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Utilizing Konjac Sponge Carrier to Enhance Polyhydroxybutyrate Production Using *Bacillus Subtilis* and Loquat Seeds in Solid-State Fermentation

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Abstract

Solid-state fermentation (SSF) is an advanced bioprocess technique with several advantages; however, various challenges including nutrient heterogeneity and limited mass transfer. To address these limitations, this study investigated the use of konjac sponge as an inert carrier for *Bacillus subtilis* in an adsorbed-carrier SSF (ACSSF) system employing loquat seed hydrolysate, and examined the effects of substrate composition, moisture content, and inoculum size, which were subsequently optimized. The results demonstrate that the adsorbed carrier system enables better contact between the microorganism and the substrate, leading to boosted mass transfer and hence Polyhydroxybutyrate (PHB) production. Under the optimized conditions (pH 7.5, 35 °C, 120 h incubation, sponge depth 15 mm, and a solid–liquid ratio of 1:30), the biomass reached 0.63 ± 0.02 g/g sponge and the PHB yield was 0.49 ± 0.01 g/g sponge using the loquat seed-derived medium.

Keywords: *Bacillus subtilis*, Solid State Fermentation, Loquat Seeds, Adsorbed-Carrier.

Introduction

Despite the promise of the solid-state fermentation (SSF) process, there are various challenges associated with the culture features including the availability of nutrients and heterogeneity, which are considered significant restrictions. To overcome these constraints, it is convenient to utilize inert carrier supports that have precisely outlined properties and are biologically inert. In this paper, results obtained from SSF observations are presentedthat show the utilization of inert carrier support material in adsorbed-carrier solid-state fermentation (ACSSF)[1]. The ACSSF system involves biologically non-active materials permeable and heterogeneous material known as an inactive support, into which the inoculum and culture media are absorbed. The growth of bacteria takes place under monitored conditions of aeration internal suitable reactors preserved at unchanging temperatures. *Bacillus subtilis* is a type of Gram-positive, rod-shaped bacterium commonly found in the soil as well as in the digestive system of animals and humans. This type of bacteria is characterized by its ability to adapt to different environmental conditions; and were classified as a model for the cell growth in different substrates. They produce various enzymes under cruel conditions using SSF technique [2].

In this study, *bacillus* was selected for the applications of solid-state fermentation as adsorbed-carrier microorganisms, based on utilization in SSF processes. And the latter contributes to effective application of several substrates, production of value-added bioproducts and sustainable application of both industrial and agricultural residues. The decomposition of cellulose produces a water-soluble fraction that contains fermentable sugars, which can be used in biological processes including biopolymers [3, 4]. Manufacturing biopolymers from cheap raw materials means overcoming challenges. The use of agricultural waste, which is considered renewable energy, has low nutritional value and is cheap at the same time. These materials are considered a good solution to reduce the problem of high carbon costs because they do not compete with foodstuffs. Loquat seeds are rich in nutrients needed for the growth of microorganisms and production during the fermentation process [5].

Referring to the literature, it was found that the seeds contain a high percentage of fat, estimated at about 7-10 %, which is saturated and unsaturated fatty acids. In addition to fats, loquat seeds are rich in carbohydrates, which range from 50-65 %, represented by sugars, fiber and starch. The moisture and ash content of these seeds ranged from 10-7 %, and 2-3 %, respectively[6]. Moreover, proteins also contribute a crucial role in the formation of loquat seeds, as they range from 15-20 % (both essential and non-essential amino acids). In recent years, interest in using loquat seeds as a source for the production of value-added and biological products has increased. Several studies have highlighted the potential applications and benefits of using loquat seed in such value-added products [7].

The konjac sponge is a common type of sponge used for skin care, as it is extracted from the roots of the Amorphophallus konjac plant, which is widely distributed in Asia. The texture of this type of sponge is soft and gelatinous, so it is frequently used as an antiseptic in medical applications. In this research, konjac sponge was used as a carrier-adsorber for a medium derived from loquat seeds and *Bacillus subtilis* as a way to overcome significant problems that face in solid state fermentation. By using this method, the process improves mass transfer and enzyme activity by enhancing the contact between microorganisms, especially bacteria, and the substrate [8].

This study highlights the use of konjac sponge as an inert carrier in solid-state fermentation, using loquat seed waste as a nutrient-rich medium to support the production of PHB by *Bacillus subtilis* [9-11]. The core idea behind this approach is to enhance the interaction between the microorganism and the substrate, which in turn improves mass transfer and boosts the overall efficiency of the fermentation process. In addition, loquat seeds, an underutilized agricultural byproduct, are introduced as a sustainable and low-cost alternative to conventional carbon sources, offering a practical solution to some of the key limitations faced in traditional SSF methods [12-16].

Materials and Methods

Raw Material Preparation

Loquat seeds

Loquat seeds were collected from a local Iraqi supermarket. The seeds were immersed in water for four hours, followed by thorough washing to eliminate any remaining date flesh. Subsequently, the loquat seeds were dried overnight at approximately 65 °C. To obtain various fractions (≤ 5 mm), the dried loquat seeds were milled and sievedusing a Fisher Scientific test sieve. The resulting fine particulate was stored at a temperature of -10 °C for future use [17].

Konjac sponge

The experiment involved cutting the hydrophilic konjac sponges into various shapes. Each sponge was then weighed. The sponges had a porosity of 0.85 with a pore size ranging from 0.3-0.6 mm. The apparent density of the sponges was approximately 0.05 g/mL. These sponges were utilized as inert support materials for adsorbed-carrier solid-state fermentation. Prior to use, they underwent a thorough washing with distilled water and were subsequently dried in an oven at 65 °C for 40 [18].

Bacillus subtilis

The bacterial strain utilized in this research was *Bacillus subtilis* (GenBank Accession Number: NC_000964.3), which was obtained from the Department of Biotechnology, College of Science. To establish a master stock, the following procedure was carried out: a colony of *Bacillus subtilis* was reactivated by cultivating them, in 12 mL of nutrient broth number 2 and incubating them at 35 °C for 24 h. Subsequently, under aseptic conditions, the culture was moved into a 500 mL shake flask containing 120 mL of nutrient broth and incubated for 25 h at 35 °C. Cryobank bed system vials from Copan Italy were prepared and stored at -90 °C in a deep freezer at ultra-low temperatures[19].

Loquat seed-derived culture medium for bacillus subtilis growth and PHB production

The extraction of the derived media from loquat seeds involved the utilization of an autoclave machine for hydrolysis under the following conditions: 20 minutes at a temperature of 121 °C and pressure of 1 bar. The solid waste was subsequently separated by filtration. After preparing the suitable media for each case and adjusting the pH to 7 with 1 N NaOH and HCl, a single *Bacillus subtilis* colony from the Petri dish was chosen to inoculate 10 mL of nutrient broth. The culture was incubated for 25 h at 32 °C and 210 rpm. The cells were then harvested, resuspended in 10 mL of working media, and subjected to a 24 h adaptation period [20, 21]. For each shake flask, 5 mL of the inoculum culture was added for inoculation, with growth conditions maintained at 33 °C and 200 rpm throughout all experiments.

The fermentation process was conducted using 1L conical flasks, with every flask containing 50 g of konjac sponge. The flasks were sterilized at 121 °C for 20 minutes. The konjac sponge was impregnated with a specific volume of medium derived from Loquat seeds. The nutrient broth and the medium's initial pH were set to 7, and the temperature was kept at 33 °C for 144 h. The medium was inoculated with a 10% v/v pre-culture obtained from a single colony on a Petri dish using nutrient broth medium, which was incubated for 25 h at 33 °C with agitation at 200 rpm. Throughout during the fermentation period, samples were collected at regular intervals, and each experiment was conducted in triplicate under the same conditions [22].

Biomass recovery from fermented konjac sponge

For biomass extraction from the fermented konjac sponge, distilled water was utilized [23]. In each instance, 1 g konjac sponge was placed in a conical flask, and 50 mL of distilled water was added. The mixture was then agitated for 15 minutes at a speed of 300 rpm. This extraction process was repeated four times to ensure the complete extraction of bacterial cells. The resulting filtrates from the four extractions were pooled together. Subsequently, the pooled filtrates were centrifuged at 8,000 rpm for 25 minutes, and the resulting pellets were subjected to two washes. The first wash was performed using a 0.7% NaCl solution, followed by a second wash with distilled water. Afterward, the biomass was subjected to lyophilization at 60 °C until a constant weight was achieved to determine the dry matter content and quantify the presence of PHB[24]. The biomass and PHB data were expressed as the total biomass (g biomass) or PHB content (g PHB) per unit weight of konjac sponge ($g_{\text{konjac sponge}}$).

Statistical Optimization for PHB Production

Plackett–burman experimental design for screening of various variables

The independent variables considered were temperature, carrier particle size and shape, pH, initial moisture content, incubation time, system depth, and container size. The responses measured were PHB yield (g/L) and dry cell weight (DCW, g/g sponge). A Plackett–Burman design, which is a resolution fractional factorial design, was applied in order to screen the main factors affecting PHB production with a total of 12 experimental runs for seven variables.

The Plackett–Burman design, a mathematical model, was carried out to determine significant variables that mainly impact PHB accumulation. Design Expert software 15 was applied for generating the experimental design, and the details are shown in Table 1. Seven individual variables were selected: Temperature, shape and size of the carrier particles, pH, initial moisture content, incubation time, depth of the system, and size of the container. Each independent variable was established at two levels: high and low and a total of 12-runs were arranged based on the Plackett-Burman matrix. All trials were carried out in triplicate, and average values were taken for PHB and biomass as responses.

Table 1. **Trials for seven individual experimental variables were used in the Plackett-Burman design software: input, observed and predicted values.**

Run	**Experimental Variables**							**Responses**	
	Particle size (mm)	***Incubation time (h)***	***Vessel size (mL)***	***pH***	***Temperature (°C)***	***Depth (mm)***	***Initial moisture***	***DCW (g/g)***	***PHB (g/L)***
1	125	4	250	6.5	27	125	5	0.30	0.15
2	125	4	250	7.5	27	2500	35	0.60	0.45
3	2000	7	1000	6.5	27	125	35	0.25	0.10
4	125	4	1000	6.5	35	2500	5	0.62	0.47
5	2000	4	1000	7.5	35	125	5	0.64	0.49
6	2000	7	250	7.5	35	2500	5	0.68	0.53
7	2000	4	250	6.5	35	125	35	0.48	0.33
8	125	7	1000	6.5	35	2500	35	0.58	0.43
9	125	7	1000	7.5	27	125	5	0.40	0.25
10	2000	7	250	6.5	27	2500	5	0.39	0.24
11	2000	4	1000	7.5	27	2500	35	0.63	0.48
12	125	7	250	7.5	35	125	35	0.52	0.37

Investigation of surface response methodology (SR) for PHB production

Based on the results obtained from Plackett–Burman matrix, all factors that indicated a substantial impact on biomass production were chosen for further examination in order to determine the optimum setup to enhance the biomass production by applying the SR depending on the central composite design. The meaningful factors were: incubation time, temperature, pH and depth. For this process, 12 experiments were carried out for each factor. The response function was developed by a second-order model.

The optimum biomass value and culture conditions were predicted and achieved. In order to validate the response surface model, the experimental results, achieved using the optimal condition, was contrasted with the predicted value of the biomass synthesis. Following the screening phase, a Central Composite Design (CCD) was implemented to optimize the significant variables identified. This second-order model allowed for the evaluation of interaction effects and the determination of optimal conditions for PHB production

Statistical analysis and Software

The analysis of ANOVA was carried out to investigate not only the model's significance but also the regression coefficients, for both Plackett–Burman matrix and the Central Composite Design (CCD) equation. The statistical meaningfulness of theutilized models and their lack of fit were examined by F-test. This test is carried out in order to compare the model variation with the residual, error, and variance: if the differences are close to a similar value, then their ratio is nearby to unity, hence it is less probable that any of the variables, terms, or deficiency of fits will have a substantial impact on the response surface. In CCD, the polynomial equation quality was assessed from the estimation and predicted coefficient (R_1 and R_2, respectively), which investigates how excellent the model predicts a response value. Design-Expert software 15 was applied in order to design the experiments, the regression, as well as a graphical analysis of the experimental data acquired [25].

Results and Discussion

Bacillus subtilis growth on konjac sponge

To investigate the growth of *Bacillus subtilis* by utilizing an inert support (konjac sponge), a series of fermentation experiments were conducted using an inert support impregnated with a medium derived from loquat waste seeds. The results of these experiments are presented in Fig. 1, which depicts the growth of *Bacillus subtilis* in the ACSSF fermentation system using a medium derived from Loquat seeds. As well as scanning electron micrographs of the konjac sponge after 168 h of fermentation.

From Fig. 1, it is evident that immobilized *Bacillus subtilis* exhibited the ability to grow on konjac sponge, achieving the highest biomass concentration of 0.06±0.01 g/g konjac sponge. Fig. 1B demonstrates a scanning electron micrograph highlighting the growth of *Bacillus subtilis* within the konjac sponge. According to the findings, the value of 0.06±0.01 g/g konjac sponge corresponds to approximately 6% of the carrier weight, which is expressed as bacterial dry mass. Thus, konjac sponge's porous structure offered effective immobilization sites for both adhesion and proliferation.

Additionally, Fig. 1B illustrates the effect of washing duration on biomass recovery from the konjac sponge after 144 h of fermentation. Biomass recovery was achieved after washing the sample three times and centrifuging at 9000 rpm for 15 m.

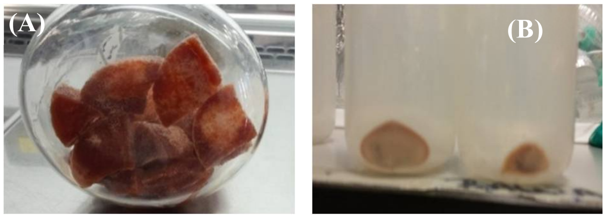

Figure 1. **Growth of *Bacillus subtilis* in a medium derived from loquat seed waste using the SSF fermentation system, with konjac sponge serving as an inert carrier support: (A) *Bacillus subtilis* immobilized and growing on konjac sponge saturated with loquat seed waste media, (B) Biomass recovered from konjac sponge**

Scanning electron microscopy (SEM) examination

Konjac sponge, due to its high internal surface area, allows for the efficient adsorption and immobilization of a large number of bacterial cells within its supporting structure. Additionally, the konjac sponge ensures a continuous and homogeneous aerobic environment throughout the entire incubation period. However, extracting bacterial biomass from the medium following fermentation remains a challenging mission. Since PHB granules, which are intracellular inclusion bodies, are produced within the cytoplasm of the microorganisms. It is crucial to efficiently extract the bacterial cells from the konjacsponge. In order to assess the effectiveness of biomass recovery from konjac sponge, SEM images were taken after fermentation, as depicted in Fig. 2.

The images clearly demonstrate the excellent growth of *Bacillus subtilis*on the konjac sponge, with minimal residual biomass remaining after three wash cycles.

Following each washing step using distilled water, the konjac sponge samples were observed and SEM images were taken. These images revealed that the repeated washing resulted in the retrieval of a significant portion of the cell biomass. Notably, the SEM images from the third wash cycle showed almost complete biomass removal. It is worth mentioning that while this method proves effective at the laboratory scale, its efficiency at the industrial scale is limited due to the large quantities of water required and the challenges associated with system control.

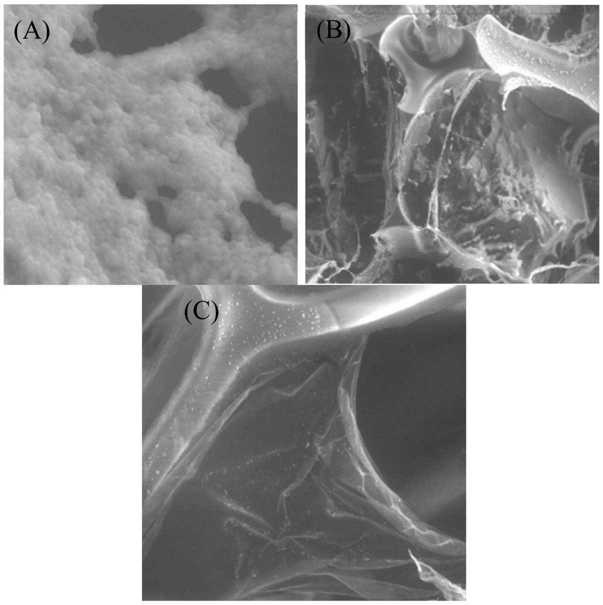

Figure 2. **Images of scanning electron micrographs: (A) konjac sponge after fermentation directly (50 µm); (B) second washing (200 µm), and (C) after third washing (200 µm)**

Impact of incubation period on cell proliferation and PHB synthesis

In *Bacillus subtilis*, determining the optimal incubation time is essential because the microorganism can produce PHB and then utilize it once nutrient depletion occurs during extended fermentation time. To investigate how incubation time affects both biomass and PHB production, the fermentation trials were carried out, in depicted, for diverse time frames, including one week. The impact of incubation period on PHB accumulation and biomass is shown in Fig. 3.

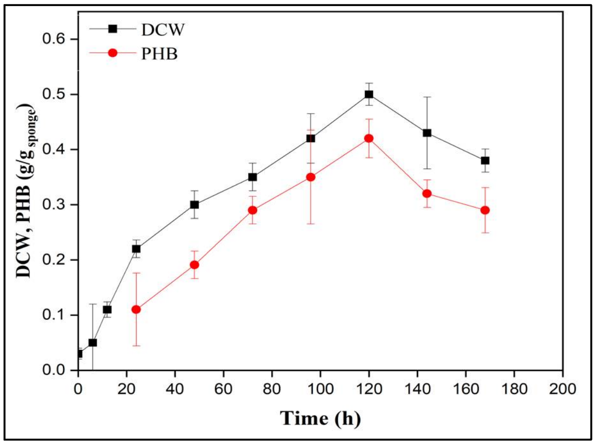

Figure 3. **Impact of fermentation time on *Bacillus subtilis* cell growth and PHB synthesis**

It is evident from Figure 3 that, in the early stages, Dry Cell Weight (DCW) and PHB concentration were minimal, around 0.23 ± 0.021 g/g sponge and 0.11 ± 0.015 g/g $_{sponge}$, respectively, through the first 24 h. In contrast, a significant growth happened subsequently, achieving a maximum value at 120 h. 0.51 ± 0.01 g/g $_{sponge}$ and 0.41 ± 0.025 g/g $_{sponge}$, followed by a reduction. The trend of PHB accumulation mirrored that of biomass, with initial production of (0.29 ± 0.013 g/g $_{sponge}$), at 48 h, gradually boosting to (0.35 ± 0.013 g/g $_{sponge}$), after 96 h.

These results showed that fermentation was extended to 168 h in order to follow the full decline profile, which in turn confirmed that 120 h was the optimal incubation period for maximum PHB yield. After this point, both biomass and PHB content dropped, most likely because Bacillus subtilis started using PHB as a reserve carbon source. It is worth noting that measurements taken after prolonged incubation were less reliable, as sampling in heterogeneous SSF systems can be challenging. For this reason, later experiments were still carried out for one week to track biomass recovery and to verify the decline pattern. The results presented in Fig. 3 represent a classical one-factor (time) experiment assessing the effect of incubation period on biomass and PHB accumulation. In contrast, CCD was applied later in the study (Fig. 6 and 7) to capture the combined influence of incubation time with other key variables such as temperature, pH, and sponge depth.

Screening effect of konjac sponge particle size on PHB

The size of particles and their specific surface area adsorber- carrier act significant roles in impacting bacterial activity and oxygen transfer rate in ACSSF. Selecting a suitable particle size is crucial to assuring an appropriate oxygen transfer rate and enhancing bacterial growth.

In this investigation, 3 g of konjac sponge with different sizes (a :125 mm^3, b: 500 mm^3, c: 1125 mm^3, and d:2000 mm^3) were used as immobilization carriers for a medium derived from loquat seeds for *Bacillus subtilis* growth and PHB accumulation. The highest biomass and PHB contents gained are shown in Fig. 4A.

Fig. 4A clearly shows that both cell biomass and PHB content increased with growing adsorber- carrier surface area and size until a specific point, after which they reduced. Generally, the findings illustrate that as the konjac sponge size expanded from size A to size C, both DCW and PHB content boosted. Nevertheless, as the size upgraded to size D, there occurred no meaningful various contrasted to size C. The maximum PHB content of 0.45 ± 0.012 g/g sponge (54%), as well as biomass of 0.072 ± 0.004 g/g sponge, were achieved with size B, whilst size A resulted in a PHB content of 0.009 ± 0.002 g/g sponge (18.6%) and cell biomass of 0.055 ± 0.003 g/g sponge. Size C yielded a maximal biomass value of 0.014 ±

0.003 g/g sponge (30%), and PHB content was 0.047 ± 0.003 g/g sponge. Finally, size D led to a PHB content of 0.014 ± 0.001 g/g sponge (29.4%) and biomass content of 0.04 ± 0.002 g/g sponge.

The effect of particle size is assigned to the opposite relationship between specific surface area and particle size. More extensive particle sizes restricted bacteria adsorption and growth because of reduced specific surface area. Conversely, lesser particle sizes result in enhanced specific surface area while decreasing the porosity. Based on the findings. It is achievable that greater particles provide lower surface area for microorganism growth whilst expanding achievable interparticle space and porosity. Conversely, lower substrate particles present the contrary a total of 12-run restricted impact. These two contrary aspects may interact and establish both the activity and bacterial growth. The oxygen transfer rate inside void spaces acts a critical role in bacterial growth, and a balance is required between substrate composition and particle sizes in order to improve mass transfer and enzyme activity. These results agree with the research by Flores-Hernández et al., 2014. [26].

Effect of konjac sponge depth on microbial growth and PHB accumulation

The depth of konjac sponge is a crucial factor that substantially impacts mass and heat transfer in SSF systems. In this investigation, the effect of four various konjac sponge depths on biomass content and PHB production was explored, and the results are displayed in Fig. 4B. The findings showed that the DCW concentration and PHB content increased with greater the konjac sponge depth and then began to reduce when the depth exceed a certain point.

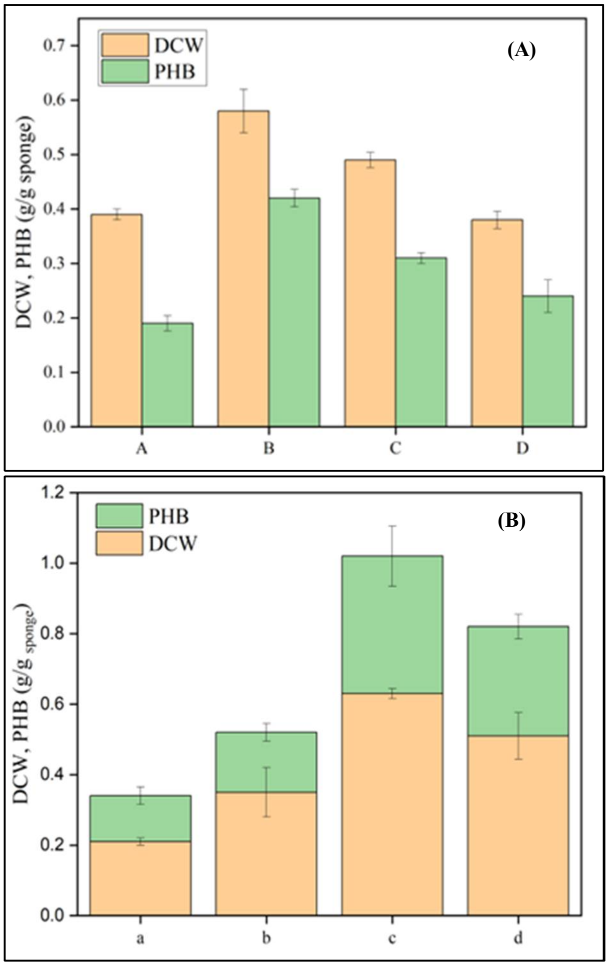

***Figure 4.* (A) Assessment of konjac sponge's effect on *Bacillus subtilis* growth and PHB accumulation in a loquat seeds waste medium (a: 5×5×5 mm^3, b: 10×10×5 mm^3, c: 15×15×5 mm^3, d: 20×20×5 mm^3). (B). Impact the konjac sponge depth on *Bacillus subtilis*growth and PHB concentration after 96 hrs. of incubation time with a medium derived from waste Loquat seeds; a: 5×5×5 mm^3, b: 5×5×10 mm^3, c: 5×5×15 mm^3, d: 5×25×20 mm^3**

The results demonstrate that both biomass content and PHB concentration enhanced as the konjac sponge depth increased, achieving a maximum at 15 mm. Nevertheless, when the depth was further increased to 20 mm, both DCW and PHB concentrations subsequently reduced. At a depth of 15 mm, the highest total DCW yield of 0.63 ± 0.021 g/g sponge and PHB content of 0.39 ± 0.01 g/g sponge were obtained. Expanding the depth of the adsorber carrier, konjac sponge, caused a meaningful enhancement in DCW and PHB content. This improvement can be attributed to the enhanced oxygen transfer rate, which promotes bacterial growth and PHB accumulation. Alternatively, beyond a certain depth, further growth negatively impacted both growth and PHB accumulation [27].The drop observed at 20 mm depth could be due to limited oxygen transfer, but this remains only a suggestion because we did not directly measure oxygen gradients or porosity. More experimen consequently inhibiting bacteria growthts will be needed to verify this explanation [28].

The reduction in cell growth and PHB accumulation with increasing depth beyond a certain threshold can be attributed to a greater negative impact on bacterial ventilation. This could be due to the expanded volume, contrasted with the surface area of konjac sponge, leading to increased oxygen transfer capability.

Initial moisture content effects on PHB production

Moisture is a significant factor in ACSSF system as bacteria need an appropriate water activity level for growth. In SSF, moisture is present in the form of absorbed or complexed internal the solid matrix, supplying benefits for growth by assisting efficient oxygen transfer. Inadequate moisture can hinder the gases as well as solutes diffusion, leading to deceleration or discontinuation of the metabolism of cells. This can happen due to insufficiency of substrate or the aggregation of restrictive metabolites nearby or within the bacterial cell.

In this investigation, multiple fermentation experiments were conducted. Utilizing various ratios of broth to konjac sponge (solid/liquid ratio ranging from 1:10 to 1:35) to study the effect of the solid/liquid ratio on biomass synthesis and PHB production. The findings, presented in Fig. 5A, disclosed that increasing the initial moisture content broadly resulted in an expansion in both biomass and PHB content. Nevertheless, there was a point at which the greatest biomass concentration was obtained, exceeding which more enhanced in moisture content led to a reduction in biomass content.

Fig. 5A spotlights that the solid to liquid ratio of 1:30 produced the maximum biomass and PHB accumulation of 0.63±0.002 g/g $_{\text{sponge}}$ and 0.49 ±0.002 g/g $_{\text{sponge}}$, respectively. This was assigned to the uniform broth absorption on the sponge surface and the lack of meaningful gradient dispersion of broth fermentation. Decreasing the solid to liquid ratio resulted in a reduction in both biomass and PHB accumulation because of the restricted number of media as well as nutrients accessible for bacterial growth. In contrast, a solid/liquid ratio of 1:35 caused the presence of free media as a result of limited water-absorbing capacity. As a result, both biomass and PHB concentration decreased to 0.51±0.002 g/g $_{\text{sponge}}$ and 0.38 ±0.002 g/g $_{\text{sponge}}$, respectively. The enhancement in liquid film thickness on the sponge surface, along with reduced porosity, limited the transfer rate of oxygen, consequently inhibiting bacteria growth. Moreover, increased moisture levels more than optimal resulted in decreased porosity and reduced oxygen transfer rate.

Investigation of Vessel size on Bacillus subtilis growth and PHB production

In order to investigate the impact of vessel size on *Bacillus subtilis* cell content and PHB synthesis, the effect of oxygen concentration that supply was further to the initial moisture study. Several fermentation trials were performed using 3 ± 0.003 g of sponge, which was supplemented with 25 mL of culture in vessels of various sizes (250 mL, 500 mL, and 1000 mL). The findings, as presented in Fig. 5B, revealed that expanding the vessel size led to enhanced growth of

Bacillus subtilis, while conversely, the PHB content reduced with greater vessel sizes.

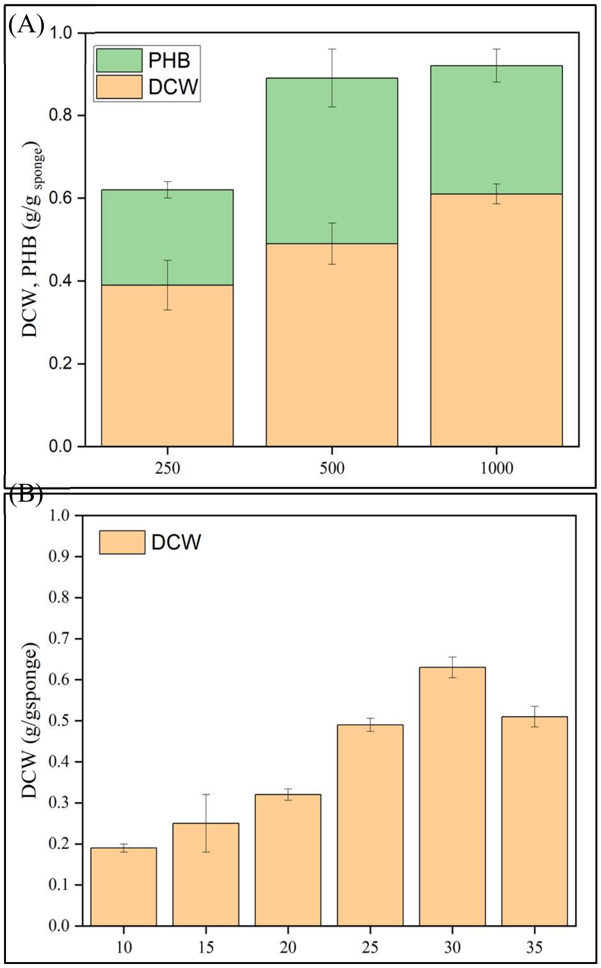

Figure 5.(A) **Effect of solid–liquid ratio on *Bacillus subtilis* biomass and PHB accumulation after 150 hrs of incubation time with a medium derived from waste Loquat seeds using ASSF.** (B) **Investigate the effect of vessel size, with PUF as a carrier support, on *Bacillus subtilis* growth and PHB production in media derived from date seed waste.**

Particularly, Fig. 5B exhibited that the highest biomass content of 0.059±0.001 g/g sponge was obtained in a 1000 mL vessel, accompanied by a PHB content of 0.31±0.002 g/g sponge. The maximum PHB content of 0.4±0.002 g/g sponge was achieved in the 500 mL vessel, with a corresponding cell biomass content of 0.49 ±0.001 g/g sponge. Oxygen performs a significant function in the bio-synthetic processes within microorganism cells. Specific bacteria, such as Bacillus subtilis, able to synthesize significant magnitudes of biopolymer, PHB, under oxygen-limited circumstances. Restricted oxygen accessibility can impact the growth and PHB accumulation of individual organisms in clear methods. Maximum oxygen transfer rates were obtained to suppress PHB synthesis [29]. Wongsirichot et al. offered that the damaging impacts of oxygen on biochemical substances because of non-specific enzyme oxidation [30]. Likewise, the effect of bacterial growth, aeration, and PHB accumulation has been presented for different bacteria in an investigation by [31].

These findings show that oxygen plays a dual role in the ACSSF system. In larger vessels, better aeration supported cell metabolism and led to more biomass. On the other hand, PHB accumulation was higher when oxygen was partly limited, since the cells redirected carbon into polymer storage. In this way, vessel size affected the balance between growth and PHB production, with intermediate aeration giving the best PHB yield.

Screening of the independent variables

The Plackett–Burman screening showed that incubation time, pH, temperature, and sponge depth were the most significant factors (Table 3). Their effects can also be seen in the response surface plots (Fig. 6) and the box plots (Fig. 7). For instance, Fig. 6A shows that higher pH and temperature improved biomass yield, while Figure 6B shows how temperature and depth worked together. In the same way, Fig. 7C points out the role of vessel size in PHB accumulation, which agrees with the ANOVA results.

The Plackett–Burman design with two levels of 12-runs was carried out to study the effect of seven individual variables: Temperature, both shape and size of the carrier particles, pH, initial moisture content, incubation time, depth of the system, and size of the container on biomass production by *Bacillus subtilis*. Experimental and predicted values of biomass synthesis are presented in Table 2. The maximum yield of DCW (0.685 g/g $_{\text{sponge}}$) was gained in run six under the following conditions: temperature 35 °C, pH 7.5, vessel size 250, depth 2500, incubation time 168 h, initial moisture 5, and with particle size 2000. While the minimum yield,0.3 g/g $_{\text{sponge}}$, was achieved at run one (temperature 27 °C, pH 6.5, vessel size 250, depth 120 µm, incubation time 96 h, initial moisture 5, and with particle size 125).

Table 3 illustrates a model F-value of 132.09, indicating that the model is statistically significant. According to Trego et al. [32], there is only a 1.29% probability that such a high F-value could be due to noise. The variable with a p-value lower than 0.05 is considered as a significant model, whilst values bigger than 0.1 mean the model terms are insignificant. The analysis revealed that variables B, D, E, and F were statistically significant factors, while variables A, C, and G were deemed insignificant. Consequently, the latter were excluded from the next phase of the study, which involved CCD experiments[33].

***Table 2*. The predicted and observed values for the experimental design of *Bacillus subtilis* biomass synthesis.**

Run Order	Actual Value	Predicted Value	Residual	Internally Studentized Residuals	Externally Studentized Residuals	Influence on Fitted Value DFFITS	Standard Order
1	0.3000	0.3000	0.0000	0.000	0.000	0.000	12
2	0.6000	0.5983	0.0017	0.186	0.162	0.228	6
3	0.2500	0.2633	-0.0133	-1.486	-1.922	-2.717⁽¹⁾	9
4	0.6200	0.6350	-0.0150	-1.671	-2.635	-3.726⁽¹⁾	5
5	0.6400	0.6333	0.0067	0.743	0.693	0.980	11
6	0.6800	0.6850	-0.0050	-0.557	-0.502	-0.710	1
7	0.4800	0.4717	0.0083	0.928	0.908	1.284	7
8	0.5800	0.5650	0.0150	1.671	2.635	3.726⁽¹⁾	2
9	0.4000	0.3917	0.0083	0.928	0.908	1.284	10
10	0.3900	0.3850	0.0050	0.557	0.502	0.710	8
11	0.6300	0.6317	-0.0017	-0.186	-0.162	-0.228	3
12	0.5200	0.5300	-0.0100	-1.114	-1.162	-1.643	4

Table 3.* Statistical analysis of the Plackett–Burman design of biomass production by *Bacillus subtilis.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.2235	7	0.0319	132.09	0.0001	Significant
A-Particle size	0.0002	1	0.0002	0.8621	0.4057	
B-Incubation time	0.0169	1	0.0169	69.83	0.0011	
C-Vessle size	0.0019	1	0.0019	7.76	0.0495	
D-pH	0.0602	1	0.0602	249.14	< 0.0001	
E-Temperature	0.0752	1	0.0752	311.21	< 0.0001	
F-Depth	0.0690	1	0.0690	285.55	< 0.0001	
G-Initial moisture	0.0001	1	0.0001	0.3103	0.6072	
Residual	0.0010	4	0.0002			
Cor Total	0.2244	11				

The interactions between two factors (pH and incubation time) with biomass synthesis were shown as a response surface in Fig. 8, which presents a visual interaction interpretation between the two variables. The 3D- response surface, Fig. 6A, shows a direct relationship between biomass synthesis, pH, and Temperature, which are diverse from (from 0 to 1.5% and from 120 h to 240 h, respectively). It is obvious that biomass yield enhanced with a raise in both pH and Temperature from 27- 35 °C and 6.5-7.5; respectively. The same pattern was obtained when the surface response, as biomass content, was plotted between temperature and depth, as presented in Fig. 6B. The maximum biomass concentration was achieved when both temperature and depth are at a high level. Fig. 6C represents the surface response, which indicates the existence of two areas of higher response, one at high temperature and the other at low pH. The maximum value obtained for the numerical factors for this response using the CCD equation is 10% for depth and 7% for pH. A reduction in biomass occurs when the pH value is raised from 6.5 to 7.5, for concentrations up to 8%, giving increase to a relative highest in the surface response. Fig. 6D indicates that biomass increases when the incubation time is at a low level, 96 h, while the DCW increases with increasing depth, reaching the highest of 0.62 g/g sponge.

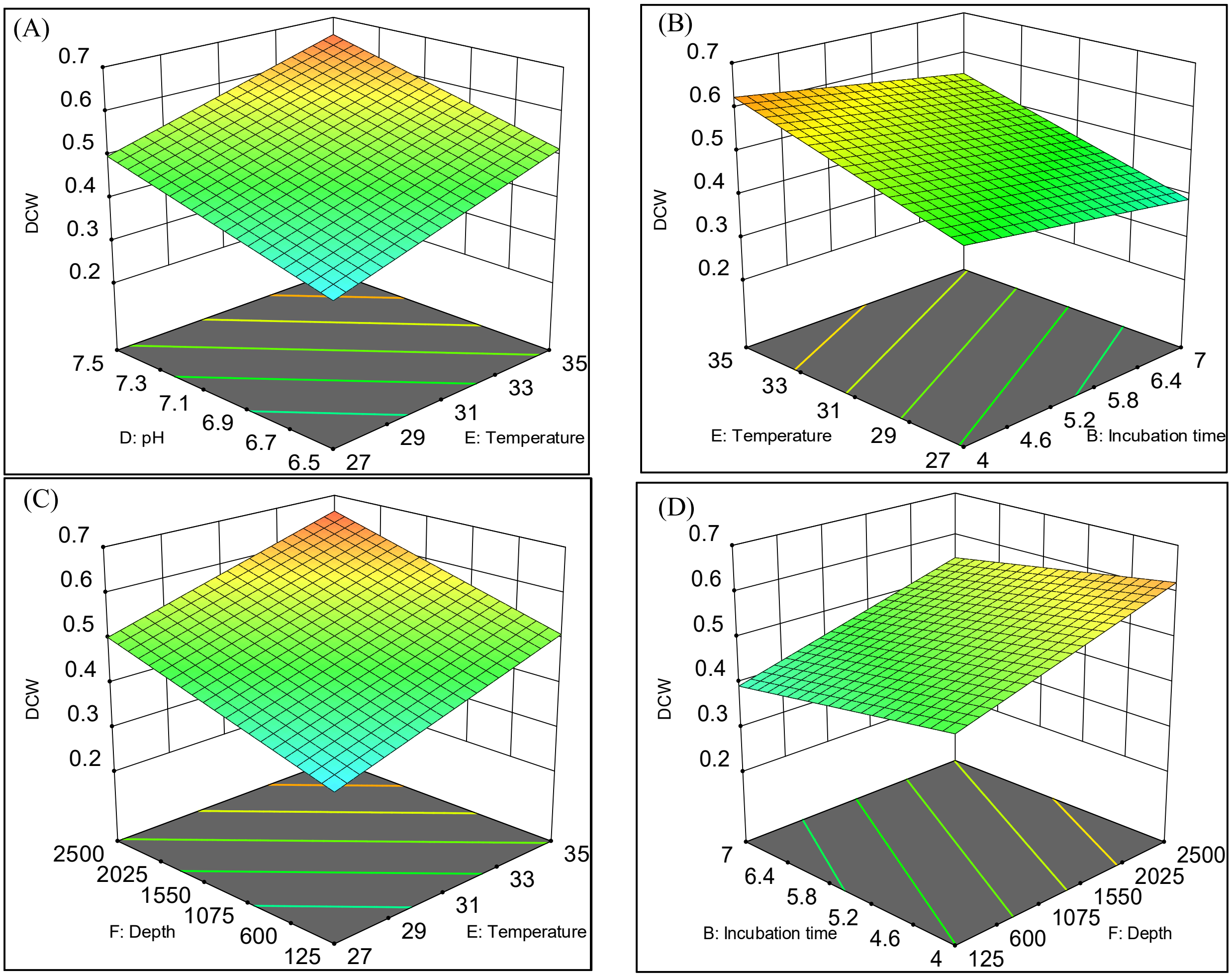

Figure 6. **3-D response surface Curves presenting the impact of interaction of different components on the *Bacillus subtilis* production (PHB accumulation) by using *Bacillus subtilis* and ACSSF system**

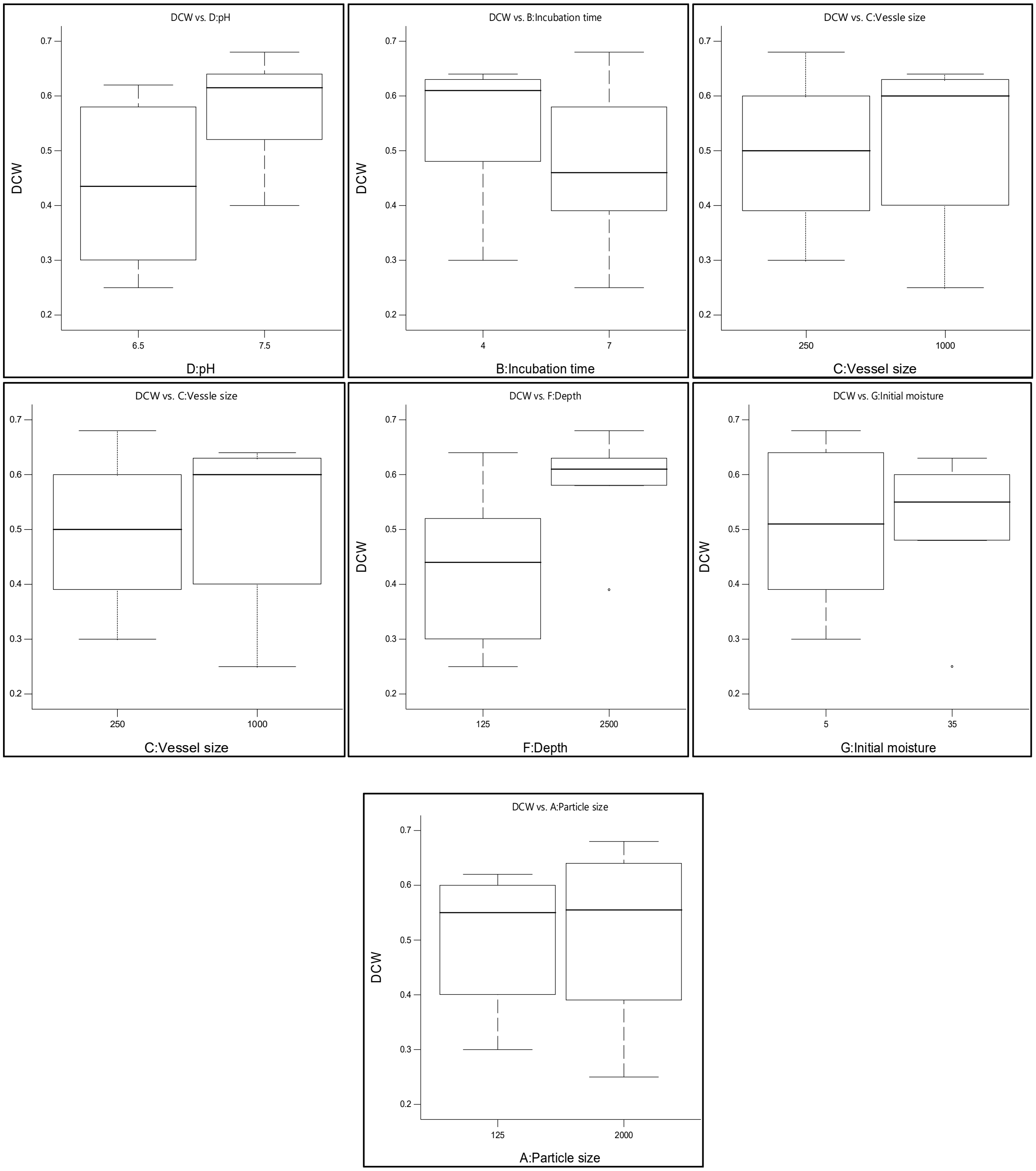

Figure 7. **Box and whisker plots of observed the impact of interaction of different components on the *Bacillus subtilis* production (PHB accumulation) by using *Bacillus subtilis* and ACSSF system**

Fig. 7 illustrates the effect of various medium components on PHB synthesis by *Bacillus subtilis* using an ACSSF system. Fig. 7D (DCW vs pH) presents a boxplot the distribution of PHB production across different pH levels. The box represents the interquartile range (IQR) of PHB production, including the middle 50% of the data at each pH level. The median line indicates the typical PHB content at a given pH, while the whiskers illustrate data variability. Any outliers in the boxplot indicate deviations in PHB accumulation at specific pH levels. Fig. 7B (DCW vs. vsincubation time): this boxplot presents how PHB synthesis fluctuates with different incubation times. The box shows the IQR for PHB accumulation at each time point, and the median line indicates the typical accumulation at that time. The whiskers extend to show the range of typical values, and any outliers would indicate unusual PHB accumulation at specific time points.

Fig. 7C (DCW vs vessel size): This boxplot displays the distribution of PHB production across various vessel sizes. The box shows the middle 50% of the data for each vessel size, and the median line presents the typical PHB synthesis for that size. The whiskers show the range of the data, while any outliers highlight unusual PHB content values for specific vessel sizes. This allows for an understanding of how vessel size influences PHB production, with the boxplot providing insight into the variability and consistency of the results for each condition.

The Plackett–Burman design (Table 3) showed that incubation time, pH, temperature, and sponge depth were the main factors affecting biomass and PHB yields. This can also be seen in the plots. For instance, Fig. 6A shows that higher pH and temperature improved biomass, while Figure 6D shows that longer incubation with greater sponge depth gave higher yields. In the same way, the box plots in Fig. 7 highlight how PHB varied with incubation time (Fig. 7B), vessel size (Fig. 7C), and pH (Fig. 7D). These visual trends match the statistical results and make the role of the key factors clearer.

Conclusion

Despite the preference for liquid fermentation in biopolymer synthesis, this study emphasizes the impact of SSF using konjac sponge on bacteria growth and subsequent PHB accumulation. It was concluded that the loquat seed hydrolysate medium immobilized on the konjac sponge is well-suited for *Bacillus subtilis* growth. Nevertheless, the choice of sponge size, depth, and initial moisture ratio is among the most significant factors to investigate. The results demonstrated that *Bacillus subtilis* effectively used loquat seeds for biopolymer accumulation, specifically PHB, demonstrating its potential as a powerful bacterium in SSF. This research provides meaningful insights into agricultural waste utilization, including loquat seeds, for sustainable bioprocessing, creating opportunities for continued investigation as well as biotechnology applications. Konjac sponge as an ACSSF of PHB synthesis offers several advantages, including efficiency enhancement, raising product yields, and tighter control of fermentation conditions, therefore, the present results indicate that SSF is considered a promising substitute for PHB production. Nevertheless, both optimization strategies and process parameters may differ depending on the microorganism applied and specific production criteria for PHB accumulation processes. The investigations also indicated that the 1.4-0.85 mm of sponge size is a feasible range of size to facilitate the maximum yield production. The greatest amount of biomass and PHB concentration was 0.169±0.03 g/g sponge and 0.4±0.003 g/ g sponge, respectively.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

References

1. L. Yafetto, Heliyon, 8 (2022) e09173. https://doi.org/10.1016/j.heliyon.2022.e09173

2. D. Marjadi and N. Dharaiya, *Eur. J. Exp. Biol.,* 3 (2013) 141.
3. G.-Q. Chen and Q. Wu, *Biomaterials,* 26 (2005) 6565. https://doi.org/10.1016/j.biomaterials.2005.04.036
4. N. V. Ramadas, S. K. Singh, C. R. Soccol and A. Pandey, *Braz. Arch. Biol. Technol.,* 52 (2009) 17. https://doi.org/10.1590/S1516-89132009000100003
5. A. K. Panda, R. K. Singh and D. K. Mishra, *Renew. Sustain. Energy Rev.*, 14 (2010) 233. https://doi.org/10.1016/j.rser.2009.07.025
6. M. Al-bachir and Y. Koudsi, *J. Food Sci. Technol.,* 27 (2021) 66.
7. R. G. Yousuf, *Novel Polyhydroxybutyrate (PHB) Production Using a Waste Date Seed Feedstock*, PhD Thesis, University of Manchester, UK (2018).
8. B. Han, C. Geng and G. Cheng, *J. Nanotechnol. Mater. Sci.,* (2018). https://doi.org/10.1155/2018/1234567
9. G.-Q. Chen and M. K. Patel, *Chem. Rev.,* 112 (2012) 2082. https://doi.org/10.1021/cr200162d
10. R. A. Verlinden, D. J. Hill, M. A. Kenward, C. D. Williams and I. Radecka, *J. Appl. Microbiol.*, 102 (2007) 1437. https://doi.org/10.1111/j.1365-2672.2007.03335.x
11. P. Zytner, A. Kowalski and M. Nowak, *RSC Sustain.,* 1 (2023) 2120. https://doi.org/10.1039/D3SU00126A
12. J. L. Castro-Mayorga, M. J. Fabra and J. M. Lagaron, *Int. J. Biol. Macromol.,* 86 (2016) 849. https://doi.org/10.1016/j.ijbiomac.2016.02.054
13. H. S. Kusuma, Biotechnol. Adv., (2024).
14. K. Khosravi-Darani, Z. B. Mokhtari, T. Amai and K. Tanaka, *Appl. Microbiol. Biotechnol.,* 97 (2013) 1407. https://doi.org/10.1007/s00253-013-4734-1
15. F. Guzman Lagunes and J. B. Winterburn, *Bioresour. Technol.,* 221 (2016) 336. https://doi.org/10.1016/j.biortech.2016.09.035
16. R. G. Yousuf and J. B. Winterburn, Bioresour. Technol., 222 (2016) 242. https://doi.org/10.1016/j.biortech.2016.09.017
17. W. Suwannasing, T. Imai and P. Kaewkannetra, *Bioresour. Technol.,* 194 (2015) 67. https://doi.org/10.1016/j.biortech.2015.06.115
18. S. Dayl and R. G. Yousuf, *Int. J. Environ. Eng. Waste Manag.,* 31 (2023) 1. https://doi.org/10.1504/IJEWM.2023.134508
19. M. Taskin and S. Erdal, *Rom. Biotechnol. Lett.,* 16 (2011) 5872.
20. Z. Gomaa, *Braz. Arch. Biol. Technol.*, 57 (2014) 145. https://doi.org/10.1590/S1516-89132014000100020
21. M. Thirumala, S. V. Reddy and S. K. Mahmood, *J. Pure Appl. Microbiol.,* 3 (2009) 735.
22. F. Matias and M. F. De Andrade Rodrigues, *Braz. J. Microbiol.,* 42 (2011) 1354. https://doi.org/10.1590/S1517-83822011000400013
23. A. Hoseinabadi, I. Rasooli and M. Taran, *Jundishapur J. Microbiol.,* 8 (2015) e19820. https://doi.org/10.5812/jjm.19820v2
24. C. Flores-Hernández, A. Colín-Cruz, C. Velasco-Santos, V. Castaño, J. Rivera-Armenta and A. Martínez-Hernández, *Polymers,* 6 (2014) 686. https://doi.org/10.3390/polym6030686
25. Y. Zhu, J. P. Smits, W. Knol and J. Bol, *Biotechnol. Lett.,* 16 (1994) 643. https://doi.org/10.1007/BF00128613

26. R. G. Bastos, F. L. Motta and M. H. A. Santana, *Appl. Biochem. Biotechnol*., 174 (2014) 708. https://doi.org/10.1007/s12010-014-1097-0
27. L. Senila, M. Roman and D. Frentiu, *Polymers,* 15 (2023) 4488. https://doi.org/10.3390/polym15234488
28. P. Wongsirichot, *Crit. Rev. Biotechnol*., (2025).
29. A. Trego, M. Hansen and R. Olsen, *Front. Syst. Biol.,* (2024).
30. P. Suriyamongkol, R. Weselake, S. Narine, M. Moloney and S. Shah, *Biotechnol. Adv*., 25 (2007) 148. https://doi.org/10.1016/j.biotechadv.2006.11.007
31. V. Ahuja, R. Singh and S. Kumar, *Microb. Cell Fact*., (2024). https://doi.org/10.1186/s12934-024-02430-0
32. T. Hahn, *Electrochem. Sci. Technol*., (2024).
33. S. González-Rojo, R. Pérez and J. Molina, *Microorganisms*, (2024).