra. This region was dominated by various stretching and deformation vibrations related to C-C, C-O stretching, C-O-H, and C-O-C deformations found in oligo- and polysaccharides.

**Microorganism population**

The growth of MSP (*Bacillus* spp., *Rhizopus* sp., and *Saccharomyces* sp.) on NA with different concentrations of inulin and various incubation times are presented in Table 3. The results showed significant differences ($P<0.05$) in the growth of probiotics at different inulin concentrations and incubation times. For *Bacillus* spp., the microbial growth at higher inulin concentrations (3 mL and 5 mL) was significantly higher than the lower concentrations (0 mL and 1 mL), with no significant difference ($P>0.05$) observed between 3 mL and 5 mL inulin at 24 and 48 hours. A similar trend was observed for *Rhizopus* sp. and *Saccharomyces* sp., whereas higher inulin concentrations led to significantly increased microbial growth. In addition, the comparison of incubation times showed that 48 hours generally led to higher microbial growth compared to 24 hours for all probiotics strains.

The growth of MSP (*Bacillus* spp., *Rhizopus* sp., and *Saccharomyces* sp.) on PDA with different concentrations of inulin and various incubation times are presented in Table 4. Similar to NA, the results showed a positive correlation between inulin concentration and microbial growth for *Bacillus* spp., *Rhizopus* sp., and *Saccharomyces* sp. Higher concentrations of inulin led to increased microbial growth, showing a potential synergistic relationship between inulin and probiotics in PDA medium. For *Bacillus* spp., there was significantly increased microbial growth with higher inulin concentrations (3 mL and 5 mL) at both 24 and 48 hours compared to lower concentrations (0 mL and 1 mL). This showed a positive correlation between inulin availability and the proliferation of *Bacillus* spp. on PDA. Similarly, *Rhizopus* sp. and *Saccharomyces* sp. showed a concentration-dependent growth pattern, showing a significant increase in microbial growth with higher inulin concentrations. The 48-hour incubation period had a more pronounced effect on microbial proliferation compared to the 24-hour duration, indicating that time played a crucial role in the growth dynamics of probiotics on PDA.

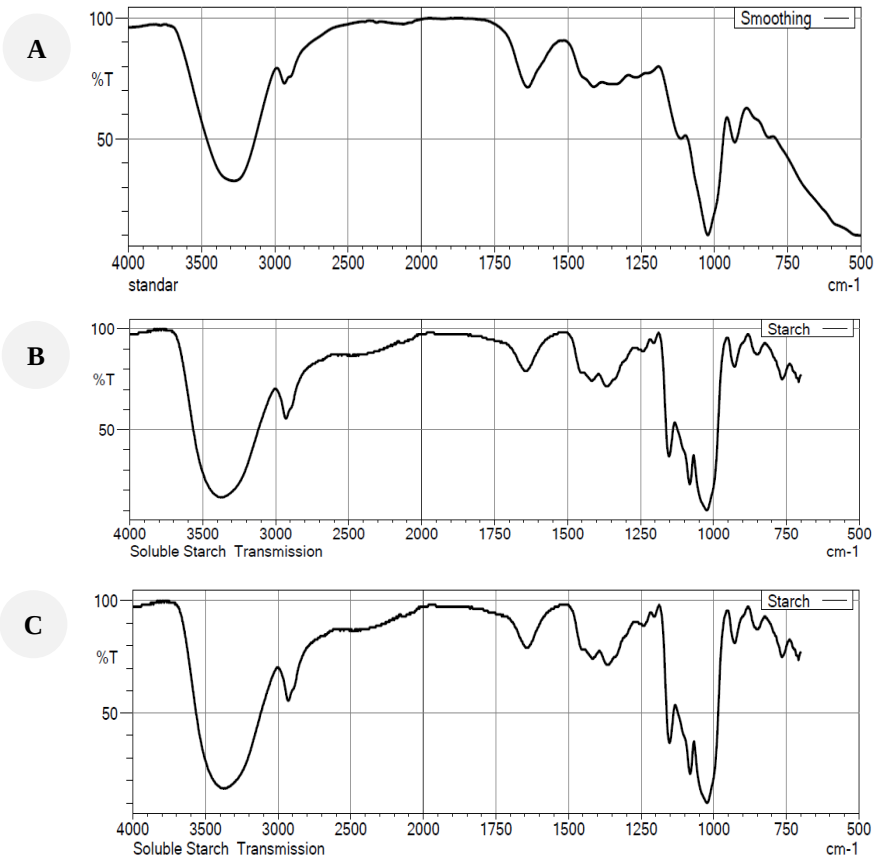


**Figure 3.** FT-IR spectra of commercial and isolated inulin from sweet potatoes. A: Commercial inulin; B: Freeze-dryer; C: Evaporator

**Digestive enzymes production**

The effect of synbiotics combinations on the production of protease and amylase enzymes is presented in Table 5. The results showed that the activity of protease enzyme was observed by a clear zone, with the biggest clear zone obtained at an inulin concentration of 3 mL, followed by 5 mL, and the smallest zone observed at the lowest inulin concentrations of 0 and 1 mL. The activity of amylase enzyme was also observed by a clear zone with the biggest zone observed at an inulin concentration of 1 mL followed by 0 mL the smallest zone observed at the concentrations of 3 and 5 mL.

**Discussion**

The concentration of inulin in sweet potatoes can vary depending on the method of measurement and the state of sweet potatoes, whether wet or dried. In this current study, inulin content in sweet potatoes was analyzed using both evaporator and freeze-dryer methods. Freeze-dryer method, which effectively reduced the water content, provided a more accurate representation of inulin concentration in dried state. Specifically, it produced an inulin of 8.75% (w/w) in the dried sweet potato samples. In contrast, evaporator method showed inulin yield of 6.88% (w/w) in the dried samples. When considering the wet weight, inulin concentration was observed to be lower due to the higher water content present in the sweet potatoes. These variations showed the importance of the drying method in accurately determining the inulin concentration, impacting the stability, shelf life, and functional properties of the isolated inulin. Previous studies have demonstrated that the choice of drying method significantly affects inulin extraction and concentration from sweet potatoes and similar sources. Freeze-drying method is widely recognized for producing higher inulin yields and superior quality compared to other methods (Savedboworn et al. 2019; Zhu et al. 2019).

Freeze-dryer method resulted in slightly lower water content compared to evaporator method, showing that freeze-dryer process effectively removed more water, beneficial for the stability and shelf life of inulin (Bhatta et al. 2020). Freeze-dryer method also produced slightly lower values for crude protein and crude fat, which could be attributed to the gentle drying conditions retaining more of the native protein and lipid content. However, further studies are needed to assess the potential impact of these differences in protein and fat content on the functional properties of the isolated inulin.

Both methods produced negligible crude fiber content in the isolated inulin, showing that the fiber-rich components of sweet potatoes were effectively removed during the isolation process, resulting in a purified form of inulin. The absence of significant fiber content is applicable where pure inulin is desired, such as in the food and pharmaceutical industries (Andrianto et al. 2022). Iinterestingly, freeze-dryer method produced a higher NFE value compared to evaporator method, confirming a higher concentration of carbohydrates in the isolated inulin. This result showed freeze-dryer process preserved the carbohydrate content more effectively, leading to a higher yield of inulin with a greater nutritional value.

**Table 5.** Diameter of zones for protease and amylase enzymes activity (Mean ± SD)

| Inulin concentrations (mL) | Clear zone diameter (mm) | |
|---|---|---|
| | Protease | Amylase |
| 0 | 0.00 ± 0.00 | 6.82 ± 1.37 |
| 1 | 0.85 ± 0.78 | 8.83 ± 0.89 |
| 3 | 7.00 ± 0.42 | 3.48 ± 0.31 |
| 5 | 0.98 ± 0.06 | 3.44 ± 0.45 |

**Table 3.** The growth of MSP (cfu/mL) on NA medium with different concentrations of inulin and incubation time

| Multi-strains probiotics | Incubation time | Inulin concentrations (mL) | | | |
|---|---|---|---|---|---|
| | | 0 | 1 | 3 | 5 |
| *Bacillus* spp. | 24 hours | 51.50 ± 4.95$^{a}$ | 59.50 ± 0.71$^{b}$ | 68.50 ± 2.12$^{c}$ | 72.00 ± 1.41$^{c}$ |
| | 48 hours | 52.50 ± 4.95$^{a}$ | 61.50 ± 0.71$^{b}$ | 73.00 ± 1.41$^{c}$ | 74.00 ± 1.41$^{c}$ |
| *Rhizopus* sp. | 24 hours | 2.00 ± 1.41$^{a}$ | 7.50 ± 2.12$^{b}$ | 14.00 ± 2.83$^{c}$ | 16.00 ± 1.41$^{c}$ |
| | 48 hours | 7.00 ± 1.41$^{a}$ | 13.00 ± 1.41$^{b}$ | 17.00 ± 1.41$^{c}$ | 20.50 ± 4.95$^{c}$ |
| *Saccharomyces* sp. | 24 hours | 14.50 ± 2.12$^{a}$ | 20.50 ± 6.36$^{b}$ | 21.50 ± 3.54$^{b}$ | 31.00 ± 8.49$^{c}$ |
| | 48 hours | 21.00 ± 1.41$^{a}$ | 31.00 ± 1.41$^{b}$ | 30.50 ± 2.12$^{bc}$ | 35.50 ± 0.71$^{c}$ |

Note: Different superscript letters indicate significant differences between treatments at the 95% confidence level (P<0.05)

**Table 4.** The growth of MSP (cfu/mL) on PDA medium with different concentrations of inulin and incubation time

| Multi-strains probiotics | Incubation time | Inulin concentrations (mL) | | | |
|---|---|---|---|---|---|
| | | 0 | 1 | 3 | 5 |
| *Bacillus* spp. | 24 hours | 46.50 ± 0.71$^{a}$ | 57.50 ± 0.71$^{b}$ | 65.00 ± 11.31$^{c}$ | 71.50 ± 2.12$^{c}$ |
| | 48 hours | 49.50 ± 0.71$^{a}$ | 54.50 ± 2.12$^{b}$ | 60.50 ± 3.54$^{c}$ | 62.50 ± 0.71$^{c}$ |
| *Rhizopus* sp. | 24 hours | 19.00 ± 1.41$^{a}$ | 29.50 ± 4.94$^{b}$ | 34.00 ± 4.24$^{c}$ | 34.50 ± 0.71$^{c}$ |
| | 48 hours | 28.00 ± 3.54$^{a}$ | 36.00 ± 1.41$^{b}$ | 42.50 ± 7.78$^{c}$ | 42.50 ± 0.71$^{c}$ |
| *Saccharomyces* sp. | 24 hours | 19.50 ± 3.54$^{a}$ | 23.00 ± 2.82$^{b}$ | 25.50 ± 4.95$^{b}$ | 29.50 ± 2.21$^{c}$ |
| | 48 hours | 24.50 ± 3.54$^{a}$ | 28.00 ± 2.83$^{b}$ | 33.50 ± 1.41$^{b}$ | 34.00 ± 1.41$^{c}$ |

Note: Different superscript letters indicate significant differences between treatments at the 95% confidence level (P<0.05)

Based on a study, comparing the effects of freeze-dryer and foam mat drying on the characteristics of inulin from gembili found that freeze-dryer was a more effective method for extracting inulin from gembili. The results showed that samples dried using a foam mat drying contained about 9.38% inulin, while those subjected to freeze-dryer had a higher inulin content (Indah et al. 2020). Inulin production was influenced by evaporation, with freeze-dryer obtaining 8.75% and evaporator resulting in 6.88%. Freeze-dryer is a highly effective method for extracting bioactive compounds from plant material, such as sweet potatoes. This process extracts up to 98% of water from samples, significantly more than traditional drying methods typically extracting only 70-80%. Also, freeze-dryer products retain more vitamins and nutritional value compared to conventionally dried products (Krakowska-Sieprawska et al. 2022). This shows the potential benefits of using freeze-dryer as a method for extracting compounds from samples, including inulin from sweet potatoes.

FT-IR spectra of sweet potato inulin samplesshowed the absorption numbers and results were very similar to those of commercial inulin. This was consistent with Melanie et al. (2015) and Akram and Garud (2020), reported similar bands for inulin. The absorption numbers of the sweet potato samples are in the range of absorption waves of functional groups, confirming the presence of inulin in the sample. According to Melanie et al. (2015), the hydroxyl group (OH) shows the main characteristics of inulin. This hydroxyl group is in the absorption range between 3550-3230 $cm^{-1}$ and has a bond band with an asymmetrical shape. Furthermore, the bands observed at 1590-1650 $cm^{-1}$, 1000-1100 $cm^{-1}$, 900-950 $cm^{-1}$, and 1030 $cm^{-1}$ were characteristic of stretching vibrations of (C–C), (C–O–C), and (C–O) groups, as reported by Grube et al. (2002). These results provided significant evidence for the presence of inulin in sweet potato samples.

Prebiotics can selectively promote the growth of probiotics (Zakariaee et al. 2021). Based on the present study, there was no information on the synbiotics combination between inulin and MSP (*Bacillus* spp., *Saccharomyces* sp., and *Rhizopus* sp). According to Oliveira et al. (2022), dietary administration of synbiotics between *Bacillus subtilis* and inulin improved growth in *Pseudoplatystoma reticulatum*, showed greater weight gain and growth performance compared to control diets. The significance of interaction dynamics between inulin and MSP was observed in the potential synergistic relationship, which could have implications for the development of synbiotics products. The results showed that higher inulin concentrations significantly increased microbial growth for MSP (*Bacillus* spp., *Saccharomyces* sp., and *Rhizopus* sp.) on both NA and PDA. In addition, the comparison of incubation times showed that a 48, hour incubation period generally resulted in higher microbial growth compared to 24 hours for all probiotics strains. Therefore, the combination of 3 mL and 5 mL of inulin concentration and a 48-hour incubation time tended to be favorable for promoting the growth of MSP on both types of agar, showing a potential synergistic relationship between inulin and these probiotics (Pandey et al. 2015; Kwoji et al. 2021). This aligns with previous findings, where inulin was reported to promote the viability of beneficial bacteria such as *Lactobacillus rhamnosus*, yielding a survival rate of 89.52% under simulated gastrointestinal conditions (Meral et al. 2024). Furthermore, the incorporation of inulin into milk with *Lactobacillus acidophilus*, *Bifidobacterium* sp. and *Lactobacillus casei* resulted in improved microbial resilience, particularly at a 4% concentration (Chutrtong et al. 2023).

The observed concentration-dependent growth pattern, as well as the positive correlation between inulin availability and MSP proliferation, showed that inulin could be a suitable substrate for the growth. Several studies supported the hypothesis that inulin acts as a prebiotic, improving the growth and counts of probiotics, and inulin supplementation can increase the relative abundance of bifidobacteria without altering microbial diversity. García-Núñez et al. (2022) and Rubin et al. (2022) found that inulin stimulated the growth of lactic bacteria and considered inulin as essential prebiotics for creating functional dairy products. Adebola et al. (2014) also examined the ability of inulin to support the growth of five probiotics lactobacilli, further supporting the potential as prebiotics. Kaewarsar et al. (2023) observed that mixed prebiotics, including inulin, fructooligosaccharides, and galactooligosaccharides, significantly improved the growth rates of *Lacticaseibacillus rhamnosus* and *Bifidobacterium*.

The mechanism behind this growth is enzymatic hydrolysis of the non-digestible polysaccharides, followed by the uptake of hydrolysis products, or even the direct uptake of small oligomers (Huynh et al. 2017; Nunpan et al. 2019; Massa et al. 2020). The one prerequisite of synbiotics is the ability of the probiotics bacteria to ferment the prebiotics components. This ability is highly specific to the probiotics bacteria and not only species- and strains-dependent but also significantly influenced by the dose, composition, and origin of the prebiotics (Zoumpopoulou et al. 2018; Zakariaee et al. 2021).

In terms of extracellular protease and amylase enzymes production, the synbiotics combinations between inulin and MSP used in this study showed distinct patterns in the production of protease and amylase enzymes. Halo zones around the colonies were indicative of positive results. Higher doses of inulin (3 and 5 mL) were found to improve protease enzyme production, attributed to the increased availability of nutrients at higher inulin concentrations. This caused probiotics bacteria to ramp up metabolic activities, including the production of protease enzymes, to support growth and proliferation. On the other hand, the production of amylase enzymes showed a different trend, with lower doses of inulin (0 and 1 mL) proving to be more conducive to enzyme production compared to higher doses. This inverse relationship showed a possible regulatory mechanism wherein excess inulin concentration could inhibit the expression or activity of amylase enzymes in probiotics strains. This inhibition could originate from metabolic feedback mechanisms or the diversion of resources toward other metabolic pathways under conditions of excess nutrient (Pereira et al. 2023). These results showed the intricate relationship between inulin concentration, probiotics growth, and enzyme production in a synbiotics context.

The observed dose-dependent responses also showed the importance of carefully optimizing the concentration of prebiotics substrates in synbiotics formulations to maximize the beneficial effects on probiotics growth and functionality. Previous studies showed that *Bacillus* probiotics species such as *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* were well known for the ability to produce high extracellular amylases, glucoamylases, proteases, cellulases, xylanases, pectinases, and lipases in their vegetative form, improving nutrient digestion and absorption in the gut (Elshaghabee et al. 2019). The growth-enhancing properties of dietary probiotics are related to the ability to secrete extracellular enzymes (protease, amylase, cellulose, etc.) (Park et al. 2022; Pawar et al. 2023). The findings of this study further supported the notion that inulin could have a significant impact on the production of these enzymes by probiotics strains, showing the importance of considering the prebiotic substrate concentration when designing synbiotics formulations.

This study concluded that there is a potential synergistic relationship between inulin extracted from sweet potatoes and MSP (*Bacillus* spp., *Saccharomyces* sp., and *Rhizopus* sp.). The extraction of inulin was more effective using freeze-dryer method, producing 8.75% (w/w) compared to 6.88% (w/w) with evaporator method. Freeze-dryer method was also superior in preserving inulin content, leading to a more stable and nutritionally valuable product. In in vitro evaluation, higher inulin concentrations (3 and 5 mL) significantly promoted microbial growth on both NA and PDA, with a 48-hour incubation period resulting in higher microbial proliferation compared to 24 hours. The optimal concentration for promoting probiotics growth and enzyme production was found at 3 mL and 5 mL of inulin with 48 hours of incubation. Distinct patterns in enzyme production were observed, namely higher doses of inulin improved protease enzyme production, while lower doses (0 and 1 mL) were more conducive to amylase enzyme production. This showed the concentration of inulin played a crucial role in regulating the metabolic activities of probiotics, impacting enzyme production.

Future studies are recommended to explore more methods for extracting inulin to optimize yield and quality. It is also crucial to conduct longer incubation studies to examine the effects beyond 48 hours and to test a wider range of inulin concentrations for the best synbiotics formulations. In addition, evaluating the practical applications and functional benefits of these synbiotics, as well as conducting in vivo studies to assess the impact on gut health and growth of fish and shrimp, provide valuable insights.

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