Optimization of ultrasound-assisted production of ergosterol from *Penicillium brevicompactum* by Taguchi statistical method

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Abstract Ergosterol as a primary metabolite and precursor of vitamin D2, is the most plentiful mycosterols in fungal cell membrane. Process optimization to increase the yield and productivity of biological products is a topic of interest. Ultrasonic waves have many applications in biotechnology, like cell disruption, and enhancement of primary and secondary metabolites production. This study disclosed an optimal condition for ultrasound-assisted production (UAP) of ergosterol from *Penicillium brevicompactum* MUCL 19,011 using *L* *₉* Taguchi statistical method. The intensity (IS), time of sonication (TS), treatment frequency (TF), and number of days of treatment (DT) were allocated to study the effects of ultrasound on ergosterol production. The results were analyzed using Minitab version 19. The maximum ergosterol, 11 mg/g cell dry weight (CDW), was produced on the tenth day while all factors were at a low level. The days of treatment with a contribution of 45.48% was the most significant factor for ergosterol production. For the first time, this study revealed the positive effect of ultrasound on the production of ergosterol. Ergosterol production increased 73% (4.63 mg/g CDW) after process optimization. Finally, a mathematical model of ultrasound factors with a regression coefficient of $R^2=0.978$ was obtained for the ergosterol production during ultrasound treatment.

Keywords Ergosterol · Optimization · *Penicillium brevicompactum* · Ultrasound-assisted extraction (UAE) · Design of experiments (DOE) · Taguchi statistical method

Introduction

Ergosterol, a 5,7-diene oxysterol, is a primary sterol in cell membranes of filamentous fungi (Pasanen et al. 1999). Moreover, it is a precursor of vitamin D2 (ergocalciferol) which has a significant role in human body absorbing Ca$^{2+}$, and PO$_4^{3-}$ (Lavová et al. 2020). Measuring ergosterol content is a proper method for determination of total fungal biomass due to its vigorous relation to cell dry weight (CDW) of fungi (Pasanen et al. 1999). The amount of ergosterol depends on fungal species, age of the culture, growth...
phase, hyphal formation, sporulation, and growth conditions such as growth media, pH, and temperature (Pasanen et al. 1999; Tešanović et al. 2017). Ergosterol content range has been varied from 2.3 up to 11.9 µg of ergosterol/mg of dry mycelium for hyphomycetes and ascomycetes (Gessner and Chauvet 1993; Newell 1994). Furthermore, the ergosterol content range for Aspergillus, Penicillium, Fusarium, Rhizopus, Cladosporium, Candida, and Alternaria species has been reported from 0.4 to 14.3 µg/mg of dry weight (Pasanen et al. 1999).

Filamentous fungi contain a crucial crowd of industrial microorganisms. They are used for the production of a vast array of products including primary and secondary metabolites (Pazouki and Panda 2000). Some examples of primary metabolites are sterols, amino acids, organic acids, nucleotides, polysaccharides, fatty acids, alcohols, and enzymes. Primary metabolites, significant metabolites for the growth of microorganisms, are produced in the logarithmic phase of growth (Singh et al. 2017).

The morphology of P. brevicompactum in submerged cultures is divided into two main categories: the filamentous form and the spherical or ellipsoidal colonies. The second category is known as pellets. For cellular and hyphal growths, many processes take place at the tip of the hyphae, i.e. the formation of the youngest cell (Pazouki and Panda 2000). Through time, cell autolysis occurs and the filamentous state prevails (Cui 1997). Filamentous growth causes a highly viscous broth with non-Newtonian and pseudoplastic flow behavior (Kristiansen 1988). High viscosity has a negative impact on mass transfer properties of broth, especially the gas-liquid mass transfer rate. Pelleted growth displays low viscosity and Newtonian flow behavior (Chain et al. 1966). Pellets can be classified into three groups: (a) fluffy loose pellets, the center is compact and the outer zone is much looser, (b) compact smooth pellets, the whole pellet is compact and the outside of the pellet is smooth, and (c) hollow smooth pellets, the center of the pellet is hollow through autolysis and the outside is smooth (Metz and Kossen 1977). This classification is in agreement with the observations of Steel and Clark (Clark 1962; Steel et al. 1954).

Type and amount of inoculum, medium composition, pH, and temperature might affect morphology, rheology, and fungi growth (Pazouki and Panda 2000). Ultrasonic irradiation is a physical factor which stimulate filamentous fungi to enhance some fermentation products (Behzadnia et al. 2020).

Ergosterol extraction can be performed via various methods such as homogenizing and refluxing in methanol (Newell et al. 1988), maceration, Soxhlet, ultrasound-assisted extraction (UAE), supercritical fluid, microwave-assisted extraction, and accelerated solvent (Wang and Weller 2006). Even though UAE consumes a large number of samples, it is a cost-effective and a rapid technique (Wang and Weller 2006).

Ultrasonic waves have a frequency range above 20 kHz which are inaudible for humans and have a great ability to enrich biotechnological products (Chisti 2003). The frequency of ultrasound is classified into three parts: (1) power ultrasound with a frequency of 20 up to 100 kHz; (2) high-frequency ultrasound which is beneficial for sonochemistry with a range of 20 kHz up to 2 MHz; and (3) diagnostic ultrasound with a frequency of more than 1 MHz which is exploited in medical imaging (Behzadnia et al. 2020). Low-frequency ultrasound, below 100 kHz, can increase the production of metabolites in microbial cultivations while ultrasound with a frequency higher than 100 kHz is extensively used for cell disruption applications (Vargas et al. 2004). Ultrasonic waves are usually generated by three devices: (1) ultrasonic bath (low frequency, 20–45 kHz) contacted with culture indirectly with numerous applications in biotechnology, pharmacy, and food industry (Kwiatkowska et al. 2011), (2) sonicator or ultrasonic probe (operated at a frequency of 350 kHz or higher) contacted with culture directly which can be used for cell disruption (Santos et al. 2009), and (3) the modern system named low-intensity pulsed ultrasound (LIPUS) which can be applied to stimulate the cells (Fontana et al. 2021) and to enhance the production of biological medicines. Recently, this system has been used for the improvement of secondary metabolites production such as mycophenolic acid, tissue engineering application, biomass treatment to release sugars for fermentation process (Zhao et al. 2012), enhancement of monoclonal antibody production in Chinese hamster ovary cells (Zhao et al. 2014) as well as stimulation of pre-osteoblast bone cell mineralization (Tassinary et al. 2018).

The application of ultrasound in low and medium intensity, which is not pernicious for microorganisms and is useful for improving microorganism
activity, is one of the most attractive aspects of ultrasound applications (Sinisterra 1992). Ultrasound irradiation to fermentation broth remarkably increased the production of various products such as mycophenolic acid from *P. brevicaules*um, fibrinolytic enzyme from *Bacillus sphaericus*, daunorubicin from *Streptomyces peucetius*, bioethanol from *Saccharomyces cerevisiae*, cellulase from *Trichoderma reesei* (Avhad and Rathod 2014; He et al. 2021; Raskar et al. 2014; Subbedar and Gogate 2015; Zhao et al. 2012). Newell et al. revealed a reliable method in the field of ergosterol extraction. They disclosed that homogenization (two times for 2 min) and reflux operation (2 h in methanol) are two equally efficient methods in the process of ergosterol extraction (Newell et al. 1988).

Variability in process parameters has an impact on the performance of the process and the quality of products. Therefore, monitoring and controlling the process parameters are prerequisites to ensure the optimum production with desired quality. Enhanced characterization of the process aligns with the design of experiments (DoE) concept which requires understanding the influence and causality of factors and parameters. Taguchi’s design is a well-planned optimization tool for the robust design which offers a simple and systematic approach to optimize the design for performance, and quality as well as the cost. As a rule, the Taguchi method relies on an orthogonal array which decreases the experimental runs and thus optimization cost is reduced, while the entire set of parameters involved in the process is studied (Montgomery 2017). Statistical analysis based on univariate testing such as analysis of variance (ANOVA) (Box et al. 1978) can be used to evaluate the influence of investigated factors.

In the present work, ultrasound-assisted production (UAP) of ergosterol from *P. brevicaules*um MUCL 19,011 was studied. The aim of this research is to find the optimized ultrasonic parameters to achieve increased ergosterol production. For this goal, the effects of treatment frequency (1, 2, and 3 times treatment per day), number of days of treatment (2, 3, and 4 days), intensity (low, medium, and high), and time of sonication (5, 10 and 15 min) on ergosterol production were studied. Using L9 Taguchi statistical method, the effect of the aforementioned factors on ergosterol production was described. The results show that the highest ergosterol amount for *P. brevicaules*um among filamentous fungi was 11 (mg/g CDW).

**Materials and methods**

**Microorganism and inoculum preparation**

*Penicillium brevicaules*um MUCL 19,011 was prepared from the Belgian coordinated collection of microorganisms (BCCM, Brussels, Belgium). The stock culture was maintained on potato dextrose agar (PDA, Merck, Kenilworth, New Jersey, United States) slant, and stored at 4 °C. The cultures were carried out in a 250 mL shake flask containing 50 mL of Ozapeck (g/L) (glucose, 80; glycine, 20; enzymatically hydrolyzed casein, 15; KH2PO4, 3; MgSO4·7H2O, 0.5 and 1 mL/L of trace elements mixture including (g/L): FeSO4·7H2O, 2.2; CuSO4·5H2O, 0.3; ZnSO4·7H2O, 2.4; MnSO4·4H2O, 0.16; and KMnO4, 0.2); YEG (g/L) (yeast extract, 20; glucose, 100; KH2PO4, 5; MgSO4·7H2O, 0.5; and 1 mL/L of trace elements mixture of Ozapeck medium); YEGI (g/L) (yeast extract, 20; industrial glucose, 100; KH2PO4, 5; MgSO4·7H2O, 0.5; and 1 mL/L of trace elements mixture of Ozapeck medium); YEGB (g/L) (yeast extract, 20; sucrose, 150; MgSO4·7H2O, 0.5; CuSO4·5H2O, 0.005; ZnSO4·7H2O, 0.01).
Apotex (Zhao et al. 2012) (containing (g/L): glucose, 150; glycine, 14; KH₂PO₄, 1; Citric acid, 2; MgSO₄·7H₂O, 1 and 1 mL/L of trace elements mixture, which included (g/L): FeSO₄·7H₂O, 1.714; CuSO₄·5H₂O, 2; ZnSO₄·7H₂O, 5.906; MnSO₄·4H₂O, 0.171; Na₂MoO₄·2H₂O, 3 and Boric acid), ZS (containing (g/L): glucose, 80; glycine, 9; enzymatically hydrolyzed casein, 15; KH₂PO₄, 5; MgSO₄·7H₂O, 1 and 1 mL/L of trace elements mixture, which included (g/L): FeSO₄·7H₂O, 2.2; CuSO₄·5H₂O, 0.3; ZnSO₄·7H₂O, 2.4; MnSO₄·4H₂O, 0.16; and KMoO₄, 0.2), BWY (containing (g/L): sweet whey as a source of nitrogen, 20; wheat paddy as a carbon source, 150; MgSO₄·7H₂O, 0.5; ZnSO₄·7H₂O, 0.01; CuSO₄·5H₂O, 0.005) and CWY (containing (g/L): sweet whey as a source of nitrogen, 20; corn as a carbon source, 150; MgSO₄·7H₂O, 0.5; ZnSO₄·7H₂O, 0.01; CuSO₄·5H₂O, 0.005) were also used to study the morphology of the fungus.

In each experiment, except for glycine, glucose, and trace elements, medium components were autoclaved separately at 121 °C for 15 min. To prevent the occurrence of caramelization, glucose was autoclaved at 110 °C for 10 min. Glycine and the trace elements mixture were sterilized by a 0.2 µm filter (Millipore, Billerica, MA, USA). The pH values of the prepared media were adjusted to 6.0 using 2 N HCl or NaOH solutions (Ardestani et al. 2010).

Fermentation process

A rotary shaker incubator (Jeitech, Geumcheon-gu, Seoul, Republic of Korea) was used for batch cultivation. Experiments were carried out in a 250 mL flask containing 50 mL culture media inoculated with 0.5 mL of the spore suspension (∼2.5 × 10⁷ 1/mL) and incubated at 28 °C with an agitation rate of 200 rpm for 12 days.

Sampling and sample preparation for analysis

The flasks were sampled consecutively once a day to obtain the CDW. The medium with maximum CDW was selected as the main medium for further studies. Ergosterol concentration was measured on the tenth day for the selected medium. At each stage, a flask with 50 mL of liquid medium was used as a sample. The desired flask was withdrawn from the fermentation process at appropriate time intervals and then the sample was filtered using a filter paper (Whatman, Maidstone, Kent, United Kingdom). The pellets, which represented the biomass, were used for CDW measurements and ergosterol assay. Supernatants were stored at −20 °C for the following experiments such as glucose assay.

Ergosterol extraction

Ergosterol extraction was carried out by the UAE method using an ultrasonic probe device (Model UP400S, Hielscher, Teltow, Germany). The filtered biomass samples (from the former step) were placed in glass plates and freeze-dried. Then, three grams of the freeze-dried powder, sieved by a twenty-mesh, was dissolved in 100 mL pure ethanol. Then, they were subjected to the ultrasonic device with a frequency of 24 kHz, 80% power, and 70% cycles for 15 min. After this step, the solution was poured into a sterile plate. The prepared sample was placed in an anaerobic jar connected to a condenser and vacuum pump (Millipore, model: XX5500000, EMD Milipore Corporation, Billerica, USA) to evaporate the ethanol solvent at low pressure. The precipitant was collected and powdered with mortar. Then 30 mg of the powder was dissolved in 1 mL chloroform and centrifuged at 13,000 rpm for 10 min. The supernatant was passed through a 0.2 µm filter (Millipore, Billerica, MA, USA) and preserved in a freezer at −20 °C until high-performance liquid chromatography (HPLC) analysis (Heleno et al. 2016). These steps were implemented for all specimens of L9 Taguchi design.

Analytical methods

All analyses were carried out in triplicate (n=3) and results are given as mean values with error bars (Table S1).

Ergosterol concentration

The analyses were performed by high performance liquid chromatography separation and ultraviolet detection (HPLC–UV) (Column: Ultra C18 5 µm (250×4.6 mm) Restek—USA, Agilent 1260 infinity quaternary pump VL, Agilent 1260 infinity variable wavelength detector, Agilent 1260 infinity manual injector). The column temperature was 35 °C.
standard solvent for ergosterol was chloroform. The mobile phase was methanol/acetonitrile (70:30, v/v), at a flow rate of 1 mL/min and the injection volume was 20 μL. The detection was performed at 280 nm (Heleno et al. 2016). The peak of ergosterol was observed at 9–10 min after the injection. A calibration curve for ergosterol concentration (0.125, 0.225, 0.5 mg/mL) with $R^2 = 0.998$ was obtained.

Glucose concentration

The glucose concentration was measured in the culture supernatant as reducing sugars by dinitrosalicilic acid (DNS) method (Miller 1959). A suitably diluted sample (1 mL) was mixed with 1 mL DNS reagent, held at 90 °C for 15 min, and cooled down to 25 °C. The absorbance was measured by a spectrophotometer (BECKMAN, model: DU530 UV/VIS, Pasadena, California, USA) at 540 nm against a blank of deionized water treated the same way as the sample. The measured absorbance and the dilution factor were used to estimate the reducing sugar concentration by comparing with a calibration standard curve which was obtained from standard aqueous solutions of glucose.

Cell mass concentration

Cell masses were dried in an oven (Memmert, model: UM110, Schwabach, Germany) at 60–65 °C for at least 24 h until reaching constant weight (weight remained constant at two consecutive weights for 1 h). CDW was calculated based on the dried biomass weight.

Ultrasound treatment set-up

Ultrasound treatment of the fermentation broth was carried out in an ultrasonic water bath with an internal dimension of 295 × 240 × 160 mm (Model UCP-10, 2010, Jeitech, Geumcheon-gu, Seoul, Republic of Korea). Erlenmeyer flasks were immersed and sustained at a height of 2 cm from the bottom of the ultrasonic bath all over the experiments.

The bath was equipped with four magneto-strictive transducers adjusted in a zigzag position with a maximum power output of 450 W, which was regarded as 100% power. The flasks were irradiated with low, medium, and high-intensity ultrasound of 60 kHz frequency.

Design of experiments (DoE) using Taguchi methodology

Different parameters of intensity (IS), time of sonication (TS), treatment frequency (TF), and number of days of treatment (DT) were considered as important factors for investigating the ergosterol production in the Ozapeck medium. The optimization and evaluation of these four parameters of ultrasonic treatment in ergosterol production were implemented by Taguchi method due to its effective orthogonal design. In the present study, an $L_9$ orthogonal array was chosen considering three different levels for the mentioned factors. The range of chosen levels of all parameters was summarized in Table 1.

Statistical analysis and mathematical modeling

The analysis of variance (ANOVA) for Taguchi experimental data was done using Minitab 19.0 software (version 19, Minitab LLC, Pennsylvania State, USA) based on the S/N ratio. Mean values were considered significantly different when $p < 0.05$. Furthermore, an attempt was made to develop a mathematical model relating to the effect of ultrasound on the production of ergosterol per g CDW.

| Symbol | Process parameters | Levels |
|--------|--------------------|--------|
| $IS^a$ | Intensity of sonication (mw/cm²) | Low Medium High |
| $TS^b$ | Time of sonication (min) | 5 10 15 |
| $TF^c$ | Treatment frequency per day | 1 2 3 |
| $DT^d$ | Days of treatment | 2 3 4 |

$a$ Intensity of sonication

$b$ Time of sonication

$c$ Treatment frequency

$d$ Days of treatment
Results and discussion

Fungal morphology study

The vegetative form of *P. brevicompactum* MUCL 19,011 as white mycelia appeared after 24 h on the potato dextrose agar (PDA) petri dish. After 48 h, germination was apparent by the formation of green spores. These green spores were observed as circular particles under the microscope (ZEISS, Jena, Germany) adhered together. The *P. brevicompactum* MUCL 19,011 grew in pellet form after 24 h following inoculation with the spore suspension. The pellet growth form, composed of branched hyphae, interknitted to a stable aggregate which led to less viscous fluids than the filamentous form. During the logarithmic phase of the fermentation, the pellets are proliferated, and their amount were increased. These pellets survived during fermentation for 280 h (up to the end of the stationary phase) and eventually broke and lysed. For the mycelial morphology of *P. brevicompactum* MUCL 19,011, there were many hyphae adhered together increasing the surface area of fungus and contact with the substrate. The newly grown tips in a young branch of hyphae and finally separated from the main hyphae at the septum to become a new hyphae. Generally, the number of tips (or hyphae) represents the amount of biomass accumulated in the medium (Zhao et al. 2012).

In defined media such as Apotex, ZS, and YES, the normal shape of *P. brevicompactum* (pelleted or filamentous form) could be observed, but in complex media such as YEGI, CWY, and BWY odd shapes are appeared (Fig. S1). The reason for these aggregates’ appearance is not completely understood. The basic morphology of fungal pellets in the Ozapeck medium is described by the compressed inner core composed of thickly packed hyphae. As it is shown in Fig. S2, on the first day of the logarithmic phase, the core is surrounded by a less dense hyphal layer, recognized as the hairy region. After some days, this hairy region had more fluffy hairs and the core region is more enormous than the first day. These observations are in good agreement with Vieter and Krull’s research in the field of filamentous fungi (Krull et al. 2010; Veiter et al. 2018).

In this study, the core area was investigated according to the days of logarithmic phase. Table 2 shows that the size of *P. brevicompactum* became approximately quadrupled from the beginning of the logarithmic phase to its end.

**Evaluation of fungal growth in different media**

The growth curves in different media for *P. brevicompactum* MUCL 19,011 were analyzed (Fig. 1 and Table S1). By sampling one time per day, two distinct phases were observed, namely, the logarithmic phase (exponential phase), and the stationary phase, while the lag phase could be observed with more numbers of sampling during the first day. It can be also concluded that the cells did not reach the death phase until the 12th day. Investigation of two observed phases in four media showed that:

### Table 2 Size of *Penicillium brevicompactum* MUCL19011 pellets in Ozapeck medium during logarithmic phase

| Day | Size of pellets (mm²) |
|-----|----------------------|
| 2   | 5                    |
| 3   | 14                   |
| 4   | 14                   |
| 5   | 22.5                 |

![Fig. 1](image-url) The growth profiles of *Penicillium brevicompactum* MUCL 19,011 in a 250 mL shake flask containing 50 mL of different media, a YEGI, b YEG, c YEGB, d Ozapeck, inoculated with 0.5 mL of the spore suspension (∼2.5×10⁷ 1/mL) and incubated at 28 °C with an agitation rate of 200 rpm for 12 days. All fermentations were performed in biological triplicates (n = 3). The glucose consumption (g/L) is shown as black lines (left Y-axis), and ln (cell dry weight (CDW)) values are shown as a mean with standard deviations as gray lines (right Y-axis).
• Logarithmic phase (from the beginning of the fermentation process up to day 5 for YEGI and Ozapeck/up to day 3 for YEG and YEGB): this phase concluded two parts; during the first part of the logarithmic phase the cells grew and the CDW increased with a constant slope, while the carbon source decreased rapidly. For YEGI, YEG, and YEGB, the first part of the logarithmic phase with a constant slope was observed from the beginning of the process up to the second day, while for the Ozapeck medium, the first part of this phase lasted one more day. During the second part of this phase, the slope of the growth curve was continuously decreasing until the stationary phase was reached. The results in all media indicated that the *P. brevicanentum* MUCL 19,011 consumed most of the glucose during 5–6 days after the beginning of the fermentation process.

• Stationary phase (from day 5 up to day 12 for YEGI and Ozapeck/from day 3 up to day 12 for YEG and YEGB): after consumption of the glucose, the culture reached the stationary phase. The CDW was in a constant range in the stationary phase which the growth rate is equal to the death rate. It can be perceived that the stationary phase was started approximately from day 5, however, in the YEG and YEGB medium, the stationary phase was started earlier. In the stationary phase, concentration variations of YEGB were less than that in YEG. Thus, it could be concluded that phosphate buffer could modify the growth profile. The effect of phosphate buffer on growth varied in different fungal species (Hilger et al. 1986).

Ozapeck medium was accepted for ultrasound treatment experiments because it was a defined medium and showed less concentration fluctuation in the growth graph. Moreover, in the Ozapeck medium, the appearance of equivalent spherical pellets and the formation of a low viscosity culture were reported in the literature which made this medium suitable for exploring the effect of ultrasound on ergosterol production (Grimm et al. 2005; Olsvik and Kristiansen 1994).

Optimization of ultrasound treatment parameters on ergosterol production

Ultrasound is a popular technique in the fermentation process to reduce fermentation time and enhance product yield and quality. Table 3 shows the effect of ultrasound irradiation on microbial metabolites in different studies. It is revealed that the application of ultrasounds with a low level of power, time, and treatment repetition has a positive effect on improving biological products (Avhad and Rathod 2014; Raskar et al. 2014; Subbedar and Gogate 2015; Zhao et al. 2012).

To maximize the ergosterol production, an L₉ Taguchi orthogonal array with the signal to noise (S/N) ratio was used. The S/N ratio refers to the ratio of mean value of the objective function (g ergosterol/g CDW) to its standard deviation was used to determine the deviation of the ergosterol production from the desired value. As a rule, S/N ratio can be evaluated by three different equations based on the desired objective function. As this study was intended to maximize ergosterol production, the S/N ratio was calculated by the following equation (Eq. 1) (Karna and Sahai 2012).

\[
\frac{S}{N} = -10 \log \left( \frac{1}{n} \sum_{i=1}^{n} \frac{1}{O_i^2} \right)
\]  

(1)

where, \( n \) represents the total number of repetitions in each experimental run, and \( O_i \) represents the percentage of ergosterol per CDW detected in the \( i \)th repetition of the experimental run.

The obtained experimental results and their S/N ratio were listed in Table 4. Experimental results were obtained by three replicates.

Table 5 showed the obtained average S/N ratio response at different levels of the process parameters and the ranking of parameters for ergosterol production. The delta values were calculated by subtracting the largest value from the lowest among the values in each row and the parameters were ranked based on the delta value. Figure 2 represented the mean S/N ratio graph for ergosterol production obtained in Minitab software version 19. Due to the orthogonal experimental design, the effect of each process parameter on the S/N ratio at different levels could be separately evaluated. A higher S/N ratio represented the minimum variance between the ergosterol production and the desired value. The response graphs in Fig. 2 showed the change in the ergosterol production when an experimental parameter changes from a lower to a higher level. The line slope of the response graphs determined the power of the parameters (i.e., IS, TS,
### Table 3 The effect of ultrasound irradiation on microbial metabolites production in different studies

| Product           | Strain                  | Description                                                                                           | Before UAP (mg/L)\(^a\) (U/mL)\(^b\) | After UAP (mg/L)\(^a\) (U/mL)\(^b\) | Increased (%) | References                  |
|-------------------|-------------------------|--------------------------------------------------------------------------------------------------------|----------------------------------------|--------------------------------------|---------------|-----------------------------|
| Mycophenolic acid | *Penicillium brev-compactum* | LIPUS system, 200 mW/cm\(^2\), 10 min, 8 times treatment per day                                       | 1279\(^a\) 2055\(^a\)                | 60                                   | (Zhao et al. 2012) |
| Riboflavin        | *Ecemotheicium ashbyii*  | Low ultrasonic system at 24 kHz, one time treatment per 12 h, 30 s on and 30 s off                    | 159\(^a\) 686\(^a\)                 | 330                                  | (Chuanyun et al. 2003) |
| Monoclonal Antibody (mAb) | Hybridoma cells | LIPUS system, 10 min per day for 4 days                                                              | – –                              | 60                                   | (Xing et al. 2012) |
| Uricase           | *Bacillus licheniformis* | Ultrasonic irradiation of 25 kHz, 60 W for 15 min of duration having 40% of duty cycle              | 0.434 0.825                        | 90                                   | (Pawar and Rathod, 2018) |
| Alkaline protease | *Bacillus licheniformis* | Ultrasonic irradiation of 25 kHz, 60 W for 15 min of duration having 40% of duty cycle              | 0.308 0.646                        | 110                                  | (Pawar and Rathod, 2018) |
| Pectinase         | *Bacillus subtilis*     | Sonication of 90 W, 25 kHz frequency with 70% duty cycle for 5 min at 6 h of bacterial growth phase | 55 87                             | 58                                   | (Yadav et al. 2020) |
| Cellulase         | *Bacillus subtilis*     | Sonication of 90 W, 25 kHz frequency with 70% duty cycle for 5 min at 6 h of bacterial growth phase | 10 22                             | 120                                  | (Yadav et al. 2020) |
| Xylanase          | *Bacillus subtilis*     | Sonication of 90 W, 25 kHz frequency with 70% duty cycle for 5 min at 6 h of bacterial growth phase | 106 138                           | 30                                   | (Yadav et al., 2020) |
| Bioethanol        | *Saccharomyces cerevisiae* | 10 min ultrasonic irradiation to fermentation broth at 12 h of growth phase with 25 kHz frequency, 160 W power and 20% duty cycle | 7800\(^a\) 14100\(^a\)            | 80                                   | (Subhedar and Gogate 2015) |
TF, and DT) which influenced on the response. While the parameters DT, IS, and TF show a larger impact on the ergosterol production, TS did not show a major impact. The highest mean S/N ratio was observed at 2 days of treatment, low intensity, and one time treatment per day for DT, IS, and TF parameters, respectively. Moreover, it could be observed that there was a minor difference between level 1 and level 2 for TS which were 5 and 10 min, respectively and there was a small reduction in the mean S/N ratio at level 3 (i.e., 15 min).

Furthermore, the results were visually displayed in a two-dimensional contour plot. Figure 3a–f showed the relation between ergosterol production and the important process parameters of DT, IS, and TF. These contour plots showed the interaction between multiple process variables with fewer experimental trials. The highest ergosterol production (dark
green) was observed at the left bottom corner of each graph. It illustrated that high ergosterol production (dark green) could be attained almost at low levels of all parameters, which was associated with 2 days of treatment, low intensity, and one time treatment per day for DT, IS, and TF parameters.

ANOVA for ergosterol production

To find the relative contribution of every parameter on the ergosterol production, a statistical analysis through ANOVA was implemented. ANOVA could find the process parameters which largely affected ergosterol production and therefore have a significant effect on the S/N ratio. Table 6 shows ANOVA results that were obtained for optimization of the ergosterol production. The results revealed that ergosterol production was largely influenced by DT (45.48%) followed by the IS (33.05%) and TF (19.65%), respectively. A low impact of the TS (1.82%) parameter could be observed. The processing condition for attaining optimal ergosterol production was the lowest level of DT, IS, and TF.

Empirical modeling

The regression analysis was applied to find a predictive mathematical model for the dependent variables of ergosterol as a function of DT, IS, TF, and TS. All variables were mean-centered. The equation was achieved from the polynomial model for ergosterol production with an $R^2$ value of 0.978, $Q^2$ value of 0.78 and a residual standard deviation (RSD) value of 0.43 (Eq. 2). TS was not significant and therefore was not considered as a term in the model. In the mathematical model, variable coefficients of the largest impact on ergosterol production was for DT with the values of 1.293. The relationship between observed and predicted ergosterol was visualized in Fig. 4.

**Table 5** Mean S/N ratio response table for optimization of the ergosterol production in Ozapeck medium from L9 orthogonal array 10 days after beginning the fermentation at 28 °C and 200 rpm

| Ultrasound treatment factors | Mean S/N ratio | Level 1 | Level 2 | Level 3 | Max–Min | Rank |
|-----------------------------|----------------|---------|---------|---------|---------|------|
| IS$^a$                      | 18.56          | 18.28   | 16.20   | 2.36    | 2       |      |
| TS$^b$                      | 17.83          | 17.89   | 17.34   | 0.55    | 4       |      |
| TF$^c$                      | 18.65          | 17.73   | 16.67   | 1.99    | 3       |      |
| DT$^d$                      | 18.84          | 18.24   | 15.97   | 2.86    | 1       |      |

$^a$Intensity of sonication

$^b$Time of sonication

$^c$Treatment frequency

$^d$Days of treatment

**Fig. 2** Main effect plots of mean S/N ratio of ergosterol production from L9 orthogonal Taguchi array 10 days after beginning the fermentation at 28 °C and 200 rpm. IS represents intensity of sonication, TS represents time of sonication (min), TF represents treatment frequency (times per day), and DT represents days of treatment (days)
From the morphological view, as it was discussed in literatures, the number of tips (or hyphae) represented the biomass accumulated in the medium. Low intensity enhanced primary and secondary metabolites production, cells with and without ultrasound treatment were viewed by a scanning electron microscope (SEM) to compare the morphologic changes induced by ultrasound. Some literatures scrutinized and studied the hyphae by counting the number of hypha tips (Ahamed and Vermette 2009) to correlate the ultrasound treatment effect with the cell’s morphology. They found that the number of hypha tips after ultrasound treatment was greater than that of the control experiment (experiment without ultrasound treatment), which could be the reason for higher metabolites production (Zhao et al. 2012). Ultrasonic by indirect mode (ultrasonic bath) caused milder shock wave and moderate mechanical shear to fungal cell which was sufficient to improve its porosity. Furthermore, increased porosity accelerated the cellular uptake of nutrient components and enhanced metabolites productivity (Avhad and Rathod 2014). So, low level of IS (low intensity) can improve number of hyphae in fungal morphology, then ergosterol productivity will increase while the enhancement of cell dry weight will occur. In addition, high levels of DT (number of days of treatment) led to higher tension for cells which finally caused cell disruption and the medium would be more viscose.

Comparing these results with previous reports, revealed that the ergosterol which was produced on the tenth day, was acceptable and higher than some previous reports. The results showed that the ergosterol which was produced on the tenth day of this study is in the range of 6–11 mg/g CDW. The enhancement of ergosterol production (4.63 mg/g CDW) in experiment number one (run 1) was compared to the control experiment (control run in Table 4) and the result was significant. Therefore, controlling the parameters such as IS, DT, and TF was essential due to the effect of these factors on the production of ergosterol on the tenth day. In comparison

$$Ergosterol \left( \frac{mg}{gCDW} \right) = 8.75 - 1.135 \times IS - 0.962 \times TF - 1.293 \times DT - 0.662 \times IS^2 - 0.717 \times DT^2$$

(2)

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to Samson’s research in 1981, the amounts of ergosterol produced by the strains of *P. brevicompactum* without ultrasonic treatment (Samson et al. 1981) and with ultrasonic treatment in this study were considerable. Therefore, ultrasounds with low levels of parameters such as days of treatment, the intensity of sonication, and treatment frequency can be a beneficial treatment for the enhancement of ergosterol production.

Ultrasound waves due to cavitation effects in the fermentation broth can change cell pore size, and increase the gaseous transport. Moreover, the cellular uptake diminishes the mass transfer endurance into the cells. Therefore the microbial uptake and metabolite production can be increased due to the approval of cell permeability (Yadav et al. 2020). The application of ultrasound during the fermentation process has also some challenges. Further investigations are needed to understand the effect of the sonication procedure at the cell molecular level and the biological impacts of ultrasound treatment on the microbial cells during the fermentation process. The interaction of acoustic cavitation and enzymes is also needed to be investigated for cell growth and cell proliferation in the fermentation process.

**Conclusion**

A proper sonication procedure can accelerate the product accumulation and also enhance the ultimate yield of products. In this study, low intensity ultrasound along with fewer days of treatment were indicated to have a positive effect on the ergosterol production from *P. brevicompactum* MUCL 19,011. Sonication at high power considerably affected fungal
morbidity, whereas pelleted growth was superseded by dispersed hyphae. The optimized ultrasound conditions increased the ergosterol concentration in the cultured medium was largely affected by days of treatment and then the intensity of sonication with the contribution of 45.48 and 33.05%, respectively. The *P. brevicompactum* MUCL 19,011 after ultrasound treatment can produce 11 mg ergosterol/g CDW, which is a high amount for *P. brevicompactum* among filamentous fungi. To the best of our knowledge, optimization of ergosterol production by UAE and with the application of DoE has not been reported previously. Future investigations need to further study including the effects of the sonication procedure at the molecular level, scaling-up process, economies, and the process sustainability.

### Declarations

**Conflict of interest** The authors declare that they have no conflict of competing interest.

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