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Research Article

Total phenolic content and *in vitro* evaluation of antioxidant activity of microbial extract of defatted biomass of mutant *Pseudochlorella pringsheimii* EMM2

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Abstract
Microalgal biomass is considered to be a renewable source for organic antioxidants. The present study aimed to evaluate the defatted biomass (DB) of the mutated green microalga *Pseudochlorella pringsheimii* EMM2 for increasing total phenolic content (TPC) extraction and antioxidant activity via microbial fermentation. Three distinct bacterial strains, *Bacillus amyloliquefaciens* (Accession number: KT276356), *B. stearothermophilus* (Accession number: KT282130), and *B. subtilis* (Accession number: KT282131) were obtained from sago industrial wastewater, utilized the hydrolysate from the DB of *P. pringsheimii* EMM2 as the exclusive medium for fermentation in this work. Among these bacterial strains, *B. stearothermophilus* was chosen for further investigation due to its effectiveness in enhancing the extraction of TPC and antioxidant activity of the hydrolysate of DB through fermentation. The fermentation conditions were optimized using a classical method, identifying the optimum physical parameters as a pH of 7.0, a temperature of 55 °C, an agitation speed of 150 rpm, and a fermentation time of 40 hours for maximizing the antioxidant activity of the hydrolysate of DB. Under these optimal conditions, a 6.11-fold increase in DPPH radical scavenging activity was observed in the hydrolysate. Consequently, this study demonstrated that the DB of the mutant microalga *P. pringsheimii* EMM2 is a valuable source for TPC production through fermentation with *B. stearothermophilus*.

Keywords: Defatted biomass, Fermentation, Hydrolysate, *Pseudochlorella pringsheimii*, Total phenolic content

INTRODUCTION

Nowadays, exploring new natural antioxidants is a key research topic that is relevant to overcoming the potential health risks and toxicity of synthetic antioxidants used in various food, cosmetic, nutraceuticals, and pharmaceutical industries. Antioxidants are considered crucial protective molecules that mitigate oxidative damage in the human body when internal enzymatic processes are deficient or ineffective (Halliwell, 1995; Jomova *et al.,* 2024). Plants are an excellent source of diverse, accepted antioxidants. These antioxidants mostly come from plants that produce food. They are divided into different groups, viz polyphenols (which include anthocyanins, flavonoids, lignans, phenolic compounds, & stilbenes), xanthophylls, carotenes, vitamin B, vitamin C, vitamin D, vitamin E and sterols (Xu *et al.,* 2017).

Despite their potential, there is still limited information and records on the sensible use of various plants to meet the growing demands of a rapidly increasing population. It is crucial to explore new natural antioxidants for use in the nutraceutical and pharmaceutical industries (Asif, 2015); microalgae are highly promising in this context (Galasso *et al.,* 2019; Sansone and Brunet, 2019; Andriopoulos *et al.,* 2022). Furthermore, culture conditions can direct the high biodiversity, photosynthetic efficiency, rapid growth, productivity, and metabolic plasticity of microalgae for use in biotechnology (Coulombier *et al.,* 2021).

Microalgae's antioxidant capacity is equal to and frequently exceeds, that of fruits and plants. Certain microalgae, specifically Chlorophyta and Eustigmatophyceae, exhibit antioxidant levels between 214 and

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258 μmol g^{-1} DM, exceeding approximately 224 μmol g^{-1} DM present in Raspberry (*Rubus* sp.) fruits (Mojaddar Langroodi *et al.,* 2019). These findings highlight the strong interest in microalgae as a promising source of antioxidants for nutraceuticals and human health. They also underscore the need for further research into this vast potential. The significant antioxidant activity of microalgae is likely due to their high content and a wide variety of antioxidant compounds, making them a rich source of diverse antioxidant molecules (Banskota et al., 2019; Khan et al., 2018).

There has been an increase in interest in fermenting natural sources to produce antioxidants for use in food and medicine. This method seeks to substitute conventionally extracted naturally occurring antioxidants increasingly deemed unfavourable due to their elevated costs (Marti-Quijal *et al.,* 2021). Fermentation is among the most ancient and effective techniques for producing and preserving food. Naturally, it reduces food volume, eliminates undesirable components, boosts nutritional value by biosynthesizing vitamins and essential amino acids, enhances food appearance, lowers cooking energy requirements, and produces safer products (Qian *et al.,*2012). Moreover, fermentation enhances the bioavailability of micronutrients and facilitates the breakdown of anti-nutritional components (Bae and Kim, 2010).

The microalgal biorefinery method offers a dual benefit by generating biofuel and animal feed from microalgal biomass, hence preventing the misuse of main crop biomass for biodiesel and the manufacture of livestock feed (Lum *et al.,* 2013). Sander and Murthy (2010) demonstrated that producing 24 kg of microalgal biodiesel yields 34 kg of co-products, comprising DB, glycerin and unsaponifiable fats. The defatted algal biomass (DAB) serves as a source for biomethane synthesis (Sarat Chandra *et al.,* 2014), bioethanol (Narmatha *et al.,* 2024), biohydrogen, short-chain carboxylic acids (Kumar et al., 2018), and animal feed supplements (Sorensen *et al.,* 2017). To present knowledge, there is no published research on the antioxidant properties of DB from mutant *P. pringsheimii* EMM2 through microbial fermentation. The primary objective of the present work was to (i) screen the bacterial strain for the fermentation of hydrolysate of DB, (ii) optimize the fermentation conditions of DB using the selected strain to improve the extraction of total phenolic content (TPC), and (iii) conduct the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging experiment.

MATERIALS AND METHODS

Materials

Nutrient agar, 2,2,-Diphenyl-1-picrylhydrazyl (DPPH), reagents, and analytical grade chemicals were obtained from Himedia Chemicals, Merck Chemicals Ltd., (Mumbai, India) and Sigmae-Aldrich (Bommasandra, India).

Double mutant microalga

Native freshwater green microalga *Pseudochlorella pringsheimii* KMAS7 (Accession Number: OR879176.1) was isolated from Kannamangalam Lake (Latitude 12.75 ˚N & Longitude 75.15 ˚E), located in Arni Taluk's, Tiruvannamalai District, Tamil Nadu, India and purified in an axenic culture. The wild strain *P. pringsheimii* KMAS7 was subjected to Ultra-violet (UV-C) treatment for 15 minutes, followed by exposure to 1.75 M ethyl methane sulfonate for random mutagenesis, resulting in the double mutant strain *P. pringsheimii* EMM2. The double mutant strain was preserved and rejuvenated monthly on the modified Chu 13 medium (Tansakul *et al.,* 2005). Cultures were maintained under white fluorescent illumination (33μmol photons m^{-2} s^{-1}) under a 12:12 h light:dark phase at 25±1°C. It was utilised for biomass generation from municipal wastewater.

Biomass production from ultrasonic pre-treated municipal wastewater

Municipal wastewater (MWW) was collected from discharge of a primary sedimentation tank in a MWW treatment plant in Kanchipuram municipality, Tamil Nadu, India. The ultrasonic pre-treatment of MWW was conducted using modified technique of Dhandayuthapani *et al.,* (2022). The pre-treatment was conducted in a 250 mL stainless steel container that contained 100 mL of 75% MWW for 20 minutes at 0.35 W mL^{-1} (25 kHz) using a probe-type sonicator (Lark Innovative Fine Teknowledge, Chennai, India). Ultrasonic pre-treated municipal wastewater (UPMWW) served as the sole culture medium for mutant *P. pringsheimii* EMM2 biomass production. The experiment was performed in 5 L Erlenmeyer flasks containing 2 L of UPMWW. 10% (v/v) of a new inoculum was inoculated in UPMWW to commence growth, with the flasks maintained at an agitation speed of 120 rpm as well as 25 ± 1°C under white fluorescent illumination of 33 μE $m^{-2}s^{-1}$, adhering to a 12:12 h day-night cycle for fifteen days. At the end of the experiment, biomass was harvested and used for defatted biomass (DB) preparation.

Hydrolysate preparation from defatted biomass

The biomass was harvested by centrifuging samples from the culture broth at 14,000 rpm for 15 minutes in order to produce defatted biomass. The extracted biomass was subsequently centrifuged and washed twice with deionised water. After cleaning, the biomass was desiccated completely in a hot air oven at 60°C. The Bligh and Dyer method (1959) was employed to extract lipids from the desiccated biomass. Subsequently, the biomass defatted (lipids removed) was dried at room temperature (28±2 °C) and employed in this investiga-

tion. 25 gL^{-1} of DB was grounded well with mortar and pestle with deionized water. This mixture was considered hydrolysate and used for fermentation to extract the phenolic compound.

Bacterial strains and preparation of inoculum

Three distinct *Bacillus* species, namely *Bacillus amyloliquefaciens* (GenBank database Accession No.: KT276356), *B. stearothermophilus* (GenBank database Accession No.: KT282130), and *B. subtilis* (GenBank database, Accession No.: KT282131), were isolated from a soil sample contaminated with sago industrial wastewaster and deposited at PG & Research Department of Botany, Arignar Anna Govt. Arts College, Cheyyar. All three strains were maintained in a nutrient agar slant and revived monthly. The inoculum was prepared by transferring a loopful of bacterial strains into 50 mL of nutrient broth in aseptic conditions. Following inoculation, the broth was subjected to aerobic incubation in an incubator with orbital shaking at 30 °C with 120 rpm agitation for 48 hours. The immobilized cell preparation seed culture was developed on the newly produced inoculum.

Preparation of immobilized bacterial cells

Each bacterial cell immobilization was performed separately. Sodium alginate slurry (6%) was prepared by dissolving 6 g of sodium alginate in 100 ml deionized water and autoclaved at 121 °C for 15 minutes. 100 mL (10^7 cells mL^{-1}) of actively grown *B. amyloliquefaciens*, *B. stearothermophilus* and *B. subtilis* culture was centrifuged at 15,000 rpm for 5 min. The supernatant was discoid and the pellet was collected. The harvest yeast cells (pellet) were washed twice with deionized water and added to the alginate slurry. The slurry reached an even viscosity after 10 minutes of stirring. We then placed the slurry into a clean tube and dropped it 5 cm into a 0.2 M calcium chloride solution. It was then left to cure at 4°C for an hour. Three times, clean deionized water was used to rinse the dried beads. When not used, the beads were kept in the fridge in a 0.9% sodium chloride solution. The whole process of preparation was done in a clean environment with a laminar flow machine (Adinarayana and Ellaiah, 2004).

Batch fermentation of hydrolysate by immobilized bacterial cells

A 250 mL Erlenmeyer flask with 100 mL of hydrolysate was used for the batch fermentation. Before the immobilised bacterial cells were inoculated, 1N NaOH or HCl was used to bring the hydrolysate's pH down to 7.0. After 15 minutes of autoclaving at 121 °C and 15 pounds of pressure, the media were allowed to cool to ambient temperature. Then the hydrolysate medium was inoculated with 10 % (w/v) freshly prepared immobilized cells of *B. amyloliquefaciens*, *B. stearothermophilus* and *B. subtilis* individually. For 48 hours, the culture was continuously shaken at 120 rpm in an orbital shaker incubator set at 28±2 °C. The TPC was assessed every 8 hours following the withdrawal of the sample. The unfermented hydrolysate served as the control. Based on the fermented hydrolysate's maximum TPC, a specific strain of bacteria was chosen and employed for further optimisation research.

Fermentation conditions optimization

To determine the optimal fermentation conditions for enhancing the extraction of TPC from hydrolysate through fermentation with a selected immobilized bacterial strain, four key process parameters were studied: pH (6 to 8), temperature (30 to 60 °C), agitation speed (100 to 300 rpm), and fermentation time (0 to 48 hours). The one-parameter-at-a-time method was used to find the best settings for these variables. To ensure they were repeated and all the tests were done three times.

Total phenolic content estimation

The TPC of fermented DBH was estimated using the Folin-Ciocalteu method (Singleton and Rossi, 1965). To match the Spectrophotometer's detectable range, the samples were diluted. One mL of diluted Folin-Ciocalteu reagent (1:10) was combined with a 200 µL sample. Following a 4-minute incubation period, 800 µL of saturated sodium carbonate solution (75 gL^{-1}) was added to the mixture, and the entire mixture was allowed to stand for two hours at ambient temperature. After centrifuging the material for ten minutes at 6000 rpm, a UV-Visible spectrophotometer was used to measure the supernatant at 765 nm. The optical density of the sample was interpolated using a gallic acid calibration curve (0-500 mgL^{-1}), and the TPC was computed and reported as gallic acid equivalents (GAE) per gram of dry defatted biomass.

***In vitro* antioxidant activity**

According to the procedure outlined by Qureshi *et al.* (2010), the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging experiment was carried out. An ethanolic DPPH solution (0.2 mM) and fermented hydrolysate (1 mL each) were combined to form the mixture. Then 1 mL of an ascorbic acid solution (200 micrograms per millilitre in ethanol) was added to this mixture. In a dark incubator set at 37°C for 25 minutes, the mixture was vigorously mixed for 30 seconds. With a mixture of 1 mL ethanol and 1 mL DPPH serving as the blank, the activity of DPPH in reducing radicals was detected at 517 nm by a UV-Visible spectrophotometer. The substance that was used as a positive control was butylated hydroxytoluene. The experiment was carried out three times, and the amount of DPPH that scavenged radicals was estimated by averaged the

results using the following equation,

Radical scavenging activity (%) = [(Abs control - Abs samples)/Abs control] X 100 …..Eq.1

Statistical analysis

The data from each experiment was shown as the mean± Standard Deviation (SD) from three separate experiments. To analyze the collected data, MINITAB 12 software was used to perform a one-way analysis of variance (ANOVA). A significance level of $p < 0.05$ was set.

RESULTS AND DISCUSSION

Extraction of total phenolic content from hydrolysate of defatted biomass by microbial fermentation

In the present study, as observed in Fig. 1, extracts from hydrolysate by fermentation using three different bacterial strains showed positive results of enhancement extraction of TPC from hydrolysate of DB. Nevertheless, among the three bacterial strains, the maximum total phenolic content of 13.75±0.06 mg GAE g^{-1} DW was achieved from the hydrolysate of DB by fermentation by *B. stearothermophilus*. This was 2.55-fold higher than the ethanol/water extraction of TPC of 5.40±0.28 mg GAE g^{-1} WD from biomass of *Scenedesmus* sp. ME02 (Bulut *et al.,* 2019). This study did not use any solvent to extract TPC from hydrolysate of DB. Whereas in the control (unfermented-water extract), a very low TPC of 1.46 ±0.06 mg GAE g^{-1} WD was observed. Extraction of natural phenolics from plant materials by enzymatic treatment is a very useful technique. Because in the fermentation process, various carbohydrases like xylanase, pectinases, cellulases, etc., are produced by the microorganisms that can release the bound phenolics into soluble form (Dey and Kuhad, 2014; Bautista-Hernandez *et al.,* 2022; Narmatha *et al.,* 2022). The current investigation did not employ any solvent for the extraction of TPC from the hydrolysate of DB. The only method used was bacterial fermentation.

Antioxidant activity of fermented hydrolysate of defatted biomass

The DPPH test was utilised to assess the antioxidant efficacy of the fermented hydrolysate of DB. This experiment assessed the tested antioxidants' capacity to reduce the DPPH radical by either direct transfer of electrons or eliminating radicals via a hydrogen atom transfer. (Prior *et al.,* 2005). In the present investigation, DPPH radical scavenging activity ranged from 54.66 ± 0.04% to 67.89 ± 0.12% in the fermented hydrolysate of DB. As depicted in Fig. 2, the peak DPPH radical scavenging activity of 67.89 ± 0.12% was recorded in the hydrolysate of DB following fermentation with *B. stearothermophilus*. Hence, this strain was selected and used for further study. This was 5.45-fold higher DPPH radical scavenging activity than the unfermented hydrolysate of DB.

Effect of pH on fermentation of hydrolysate of defatted biomass

This needs to be done to better understand the factors that affect the fermentation of DBH by *B. stearothermophilus* to use it for ecofriendly extraction of natural TPC. The ideal pH for augmenting the activity of DPPH in the scavenging of hydrolysate of DB by fermentation by *B. stearothermophilus* was examined within a range of 6 to 8, rising in increments of 0.5. The pH of DB fermentation was adjusted using 1N NaOH/HCl. The present study found that the initial pH level of culture medium was one of the key factors influencing the growth of *B. stearothermophilus*. As depicted in Fig. 3, the highest activity of DPPH in scavenging of 68.12 ± 0.05% was achieved from DB fermented at pH 7.0, which favoured the growth of *B. stearothermophilus*. The maximum biomass of *B. stearothermophilus* of 15.88±0.14 g dwL^{-1} was also obtained at pH 7.0. However, Ljungqvist *et al.* (2024) found that the highest growth rate of *Geobacillus* sp. LC300 at pH 6.9. Misiou *et al.* (2021) also

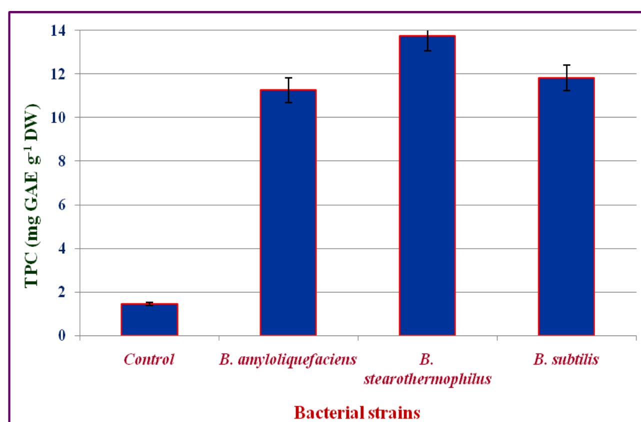

Fig. 1. *TPC of hydrolysate of defatted biomass after microbial fermentation*

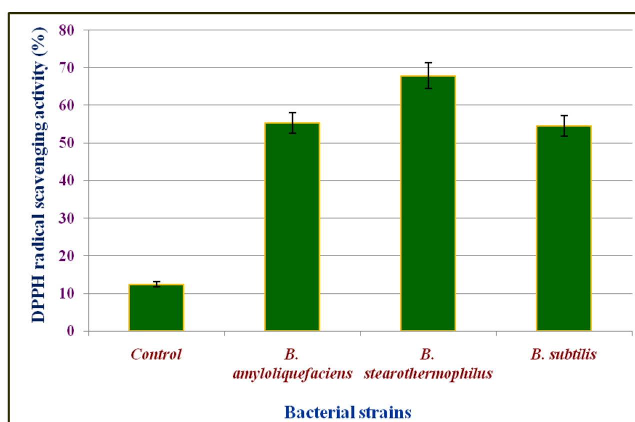

Fig. 2. *Antioxidant capacity of hydrolysate of defatted biomass after microbial fermentation*

studied the effect of pH on *Geobacillus stearothermophilus* growth rate, and they found that a pH 6.8 was the optimum for the growth of G. *stearothermophilus.* Hence, pH 7.0 was considered an optimum pH for further optimization study.

Effect of temperature on fermentation

In microbial fermentation, physical variables like temperature can directly impact productivity and product production (Reihani and Khosravi-Darani, 2019). From 30 to 60 °C, with 5 °C increments in between, the effect of temperature on improving DB's antioxidant activity via fermentation with *B. stearothermophilus* was studied. Fig. 4 shows that at 55 °C, the activity of DPPH in scavenging was at its greatest, at 73.55 ± 0.06%. Since higher temperatures hindered the growth of *B. stearothermophilus* and had a detrimental impact on fermentation, antioxidant activity declined as temperatures rose more. In addition, the biomass of *B. stearothermophilus* went up from 13.84 ± 0.05 g dwL^{-1} to 21.04 ± 0.11 g dwL^{-1} when the temperature went up from 35 °C to 60 °C. These findings imply that *B. stearothermophilus* can grow in the DB fermentation broth at a temperature of 55 °C. Generally the *G. stearothermophilus* grows at 55 to 65°C (Nazina *et al.,* 2001; Kumar *et al.,* 2021). As a result, it was determined that 55 °C was the ideal temperature, and this value was adopted for subsequent optimisation study.

Effect of agitation speed on fermentation of hydrolysate of defatted biomass

Being agitated during fermentation ensures that the fermentation media is sufficiently mixed to allow the yeast cells to more easily absorb nutrients (Rollero *et al.,* 2018). According to Mittal (1992), when the agitation speed is too high, it can cause shear pressures that harm the cell structure of microorganisms and alter their morphology, hindering product synthesis during microbial fermentation. This investigation looked at a speed range of 100–300 rpm, with 50 rpm increments, to determine the optimum agitation speed for boosting antioxidant activity in fermented DB. At 150 rpm, the agitation speed produced the highest activity of DPPH radical scavenging (74.32 ± 0.06%) and biomass (21.06 ± 0.04 g dwL^{-1}). Fermentation was unaffected by further rises in agitation speed (Fig. 5). This study is in accordance with the study of Narmatha *et al.* (2022). They also found that 150 rpm was an optimum agitation speed for extraction of TPC from defatted biomass of *Tetradesmus dimorphus* EMS2 by *B. licheniformis*

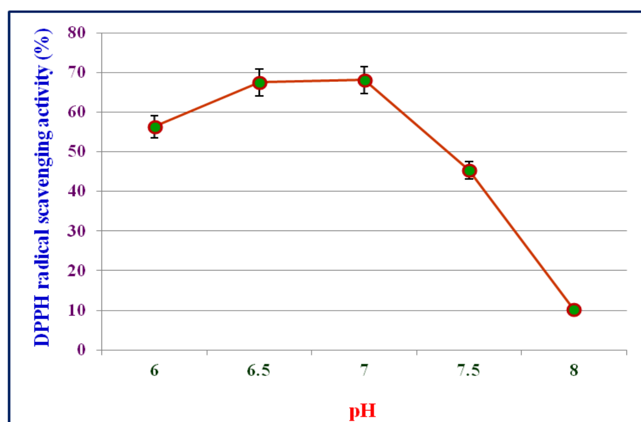

Fig. 3. *Effect of pH on fermentation of hydrolysate of defatted biomass using Bacillus stearothermophilus*

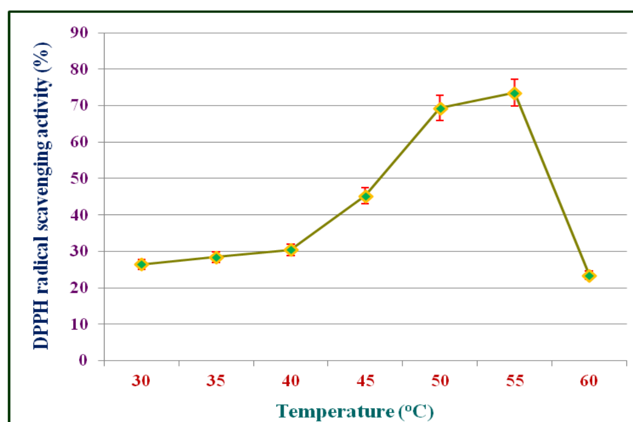

Fig. 4. *Effect of temperature on fermentation of hydrolysate of defatted biomass using Bacillus stearothermophilus*

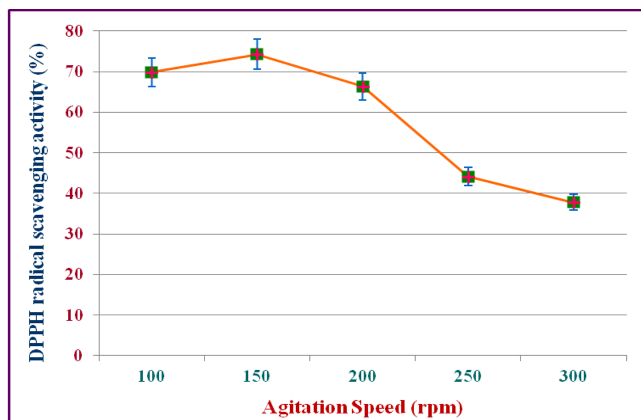

Fig. 5. Effect of agitation speed on fermentation of hydrolysate of defatted biomass using Bacillus stearothermophilus

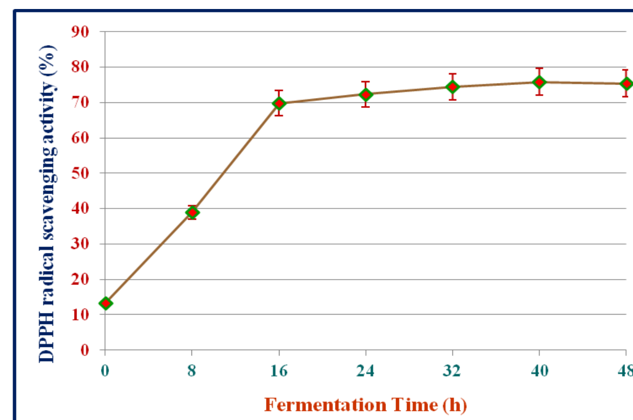

Fig. 6. *Effect of time on fermentation of hydrolysate of defatted biomass using Bacillus stearothermophilus*

SLP4 fermentation. Therefore, 150 rpm was selected as the best agitation speed for further research.

Effect of fermentation time on fermentation

The fermentation of DB was performed at 55 °C for 48 h, with continuous agitation at a speed of 150 rpm. Before fermentation, the pH of the DB was calibrated to 7.0 using 1N NaOH or HCl, followed by autoclaving at 121 °C for 15 minutes at 15 pressure. The activity of DPPH in scavenging radicals was assessed every 8 hours utilising the fermented extract. Fig. 6 shows that the peak radical scavenging activity of DPPH was 75.84 ± 0.05%, observed at a fermentation duration of 40 hours. The present observation showed no significant changes in the DPPH radical scavenging activity followed during this period. Researchers acknowledge polyphenolic compounds' antioxidant properties and consider them among the most potent antioxidants (Zakaria *et al.,* 2011; Andriopoulos *et al.,* 2022). Some studies reported that the solvent's polarity primarily influences the solubility of natural antioxidants (Bulut *et al.,* 2019; Jerez-Martel *et al.,* 2017). Narmatha *et al.* (2022) found that bacterial fermentation improved the ability to extract phenolic compounds from microalgae's DB and its antioxidant level. The results of this study demonstrated that bacterial fermentation was a superior approach for extracting phenolic compounds from microalgal biomass compared to solvent extraction.

Conclusion

In the present study, the hydrolysate was prepared from the DB of the mutant microalga *P. pringsheimii* EMM2 and used as a source for extracting TPC through microbial fermentation, with an assessment of its antioxidant activity. Based on TPC yield, *B. stearothermophilus* was identified as the best bacterial strain for extracting TPC from DB via fermentation. The optimal conditions for enhancing the antioxidant activity of DB during fermentation with *B. stearothermophilus* were found to be a pH of 7.0, a temperature of 55 °C, and an agitation speed of 150 rpm. Under these conditions, the DPPH radical scavenging activity of 75.84 ± 0.05% was observed in the fermented extract at 40 hours, representing a 6.11-fold increase compared to the unfermented (water extract). Therefore, the DB of the mutant microalga *P. pringsheimii* EMM2 can be considered a potential natural source for antioxidant production. Notably, this study did not use any solvent to extract the TPC, and *B. stearothermophilus* was the most effective organism for microbial extraction of TPC from DB.

Conflict of interest

The authors declare that they have no conflict of interest.

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