Performance of Fungal Growth Through Integrated Gompertz Model and Respiratory Quotient by Solid State Fermentation in Multi-Layer Squared Tray Solid State Bioreactor with Aeration Strategies

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Research Article

Performance of fungal growth through integrated Gompertz model and respiratory quotient by solid state fermentation in multi-layer squared tray solid state bioreactor with aeration strategies

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Abstract

A newly designed, laboratory-scaled and multi-layer squared tray solid state bioreactor (SSB), was developed and successfully operated in solid state fermentation (SSF) conditions. The bioreactor was divided into eight layers of squared perforated trays. Wheat bran was used as a solid substrate for the growth of *Aspergillus awamori* and *Aspergillus oryzae*. The SSB was equipped with an oxygen (O_2)/carbon dioxide (CO_2) gas analyser and a thermocouple. Continuous on-line monitoring of fungal growth could be performed by indirect methods that measure O_2 consumed, production of CO_2 and metabolic heat. The advantage of using this method is that there are no tedious and time-consuming sampling processes. The evolution of CO_2, which represents an accumulation term, was integrated with time and fitted to the Gompertz model in a log-like equation. The Gompertz model generated values that may be used to stimulate and verify the experimental data. Results strongly suggest that the evolved and accumulated CO_2, excellently described fungal growth. Simulated results agreed with experimental results. The respiratory quotient (RQ), which is the ratio of CO_2 evolution rate (CER) to O_2 uptake rate (OUR), was determined by the gas balance method. CER and OUR confirmed that measurements correlated to fungal activity. Each RQ values can explain the differences of each SFF process carried out. Yet, heat evolved by fungal activity also described fungal growth. The current findings is an excellent pre-liminary experimental work, evidencing that multi-layer squared tray SSB with forced moistened aeration present a promising alternative of instrumented bioreactors for SSF processes.

Key words Solid state fermentation (SSF) . Multi-layer squared tray solid state bioreactor (SSB) . Gompertz model . Metabolic activity . Respiratory quotient (RQ)

Introduction

Solid state fermentation (SSF) has shown much promise in the development of several bioprocesses and products, with high volumetric productivity, relatively higher concentration of the products, less effluent generation, and simple fermentation equipment or bioreactor systems. Since 2000, SSF has received significant attention for the expansion of industrial bioprocesses, mainly due to its lower energy requirements and association with higher product yields, lower catabolic repression, cost-effective technology and less wastewater production with a lower risk of bacterial contamination [1]. In addition, SSF can be identified as an environmentally friendly process, as it uses solid residues from agriculture industries as the source of carbon substrate.

Along with the development and modernization of technology, SSF will soon be related to a solid state bioreactor (SSB) design which is a vital development and advancement in the bioprocessing technology. A number of SSB designs exist for SSF, such as semi-continuous plug-flow [2], packed-bed [3-7], tray [8-12], trickle bed [13], multipurpose fixed-bed [14], rotating drum [9,15], horizontal drum [16], column-tray [17], novel deep-bed cubical [18], multi-layer packed-bed [19], column [20], fluidised-bed [21], hexahedral modular [22], SSF reactor (SSFR) [23], horizontal rotary drum [24], continuous counter-current [25], scrapped-drum [26], zymotis packed-bed [27], gas-solid spouted-bed [28], horizontal stirred tank [29], vertical cylindrical shaped (fed-batch SSF) [30] and cylindrical [31] bioreactors. Progress has been made in improving processes at the laboratory level, but research on bioreactor designs and process scale-ups that allow the usage of microorganisms needs further focus [32].

In any aerobic SSF processes within any type of SSB, there are a few identified factors in determining the performance of the fermentation process. They are substrates heterogeneity, water activity or moisture gradients, O_2 supply, carbon dioxide (CO_2) transfer, temperature, metabolic heat removal and the bioreactor's design itself. Operational difficulties include issues related to poor O_2 supply, moisture level, heat accumulation in the bioreactor system and controls, which needs to be highlighted [33]. During the fermentation process, heat is generated and can consequently and directly affect the metabolic activities of the microorganism, causing, amongst others, moisture loss. Moreover, if the temperature control and aeration fail, the selectivity of the biosynthesis process towards product formation could be lost. Additionally, there is potential for the process to fail completely. A good strategy is therefore needed to effectively remove heat from the system [34]. Moisture content, heat and evolvement of CO_2 are requirements strongly linked to O_2 transfer rates in aerobic processes [35-37]. Direct aeration is the principal operation that results in O_2 transfer, moisture loss, heat and CO_2 removal. However, these effects are increased when high biomass concentrations and microorganisms

(particularly, filamentous fungi) are fed into the SSF, creating a complication with its use. A strategy to control these effects is to introduce forced aeration. Forced aeration allows on-line analysis of CO_2 and O_2 concentrations in the exhaust gas that is in accordance with and controls the fermentation process [34]. In addition, analysis of the exhaust gas has been used to indirectly estimate the biomass growth [38]. An important concept in the design of bioreactors is to allow a single vessel to have as many major operations as possible. The bioreactor is the core of the biological process with four major functions. They are: (1) to contain the substrate; (2) to contain the processed microorganism; (3) to protect the processed microorganism against contamination and (4) to control parameters and environmental conditions to optimise growth and product formation [39-41].

The most important factor need to be highlighted during microbial fermentation is sterilization. According to Sindhu et al. [42], sterilization is mandatory to strictly control bio-contaminants. This was supported by Arora et al. [33] that SSF bioreactors must be completely sealed to prevent unwanted microorganisms or harmful environmental substances from entering into the bioreactor. Besides that, temperature, O_2, CO_2 and heat removal need to be carefully controlled and optimised [39]. The SSB design must consider design and operating variables, especially aeration transfer for effective diffusion of O_2 in the gas phase, effective elimination of CO_2 and metabolic heat, temperature control including the cooling system [33,34,39,43]. In SSF, O_2 transfer can often be inefficient in some designs, whereby O_2 transfer is ineffectively homogenously distributed into every particle of the solid substrate. Other difficulty would furthermore affect the temperature and water content of the solid medium, thus need to be tightly and concomitantly controlled and measured [16]. Constructive bioreactors have high biological reaction rates including microbial growth performance, substrate consumption, product formation and by-product synthesis rates [33,39,44]. Thus, it is very important to understand which of these criteria provides the best performance for bioreactors to be constructively designed. Yet, a greater understanding of the biological systems of microorganisms and biological processes in SSF are key to improve the design of bioreactors [32,45].

This work was carried out at the location where the bioreactor system was designed to generate moistened air. Here, two different arrangements were provided into the multi-layer tray SSB. The moistened air supplied water, O_2 and facilitated heat and mass transfer to collectively improve fungal growth. In order to evaluate the performance of the bioreactor, final moisture content, O_2 uptake rate (OUR), CO_2 evolution rate (CER) and evolved temperature were investigated during fermentation. As analysis of O_2 as well as CO_2 in the exhaust gas is becoming generally accepted as a standard measuring technique in bioprocessing, the analysis was also applied in this study because it does not interfere with the sterile barrier. The results obtained will be discussed and reviewed for the two fungi reacting to every study design proposed.

Materials and methods

Microorganisms and sporulation medium

The strains of *Aspergillus awamori* and *Aspergillus oryzae* were cultured and preserved in a solid sporulation medium containing 5% (w/v) whole wheat flour and 2% (w/v) agar (Sigma-Aldrich). The strains were activated in sterilised media and incubated over seven days at 32°C, then preserved at 4°C. They were sub-cultured on an interval of every two months during the study.

Preparation of fungal spores as inoculum

0.5 mL spore suspension was transferred onto the surface of 100.0 mL of the same sporulation medium in a 500.0 mL Erlenmeyer flask and incubated for seven days at 32°C. After the incubation period, 50.0 mL of sterile 0.1% (v/v) Tween 80 solution and several sterile glass beads (4 mm diameter) were added to the flask. The spores were suspended by shaking the flask gently and then collected in a bottle as a spore suspension. Concentration of the spore suspension was measured by a haemocytometer. The volume of suspension needed for inoculation of the solid substrate was calculated for each experiment to reach a concentration of approximately 1.0×10^6 spores/g solid substrate.

Preparation of wheat bran as a solid substrate and inoculum transfer procedures

Wheat bran was obtained from the Cargill Wheat Processing Plant, Manchester, United Kingdom (UK). The substrates were kept in an airtight container and stored in a cold room (4°C) for future use. Wheat bran is used without any treatment as a solid medium for growing *A. awamori* and *A. oryzae*.

Firstly, 20.0 g of wheat bran was weighed and placed into separate 250 mL flasks (in a total of eight flasks) before being sterilised at 121°C for 15 min. The substrates were allowed to cool at room temperature before inoculating with *A. awamori* and *A. oryzae* spores and being moistened with an amount of sterile distilled water to obtain the initial moisture content of 65%. About 1.2×10^6 spores/g substrate were inoculated into the flask and mixed well with a sterile spatula under aseptic conditions to uniformly distribute the spores within the substrate. After sufficient mixing, the content was distributed into each tray before being incubated at 32°C for 72 h. At the end of the fermentation period, a sample was taken for sample analysis.

Multi-layer squared tray solid state bioreactor

The bioreactor chamber consists of eight identical square trays perforated at the base and arranged in a stacked fashion. The enclosed assembly has a door attached to the main body of the bioreactor. The dimensions of a rectangular bioreactor assembled as one structure are 14.0 cm in length, 14.0 cm in width and 73.5 cm in height (Fig. 1). The main body of the bioreactor is made of aluminium while the wall at the back and the door are made of the Perspex material. The enclosing structure of the bioreactor is of a durable transparent material, thereby enabling viewing of the bioreactor chamber contained therein. In one structure, the dimensions of the tray are of 12.0 cm in length, 12.0 cm in width and 2.0 cm in height. The tray was constructed from the Perspex material and equipped with a perforated base with an aperture size of 150 µm that permits operation, sterilization and high conduction.

The bioreactor chamber's assembly was configured as a module that fits in an autoclave. The bioreactor was autoclaved for 15 min at 121°C prior to operation. After cooling to room temperature, the reactor was filled with sterile inoculated substrate in a laminar flow cabinet under sterile conditions. Each tray can hold up to about 20.0 g of solid substrate with a 1.5 cm bed height. The tray medium enters the bioreactor chamber through the door. The door is then sealed to avoid any access from the environment into the system and vice versa. Each subsequent tray was assumed to be filled in the same thus are all under equal conditions. A schematic diagram of the experimental set-up consisting of the multi-layer tray SSB with aeration, control and on-line monitoring system is shown in Fig. 2.

Bioreactor setup and fermentation experiments

The bioreactor and solid substrate were autoclaved separately at 121°C for 15 min, prior to operation. The sterilised substrate was inoculated with 1.2×10^6 spores/g substrate in another Erlenmeyer flask before loading into the sterile tray and before transfer into the bioreactor. All work was carried out in a laminar flow cabinet under sterile conditions. The bioreactor was then placed in an incubator room for 72 h at 32°C. Sterile moistened air was introduced as a fluidising medium at the bottom of and on the surface of the tray from the compressor depending on the air strategies applied to every experiment. Two air flow rates were chosen as moistened air is sparged in two arrangements. Firstly, sterilised moistened air at a flow rate of 8 L/min was passed into an air distributor at the bottom of the bioreactor before being forced through the perforated mesh tray from bottom to the top (labelled as Exp 1). Secondly, sterilised moistened air at a flow rate of 1 L/min was passed onto the surface of each fermented substrate (labelled as Exp 2). These air arrangements are described in Table 1. For moistened air, dry air from the compressor (Air compressor JUN-AIR, USA) was passed through a humidifier chamber before entering the system. The flow rate of the air into the bioreactor system was accurately measured and adjusted with a rotameter. The air was sterilised by passing it through a 0.45 µm cellulose

acetate membrane filter and then into a humidifier chamber with sterilised distilled water which was monitored in a water bath at 30°C.

Measuring metabolic activity during solid state fermentation

Online monitoring of temperature

Each tray was equipped with a thermocouple type K (Pico Lab Technology, UK) placed at the surface of the fermented substrate to continuously record the bed temperature over time. All of the on-line data were monitored on screen and recorded using a computer.

Online monitoring of respiratory gases

The system used for quantifying O_2 and CO_2 continuously measured exhaust gas compositions online. An air outlet was situated in the headspace of the highest tray. The exit gas may need to be conditioned (moisture removed) before venting into the gas analyser. Therefore, the humid exhaust air from the bioreactor was firstly dried by passing through silica gel-containing tubes before entering an O_2/CO_2 analyser (FerMac 368 Electrolab, UK). This analyzer was connected to the Electrolab eLogger data logging and translates the concentration of O_2 and CO_2 to be recorded using a computer system.

Mathematical modelling

Quantification of oxygen uptake and carbon dioxide evolution rates

In this study, O_2 uptake rate (OUR) and CO_2 evolution rate (CER) were calculated using the following equations [46].

O_2 uptake rate (OUR):

$$OUR = \frac{F_1}{V_m \times V_0} \left(X_{O_2(in)} - \frac{1 - \left(X_{O_2(in)} + X_{CO_2(in)} \right)}{1 - \left(X_{O_2(out)} + X_{CO_2(out)} \right)} \times X_{O_2(out)} \right) \tag{1}$$

CO_2 evolution rate (CER):

$$CER = \frac{F_1}{V_m \times V_0} \left(X_{CO_2(out)} \times \frac{1 - \left(X_{O_2(in)} + X_{CO_2(in)} \right)}{1 - \left(X_{O_2(out)} + X_{CO_2(out)} \right)} - X_{CO_2(in)} \right) \tag{2}$$

Where;

OUR	: O_2 uptake rate (mole/L.h)
CER	: CO_2 evolution rate (mole/L.h)
F_1	: Air flow rate of inlet gas (L/h) at 1 atm and 30°C
V_m	: Molar volume of gases = 24.88 L/mole at 1 atm and 30°C
V_0	: Working volume, solid phase (L)
$X_{O_{2(in)}}$	: Molar fraction of O_2 at gas inlet
$X_{O_{2(out)}}$	: Molar fraction of O_2 at gas outlet
$X_{CO_{2(in)}}$	: Molar fraction of CO_2 at gas inlet
$X_{CO_{2(out)}}$	: Molar fraction of CO_2 at gas outlet

This equation is based on the inert gas balance. It is assumed that CO_2 is the only gaseous product of the fermentation process [46].

The Gompertz curve as a fungal growth curve

The Gompertz model was first initiated by Benjamin Gompertz (1825) and a thorough analysis of the model has been described by Winsor, (1932). The Gompertz model is a sigmoid function of the logistic curve and has been used as a growth curve, both for biological and economic phenomena [47]. An improvement to the model and derivation based on the systems theory was done by Skiadas and Skiadas [48]. In differential equation form, the model is based on this equation:

$$(\ln x)' = -b \ln x \qquad (3)$$

Where;

    x = the function of time

    b = a positive constant expressing the rate of growth of the system

Without loss of generality, the function of x can be assumed bounded ($0 < x < 1$, with $x = 1$ corresponding to the entire population), in order that x is the probability density's function of the growth process. Direct integration of equation 3 gives a solution to the Gompertz function [48]:

$$x = \exp(\ln(x_o) \exp(-bt)) \qquad (4)$$

The integrated Gompertz model, was used to analyze the kinetic data [49-51]. In this logistics-like model, the product ($[CO_2]$) is a function of time (t) according to the following equation.

$$[CO_2] = [CO_{2_{max}}] \exp(-b \exp[-kt]) \tag{5}$$

Where;

$[CO_{2_{max}}]$: the maximum CO_2 concentration (at t ----------> ∞) (mole)

b : a constant related to the initial conditions (when $t = 0$, then

           $[CO2] = [CO_{2_0}] = [CO_{2_{max}}] \, exp \, (-b)$ (dimensionless)

k : the specific CO_2 evolution rate (h^{-1})

t : fermentation time (h)

The constants $[CO_{2_{max}}]$, b and k were estimated from the data using a non-linear regression programme. The value of k (h^{-1}) refers to the time it takes for the value of $[CO_2]$ to reach $[CO_{2_{max}}]$. Larger values of k cause $[CO_2]$ to reach $[CO_{2_{max}}]$ in a shorter time. The value of b (dimensionless) will determine the shape of the sigmoidal curve. Larger values of b cause the initial exponential growth phase to be slower and the deceleration phase to reach asymptote faster. Smaller values of b cause a rapid exponential growth phase and slower deceleration before the asymptote of $[CO_2]$ is reached [47]. Here, asymptote refers to a line that continually approaches a given curve but does not meet it in any finite distance.

Gas balance and calculation of respiratory quotient

The respiratory quotient (RQ) can be directly calculated when the composition of the exhaust gas is known according to the following equation:

$$RQ = \frac{CER}{OUR} \tag{6}$$

Where;

       RQ : Respiratory quotient (dimensionless)

       CER : CO_2 evolution rate (mole L^{-1} h^{-1})

       OUR : O_2 uptake rate (mole L^{-1} h^{-1})

Determination of final moisture content

The moisture content of the samples was determined using the oven method, by measuring weight loss after heating to a constant weight at 95°C. Samples were placed in pre-dried metallic dishes with a

known weight and immediately weighed. After being dried in the oven for 24 h, the samples were cooled for 30 min in desiccators before a final weighing.

Enzyme extraction and activity determination

After the 72 h fermentation period, samples were taken for enzyme (glucoamylase, protease, xylanase and cellulase) analysis. Fermented samples (2.0 g on a wet basis) were extracted with distilled water (40.0 mL) and agitated for 30 min on a rotary shaker (Infors A – CH 4103 Switzerland) at 250 rpm and 30 °C. Next, the solid suspensions were centrifuged at 10,000 rpm for 10 min (4 °C). The clear supernatant was used for enzyme analysis. Glucoamylase was assayed using a method as described by Ariff and Webb [52], using maltose as a substrate. Protease activity was measured using the ninhydrin colourimetric method as outlined by the European Brewery Convention with modifications made by Wang [53]. Determination of xylanase activity was conducted according to a method developed by Bailey et al. [54], while filter paper-based cellulase activity was measured according to IUPAC recommendations [55]. Here, filter paper Whatman No. 1 was employed as a substrate [56]. A standard operational procedure for enzymes analysis was developed in this study, as reported elsewhere [57].

Spore's count

About 2.0 g (wet weight) of fermented substrate of *A. awamori* and *A. oryzae* were used to harvest the spores in a 250 mL flask containing 40 mL 0.1% (v/v) Tween 80. The flasks were continuously agitated in an orbital shaker at 100 rpm for 30 min at 30°C. The spore suspensions were then filtered using a stainless-steel sieve with an aperture size 45 μm to separate the solid particles. Spores were counted using a haemocytometer.

Results and Discussion

Effect of air arrangements on final moisture content during fungal growth

Fig. 3 shows profiles of the average and final moisture content from multi-layer tray SSB. Fermented *A. awamori* in Exp 1[AA] experienced high loss of moisture content for every tray (ranging between 51.95 to 59.82%). For fermented *A. awamori* in Exp 2[AA], the moisture content loss was not as critical as in Exp 1[AA] (ranging between 59.8 to 62.22%). However, the final moisture content for fermented *A. oryzae* was above the initial level of 65%. Exp 1[AO] recorded a final moisture content within the range of 66.04 to 67.84%, while in Exp 2[AO], the final moisture content was in between 66.71 and 69.89% for all the eight trays. *A. oryzae* proved to have high ability to retain water in its cells compared

to *A. awamori*, as previously reported [58]. Thus, in combination, wheat bran and *A. oryzae* were able to retain water and resulted in a high final moisture content in the fermented *A. oryzae* product.

This system was completely sealed with no access to the air from the inside or outside. However, large gas spaces in between the trays were included. In Exp 2[AA] and Exp 2[AO]air sparged directly onto the surface of the fermented substrate provided advantages to the trays above. For example, substrate in the tray at position 2 has access to air from: (i) the surface itself and (ii) the bottom of the perforated tray on which the air was sparged. The tray at position 3 has the same advantage as the tray at position 2. It seemed that trays at positions 2, 3, 4, 5, 6, 7 and 8 were aerated both from the surface and the bottom. Only the tray at position 1 was aerated from the surface.

There are some key factors affecting the moisture content in SSF and it is important to know their influence on the processes. It could be summarised that *A. awamori* and *A. oryzae* were very versatile but also a sensitive type of fungi towards air arrangement in bioreactor systems. The use of moistened air as an alternative to provide adequate O_2, is suitable for moisture content control. The air flow through the fermented substrate was also observed to play an important role in carrying moisture from the fermented substrate. When air was forced from the bottom to the top, it had a negative effect on *A. awamori* culture but a positive effect on *A. oryzae*. When air was blown through the surface of the fermented substrate, it had a negative effect on the moisture content for both fungi. However, this is not the only factor to determine high productivity of the culture. At the initial fermentation stage, the temperature, O_2 and moisture content are expected to be the same throughout the SSF system; however, as the fermentation progresses, O_2 is consumed, heat is evolved and water is evaporated, and therefore gradients of temperature, moisture, gas, substrate and products are commonly observed in the fermented bed [14].

Gompertz curve and analysis of a distributed gas balance during fungal growth

Fig. 4[a] shows the profile of CO_2 evolved during the fermentation process for both fungi. Fig. 4[b] shows the fitting of the Gompertz model to these data for both fungi during SSF with wheat bran for the description of fungal growth. In this system, it is clear that air arrangement has a direct influence on the metabolic activity and evolved CO_2. The strategy to supply air onto the surface of the fermented substrate (Exp 2) with moistened air at a flow rate of 1 L/min greatly enhanced the evolution of CO_2 compared to Exp 1.

For Exp 2 (for both *A. awamori* and *A. oryzae*), the evolution of CO_2 was higher probably due to high concentration of O_2 at the surface of fermented substrate. With a low flow rate at 1 L/min, the accumulation of O_2 in the headspace (between the trays) was adequate to supply O_2 for the fungi.

Compared to Exp 1, the concentration of O_2 might be variable in different locations of the trays. The bottom tray might have a higher concentration of O_2 compared to the trays above. Another probable reason for these differences is that some amount of O_2 might have escaped from the system due to the built-up of high pressure. The high pressure may be a result of the high flow rate used, at 8 L/min in Exp 1.

The kinetics constants are presented in Table 2. The model was found to be adequate to describe the integrated data from CO_2 evolution, as seen from the R^2 coefficients ($R^2 > 0.997$). The arrangement of trays with air sparged onto the surface of fermented substrate (Exp 2) greatly enhanced $[CO_{2_{max}}]$ (by more than 2-fold) compared to Exp 1 for both fungi. This was expected since in this system there are eight trays with the same amount of solid substrate.

The evolution rate (k) values were 0.048 and 0.059 h^{-1} for *A. awamori* and *A. oryzae*, respectively (Exp 2). Yet, there is no clear relationship between this parameter and the amount of CO_2 produced, as characterised by $[CO_{2_{max}}]$ from all experiments. Another parameter, the *t_{max}* value, coincided with those obtained for the maximum evolution of CO_2 and in most cases correspond to those experimentally obtained.

Further, the experimental data for the cumulative evolved CO_2 by *A. awamori* and *A. oryzae* was calculated using the Gompertz model. The Gompertz model showed an excellent agreement between experimental and predicted data in growth rate for *A. awamori* and *A. oryzae*, with an $R^2 > 0.99$ (Fig. 4[c]). It was observed that fitting the growth models to the accumulated CO_2 evolution raw data was meaningful and more feasible for further analysis. Data of the CO_2 evolution were easy to interpret with this model. It was observed that the concentration of CO_2 increases during SSF over time, following a sigmoidal curve that describes fungal growth (Fig. 4 [a] and 4[b]). The variation in the patterns of the sigmoidal curve were produced in response to two different moistened air arrangements. Yet, from all experiments on CO_2 evolution, the evaluated parameters revealed that the total amount of $[CO_{2_{max}}]$ was dependent on the type of fungus and air arrangement. It is perceived that in all the cases in this study, it was practicable to use the Gompertz model to describe fungal growth in SSF based on the CO_2 evolution. Thus, this model allowed an excellent prediction of the effects of moistened air arrangement on CO_2 evolution during SSF. In this study, it can be summarised that the Gompertz model is the best to describe the growth curves of *A. awamori* and *A. oryzae*. Here, the growth *A.awamori and A. oryzae* in every experiment, followed a typical pattern with four distinct phases: (i) a lag phase, (ii) an acceleration phase, (iii) a log (exponential) phase and (iv) a deceleration phase. Yet, there was no clear stationary phase and no accelerated death phase observed.

Ultimately, given the assumptions to be considered, the Gompertz model shows potential. It has the ability to describe the varied outcomes under different culture conditions. Previously, the Gompertz model proved most suitable to fit the experimental data in a kinetic study on anaerobic treatment of a hazardous steel-mill waste known as rolling oil [59]. The biomass data obtained from the growth analysis of *Streptomyces venezuelae* for the effect of ultrasonication was well fitted to the Gompertz model [60]. Additionally, the Gompertz model predicted the microbial inactivation under varying time and temperature subjected to the surface of a food product. Here, the model required non-linear regression schemes and analyses were tested on pseudo-experimental data [61]. According to Kafle et al. [62], the modified Gompertz model fitted the experimental data better than a first-order kinetic model, during anaerobic treatment of apple waste with swine manure for biogas production. In addition, the modified Gompertz model best described the growth of *Pseudomonas* in raw pork under pallet packaging at all temperature conditions [63]. According to Augustine et al. [64], the Gompertz model does not incorporate the symmetry restriction, has a shorter period of fast growth and gave the best fit for growth kinetic study of filamentous fungi in SSF.

Based on simulation work by Mitchell et al. [65], a relevant approach to modelling the product formation kinetics in SSF is valuable, owing to the difficulty in monitoring the different variables involved in the fermentation systems. Christen et al. [51] used the Gompertz model to describe the growth of *Ceratocystis fimbriata* in SSF and reported that the integrated data of CO_2 and volatile compounds production curve gives a good fit. Soares et al. [66] used the Gompertz model to observe the growth of *C. fimbriata* to describe the total volatile compounds production during SSF on coffee husks. Whilst, Erkmen [67] used the Gompertz model to describe the growth in *Listeria monocytogenes*, aerobic bacteria and lactic acid bacteria, during ripening and storage process. Other researchers used the Gompertz model of growth curve to describe the time course of fermentation under different conditions for lipopeptide production by *Bacillus amyloliquefaciens* in SSF [68]. Braissant et al. [69] suggested the use of the Gompertz model as a growth model and potential application to microcalorimetric data. Their study further observed the use of microcalorimetry in microbiology and biology, thus has become available to the end users of isothermal microcalorimetry. Instead of SSF, Augustine et al. [64] reported that the Gompertz model can be used to describe the growth of bacteria and yeasts in liquid media. Yet, the Gompertz and other log model possess similar properties that make them useful for the empirical representation of growth [47].

Metabolic measurements during fungal growth

Quatification of OUR and CER to describe fungal growth

It is possible to estimate biomass development in SSF by considering OUR and CER, which are easily measurable parameters. OUR and and CER offer the advantage of a fast response time and are directly linked to the metabolism of the microorganism [38,70]. Fig. 5 shows OUR and CER profiles from four experiments carried out for both fungi.

The stationary growth phase for both fungi in all tray SSB systems were clearly reflected in the OUR and CER evolved heat curves. This growth phase was very short and lasted for 1 to 2 h of the fermentation time. The curves were not of a sigmoidal shape and reached the maximum value of O_2 consumption, CO_2 generation and heat evolution approximately in between 24 to 30 h of fermentation. After this peak, the gradual decrease of heat and O_2 and CO_2 concentrations indicated the beginning of the last phase, which corresponds to slower fungal growth. This is a very slow phase of progress because the active spores still consume the O_2 (plus other nutrients) and produce CO_2 and heat. During the SSF of *Rhizopus oligosporus* with rice bran as the substrate, similarly, an increase in OUR and evolved heat at around 24 h of fermentation followed by a significance decrease was reported at 72 h [71]. In this scenario, it was considered that the high content of readily available starch, as a carbon source in wheat bran, was the reason for the comparatively high OUR, CER and evolved heat. This indicated high fungal growth during the first 24 to 30 h of SSF. Furthermore, in SSF, fungi cannot grow continuously as the amount of nutrients available is finite and waste products will accumulate. These conditions might be the reason for the deceleration of fungal growth after achieving maximum activity. Growth still continues but at a slower due to thepresence of nutrients.

Heat evolution as a mechanism to describe fungal growth

Temperature is the limiting design consideration for most SSB systems. A high amount of metabolic heat is produced during growth. The temperature of SSF cultures often increases to levels incompatible with growth. As the scale of SSB increases, the mechanism of heat transfer rapidly becomes insufficient. The forced addition of air is usually necessary to allow evaporative cooling of the fermented substrate and also the system. Some form of moisture control is then required. Forced aeration with saturated moistened air can then be introduced into the system.

The effects of moistened air with two arrangements in multi-layer tray SSB are shown in Fig. 6. Fungal growth for both fungi with Exp 2 provided a great respond based on temperature profiles during the 72 h of fermentation time. The temperature reached maximum at about 27 h with 37.03 and 36.55 °C for *A. awamori* and *A. oryzae,* respectively. All experiments show a rise in temperature after 18 h of fermentation, reaching a maximum between 27 and 30 h before it started to decrease until the end of the fermentation. Exp 2 with the moistened air at a flow rate of 1 L/min tended to be suitable for growth conditions, and for both fungi, the temperature recorded to be below 40 °C. Exp 1 with a flow rate of 8

L/min successfully witnessed the movement of generated heat from each tray, from the bottom to the top due to the high flow rate.

A better growth performance was observed in Exp 2 due to adequate O_2 concentration. Furthermore, in Exp 2, the air was not forcefully blown through the bed but rather circulated through the headspace above the bed. Additionally, the substrate in the perforated mesh tray allowed better air circulation around the trays. Perforation in a tray's base allows air flow through the tray to assure uniform mass and heat transfer. The system was properly sealed with a gap of about 2.0 cm between the trays to allow better aeration and O_2 accumulation.

In the current tray system of bioreactors, it was impossible to maintain the bed temperatures at the optimum value for growth, and therefore the fungus will suffer variations in temperature during the fermentation process. It is worth mentioning that from these studies, the amount of water loss and drying of the substrate were not drastic. Another important factor is the ability of the fungus to deal with fermentation conditions. For example, in this study, *A. awamori* might have suffered higher water loss compared to *A. oryzae*. However, the growth of *A. awamori* tended to be excellent in all experiments. At the same time, inhibitory metabolites were increasing due to waste accumulation within the system. The temperature often reaches values which severely limit growth or even kill the microorganims [8,19,65,70,71]. High temperatures might affect spore germination and growth, and product formation. This can be observed in temperature profiles illustrated in Fig. 6 where a temperature of above 30°C were noted in all experiments.

The approach taken by creating moistened air flow in the system created a cooling system able to supply moisture into the fermentation substrate. At one stage, the substrate possessed enough moisture to support growth and fungal metabolic activities. The growth rate was expressed solely as a function of the current temperature according to the experimental results. The increment of temperature during SSF in the tray and the insulated packed-bed bioreactors was responsible for changes in the use of the soybean oil and in protease secretion by *Yarrowia lipolytica* [8]. A strategy that uses intermittent trickling of water in the bed bioreactor, results in good temperature control, avoiding the bed from drying, preventing substrate compaction, as well as increased *Aspergillus ficuum* mycelium growth and phytase production [13]. Evaluation of different airflow distribution techniques in a pilot-scale bioreactor by using an inner tube for air supply resulted in more homogenous enzyme production, with higher activities by the fungus, *Myceliophthora thermophila* I-1D3b [4].

Additionally, temperature can be expressed as a function of the evolved CO_2 in the system. The profile for both parameters recorded in this work can almost provide an adequate estimation of the complete growth curve including the lag, log, exponential, and stationary growth phases. However, this profile

cannot provide a complete representation that also includes the death phase. Instead, it clearly shows that the growth becomes slower after the exponential phase, as reported by Finkler et al. [72] on their investigation of heat and mass transfer done in the absence of growth. Furthermore, research on hydrodynamics under abiotic and biotic conditions in a novel bench-scaled wall-cooled tray SSB packed with moistened particles of agro-industrial waste, provided a focused understanding of the interaction with heat and mass transport mechanisms [73].

Respiration quotient and considerations about fungal growth

RQ simply explains the state of the microbial population in the fermentation process and gives an indication of the behaviour of metabolic activity. Accurate measurements of CO_2 and O_2, allow precise and instantaneous estimation of kinetic parameters associated with the respiration of the culture such as the specific OUR, CER and the RQ [38]. According to Equation 7, a theoretical RQ of the oxidation of simple sugars for aerobic microorganisms is equal to 1 [74,75].

$$Sugars\ (C_6H_{12}O_6) + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad (7)$$
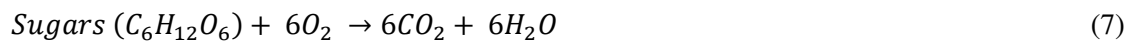
The equation shows that during glucose fermentation through aerobic fermentation, the rate of O_2 consumption is ideally six molecules, while six molecules of CO_2 are produced per glucose molecule [74,75]. These deliberations are based on simple sugars, or related carbohydrates or polysaccharides [75]. It is important to know that solid substrate used in SSF has varying nutrients composition of carbon and nitrogen. Some contain complex compositions such as hemicellulose, cellulose and lignin. In this situation, microorganisms need to utilise the entire complex of the compounds and produce simple fermentative sugars before these can be easily consumed. In the practical terms in SSF, it is impossible to obtain the RQ equal to 1, but the probability of obtaining RQ < 1 and RQ > 1 is possible as shown in Equation 8.

When RQ < 1, it is assumed that one or more non-constitutive or newly induced metabolites have been possibly produced. CO_2 was not the only product produced during the microbial fermentation. According to Equation 8, other products, such as energy (ATP), water and secondary metabolites were produced. Yet, O_2 is not only used for the respiration process but also for cell maintenance [74,75].

$$Organic\ material + nO_2 \rightarrow Biomass + sugars + nCO_2 + nH_2O + Energy \qquad (8)$$
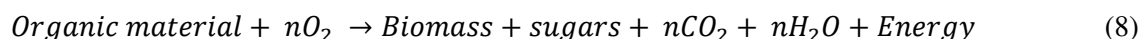
RQ > 1 indicates low O_2 uptake and the occurrence of glucose fermentation. It is also indicates anaerobic fermentation. For example, in highly aerated fermentation, it is often considered that higher

concentration of O_2 was washed out, corresponds to badly functioning aeration and the existence of anaerobic zones in the fermenter system that must be immediately resolved [38,74,75].

Further discovery of off-gas analysis is provided in this work for multi-layer squared tray SSB with two varied air arrangements. The rates of O_2 consumption and CO_2 evolution are presented along with the calculated RQ values, giving further insights into the behaviour of the fermentation process. Fig. 7[a] shows the profiles of RQ obtained using Equation 6 for both fungi in multi-layer squared tray SSB with moistened air arrangements. The RQ values are presented in Fig. 7[b]. These RQ values were generated by obtaining the slope from every experiment illustrated in Fig. 7[a]. It was observed in these studies that all RQ values obtained were below 1 (Fig. 7[b]). Exp 1, the RQ value was 0.533 and 0.578 for *A. awamori* and *A. oryzae*, respectively. Whilst, RQ values were higher in Exp 2. With the air arrangement in Exp 2, the RQ value for *A. awamori* and *A. oryzae* was 0.947 and 0.868, respectively. *A. awamori* showed RQ values around 1. The gas phase between the trays and the blowing of moistened air at 1 L/min onto the fermented surface provided a higher advantage for fungal growth. It was assumed that adequate O_2 concentration was available in the system. This situation arises because of the perforations at the bottom of the trays, that facilitate mass transfer to the gaseous surrounding, both at the top and the bottom.

In SSF, the mycelium growth of fungi is restricted by the diffusion of O_2 in the fermented solid substrate, especially in the inner depth [3]. Poor growth might occur deep within because O_2 transfer inside the solid substrate is blocked due to the compact structure of the solid particles [3]. These situations were observed in rice koji at high moisture contents. Here, poor growth of the deeply submerged mycelium was probably caused by blocked O_2 flow inside the rice grains [76]. The same observation was noted during the growth of *A. niger* in a tray SSF system that was limited by heat and O_2 transfer, with the bed height of a tray reactor being limited due to heat, had mass transfer limitations and the presence of heat transfer gradients [77]. The study suggested that by increasing the thickness of the fungal biofilm around the solid particles, the porosity of the substrate bed and diffusivity of O_2 in the bed were reduced.

According to Govind et al. [78], RQ values are close to 1 until O_2 uptake reaches a maximum level and thereafter RQ becomes higher than 1 due to utilisation of metabolite products. Becerra and Gonzalez-Siso [79] argued that changes in RQ values could be associated with the production of multiple products by *Streptomyces cattleya* during SSF. Barrios-Gonzalez et al. [80] reported that RQ of 0.7 obtained from their work indicated oil-supported metabolism for *Nocardia lactamdurans* during the production of efrotomycin in SSF. As recommended practise based on a simulation work by Rodríguez-León et al. [75], RQ patterns indicated the kind of kinetics involved in a particular fermentation process influenced by metabolite productions and microbial growth that can be categorized as (1) associated growth (RQ value close to 1); (2) partially associated growth (RQ value lower than 1 in the case the substrates are

polysaccharides from simple sugars units) and (3) non-associated growth (RQ value lower than 1 in the case sugar or related molecules are the substrate) [75]. Overall, the RQ values must be determined for particular processes and conditions [38].

The correlation between RQ and production of CO₂ during fungal growth

Fig. 8 shows exponential regression analysis of the relationship between $[CO_{2_{max}}]$ and RQ, giving a straight line ($R^2 > 0.9$) for both fungi, using data from 4 experiments. It should be noted that, changes in RQ value can be correlated with changes in the metabolic condition of the fungus. Based on the results, it was assumed that *A. awamori* and *A. oryzae* apparently adapted to aeration strategy, complete oxidation (microbial respiration) and utilisation of the available nutrients provided in the system (water, O₂ etc.). In this proposed concept, O₂ is continuously supplied to ensure a homogenously into solid substrate and RQ has proven to be an effective indicator for the measurement of metabolic conditions in SSF processes. RQ input associated with CO₂ evolved during fungal growth and biomass accumulation phases should be used throughout the development of the fermentation conditions. The RQ values of the metabolic activity may have resulted from beneficial microbial respiration by O₂ consumption and CO₂ evolution rate [38,75]. Thus, it would be convenient for a basic laboratory SSF bioreactor to have a gas analysis system at the bioreactor's outlet. A simulation work by Mann et al. [81], described the calculation of the ratio of evolved CO₂ similar to the RQ for aerobic fermentation, allows the prediction of whether acetate or ethanol is predominantly produced during syngas fermentation in quasi-continuously ventilated shake flasks using *Clostridium ljungdahlii*. Different types of solid substrates, microorganisms, SFF bioreactor systems and the overall metabolic pathway must be taken into account to obtain a proper RQ value [34,74,75]. Mendez-Gonzalez et al. [34] reported that conidia production and productivity were significantly enhanced with packed bed column with forced aeration compared to tray and plastic bag bioreactors. Forced aeration allowed online measurement of CO₂ production and proved to have an important role in determining when to harvest conidia from *Metarhizium robertsii* in SSF processes.

The correlation between heat evolution with CER during fungal growth

One of the most remarkable observational findings was the strong correspondence between the increase of temperature and the concentration of evolved CO₂ during the 72 h fermentation. A high degree of correlation was found to relate the evolving CO₂ and metabolic heat, as a result of carbon utilization. During the period of CO₂ evolution, the total amount of evolved CO₂ with time was found to correlate with changes in evolved temperature during microbial activity. Meanwhile, during respiration, a process dependent on O₂ consumption and evolved CO₂, was observed to be highly exothermic. Furthermore,

heat generation was directly related to the level of metabolic activities of the microorganisms [3]. Motivated by these observations, evaluation of CER data and temperature changes during SSF for both fungi were carried out.

Fig. 9 shows the profiles of temperature changes and CER over time for A. *awamori* and A. *oryzae*. The data shown suggest a possible linear dependence between the temperature rise and CER. A straight line with the slope of 2.224 °C/mole of CO_2. Commencement of CO_2 evolution occurred during 6 to 24 h of fermentation for all experiments. Data generated from the increase in temperature revealed a same profile as CER. When the CO_2 concentration increases, temperature rises. The relationship between temperature and evolved CO_2 is consistent with a feedback between CO_2 evolution rate (and might also correspond to oxygen uptake rate, OUR). Temperature rise and rate of CO_2 evolution reflect the metabolic activity of the fungus during the SSF process. In every experiment, the temperature rise during SSF was found to correlate with the rate of CO_2 evolution. A linear correlation exists between the temperature rise and CER irrespective of the growth rate of the fungus. The correlation between temperature rise and CO_2 evolution is very satisfactory; however, it does not permit comparison between processes.

Heat and CO_2 are microbially produced, thus indicate the existence of microbial metabolic activity, consumption of O_2, evolution of CO_2 and utilization of organic carbon [82]. In the case of aerobic microorganisms, the catabolic heat evolution rate is regarded to be proportional to the OUR and CER. According to Cooney et al. [83], if the heat evolution rate transfer ratio is constant, the change of temperature can be calculated by monitoring O_2 consumption or CO_2 evolution. The rising CO_2 levels correlated with rising temperatures because CO_2 is believed to trap natural heat from the surface warming of fermented substrate [82]. The study further elaborated that CO_2 would enclose the surface and trap heat, causing the fermented bed surface to become insulated and ultimately causing a temperature rise. In addition, the evolution of heat during microbial activity can provide useful data, such as growth rate, lag phase, or maximum growth [69]. In addition, encouragingly, the use of this technique in the biological and biomedical field has been increasing. Examples include detection and characterization of pathogens, drug testing, parasitology and tissue engineering [69].

Effect of air arrangement on spore and enzyme production

An average value of spore production and levels of enzymatic activities resulted from the moistened air arrangement with different flow rate are shown in Fig. 10 and Fig. 11, respectively. From these observations, A. *awamori* is an excellent fungus for spores and glucoamylase, xylanase and cellulase production. However, A. *awamori* is a poor protease producer. A. *oryzae* is excellent in protease production but poor in glucoamylase, xylanase and cellulase production. This might be because A.

oryzae seemed to favour a solid substrate with high protein content (as a nitrogen source) such as rapeseed meal, and does not favour wheat bran as a main substrate [57]. *A. awamori* seemed to favour a solid substrate with a high carbohydrate content (as a carbon source) [57].

In all experiments carried for both fungi, moistened air at a flow rate of 1 L/min (Exp 2) favour spores and enzymes production. This strategy was believed to create homogenous conditions in term of an enough supply of O_2 concentration while maintaining moisture contents and avoiding bed drying. In Exp 1, a high flow rate of 8 L/min tend to create stress in the bioreactor's system, whereby most O_2 was "washed" out and concentrations of O_2 might be low. A high flow rate also gave some negative effects on the moisture content, especially for *A. awamori* (Fig. 3). We conclude that with proper strategy to supply air into the bioreactor system for SSF, is advantageous to the fermentation's performance and fungal activity. The strategy suggests that reasonable control of the O_2 concentration, bed temperature and moisture content can be achieved by manipulating the flow rate of moistened air. As reported by Perez et al. [4,5], using an inner tube for air supply resulted in more homogeneous enzyme production, with higher activities and solving overheating and gaseous exchanges. However, our results contrast with previous reports by Castro *et al*., 2015 [84]. In this study SSF in SSB with forced aeration showed high levels of a cocktail of hydrolases (amylases, cellulases, xylanases and proteases) by *A. awamori*. This was supported by Mendez-Gonzlez et al. [34] which showed that forced aeration during SSF in SSB allows high conidia production and productivity. However, for a better monitoring and good comparison, other solid substrates, different types of microorganisms or fungi and different types of bioreactors, should be investigated.

Conclusion

This work presented two different moistened aeration strategies in newly-designed multi-layer eight squared tray SSB containing wheat bran to cultivate the fungus *A. awamori* and *A. oryzae* by solid state fermentation. Experiments were carried out under sterile conditions, equipped with an online gas analyse and temperature recorder, for continuous O_2, CO_2 and metabolic heat measurement. Continuous on-line monitoring of fungal growth could be performed by indirect methods, including monitoring O_2 consumption, CO_2 and heat evolution during SSF, is very useful to assess the physiological state and respiration rate of culture. Yet, it can directly reveal the activity of the *A. awamori* and *A. oryzae* culture in SSF and can be observed as indirect techniques for biomass estimation. Reasonable measure of the final moisture content can be achieved through strategy with moistened air at inlet with a flow rate 8 L/min and 1L/min. The air flow distribution strategies and the bioreactor with multi-layered trays contributes to an effective air distribution and optimal temperature maintenance. Even though, the final moisture content and temperature profiles for both fungi were little affected by the alternative of airflow

distribution, one might conclude that the introduction of moistened air produced a better distribution of gas within the bioreactor. CO_2 that evolved as a result of metabolic activity during *A. awamori* and *A. oryzae* SSF on wheat bran is easy to handle with a simple Gompertz model and fit the integrated CO_2 data and predict the quantity of CO_2 evolution in all experiments. Measurement of OUR and CER will lead to determination of RQ that gave important information about the fungal growth. In all respective experiments carried out in this work, the RQ values obtained are less than 1. Yet, a strategy with different aeration arrangements improved productivity of fungi, including production of spores and consortium of enzymes (glucoamylase, protease, xylanase and cellulase).

Even though the present study is on a small scale, it is hoped that the multi-layer squared tray SSB systems will provide a greater and general understanding information when looked at in conjunction with other studies in open literature. This work suggests that there will be a remarkable improvement in bioreactor productivity and better fungal growth. Information obtained could have several potential advantages including, contributing to future designs and applications. Some more efforts must be done to improve the performance of the bioreactor; for example further investigations of mass transfer and possibly to scale-up into larger bioreactor by solving problems of overheating and gaseous exchanges.

Authors' contributions Dr Musaalbakri Abdul Manan was carried the experiments in the laboratory and wrote the article as part of their Ph.D work. Prof. Colin Webb supervised the research activities and helped to review and edit the article before submission. Both authors read and approved the final manuscript.

Conflicts of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval This research articles do not contain any studies with human participants or animals performed by any of the authors. Hence, no format consent is required.

Consent to Publish The authors give their consent to publish this research article.

Consent to Participate The authors give their consent to participate in this research article.

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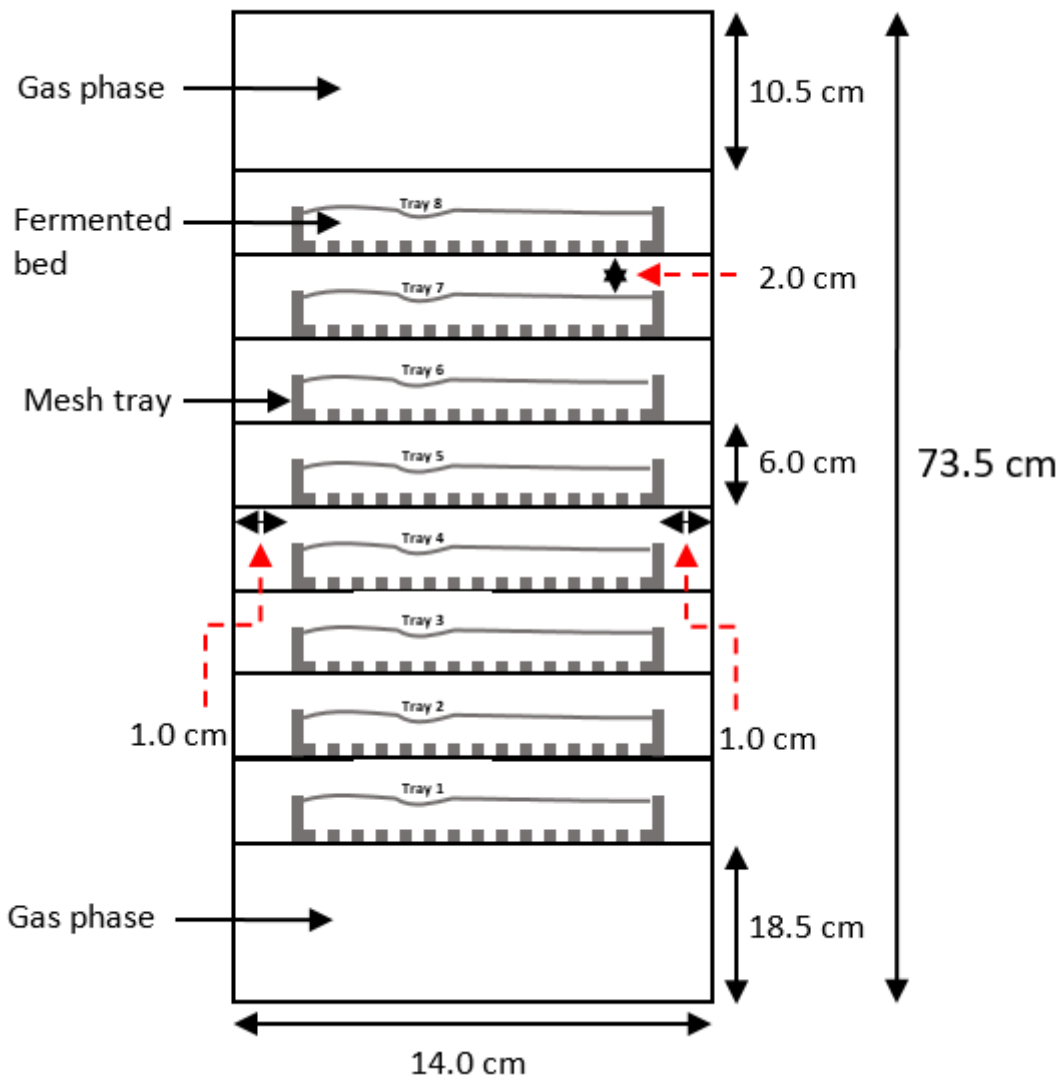
Figures

Figure 1

A schematic of a multi-layer tray SSB viewed from the side, with emphasis on the location of the tray
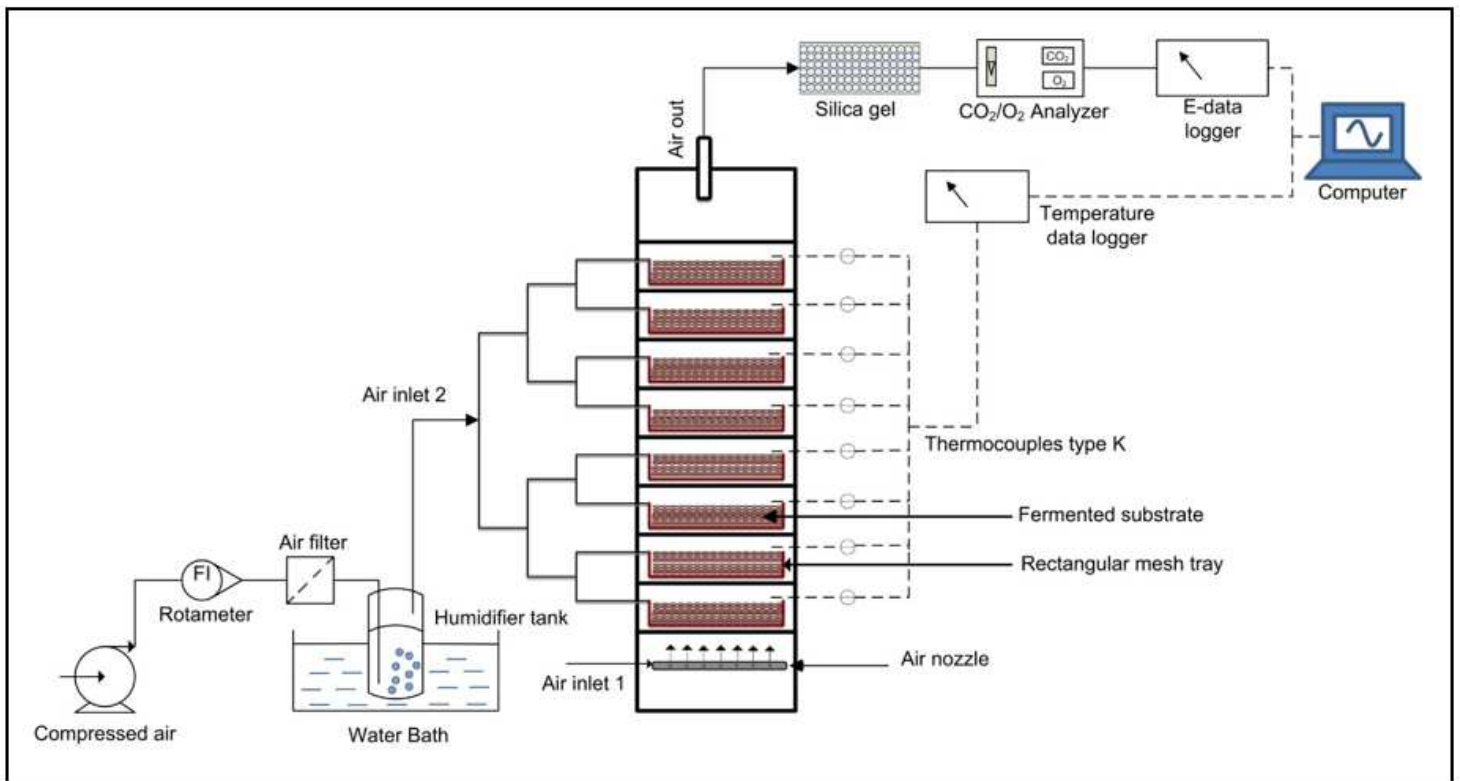
Figure 2

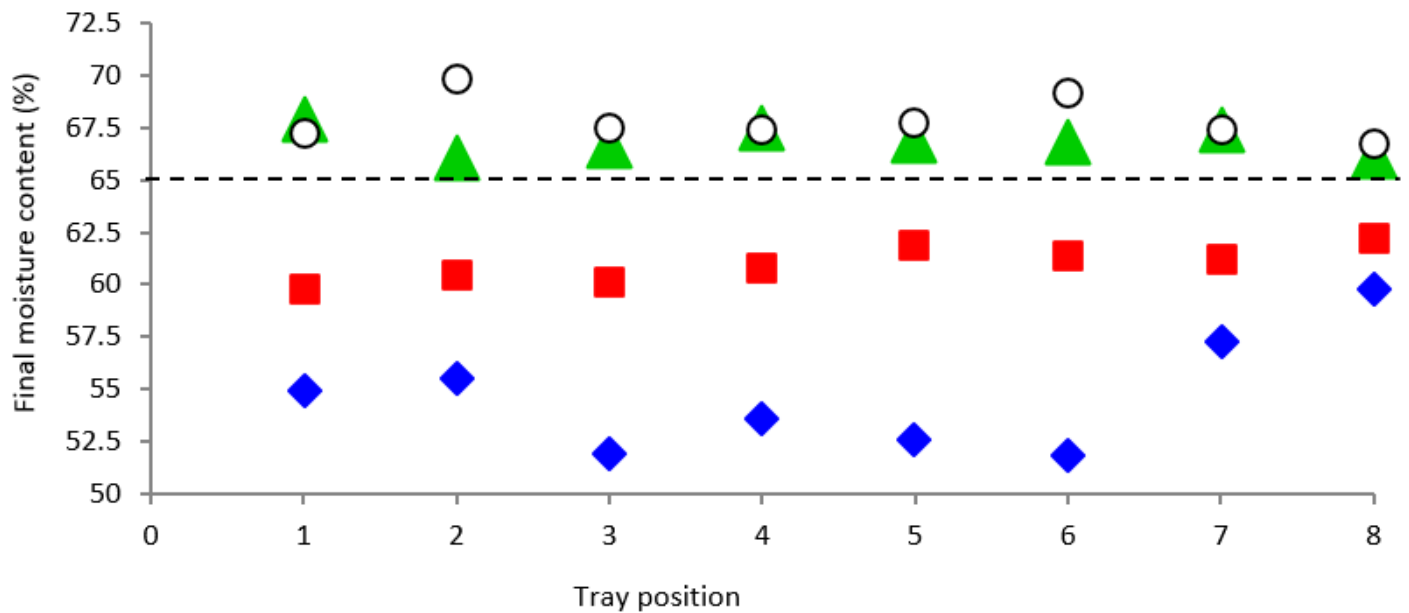A schematic diagram of the experimental set-up of a multi-layer squared tray SBB

Figure 3

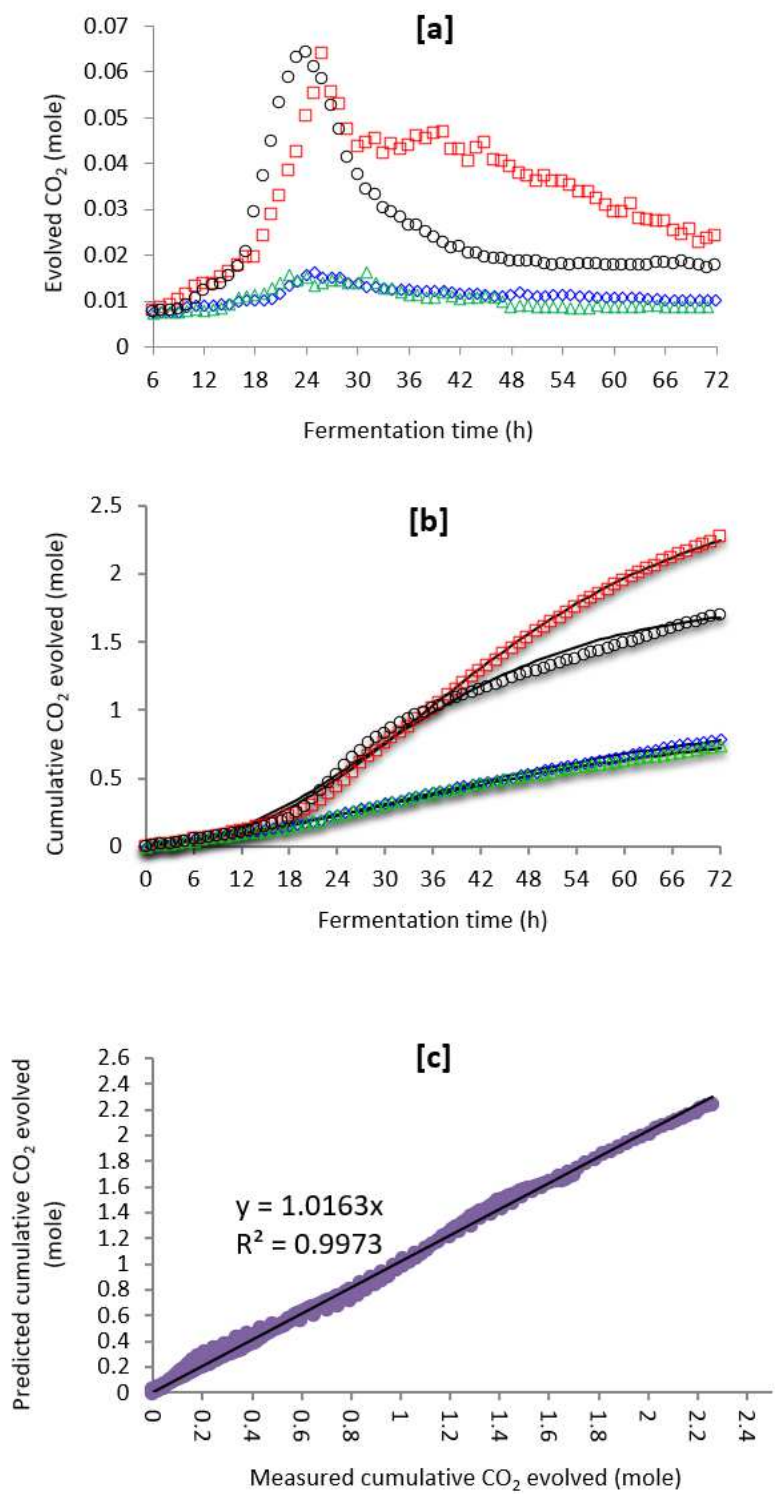"See the Supplemental Files section for the complete figure caption"

Figure 4

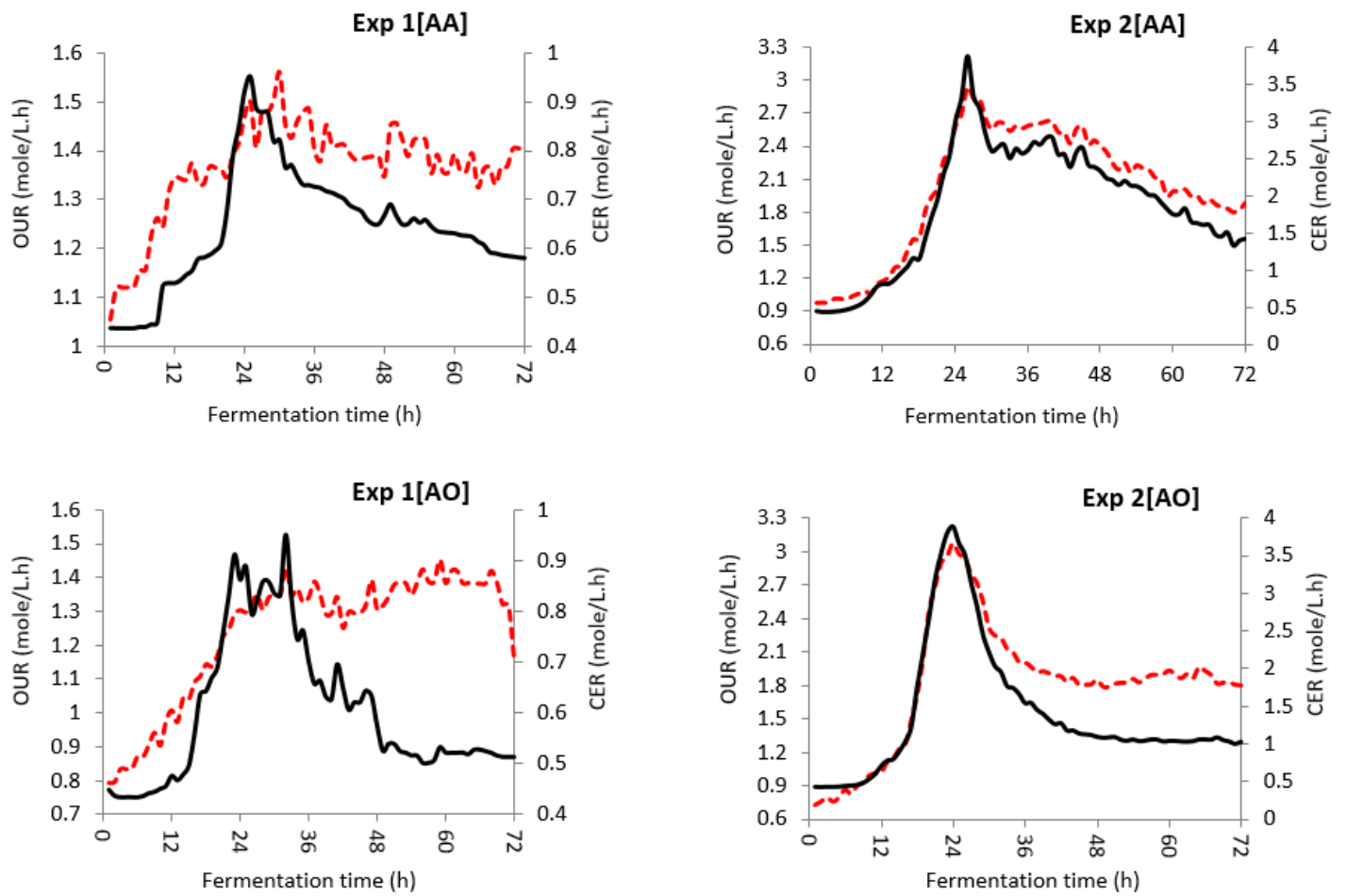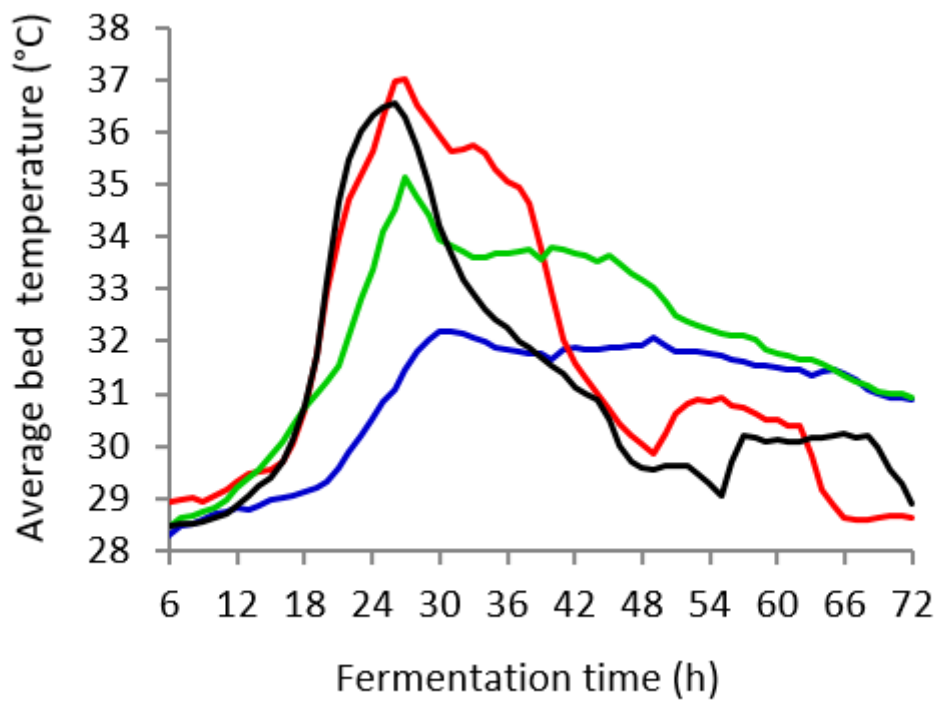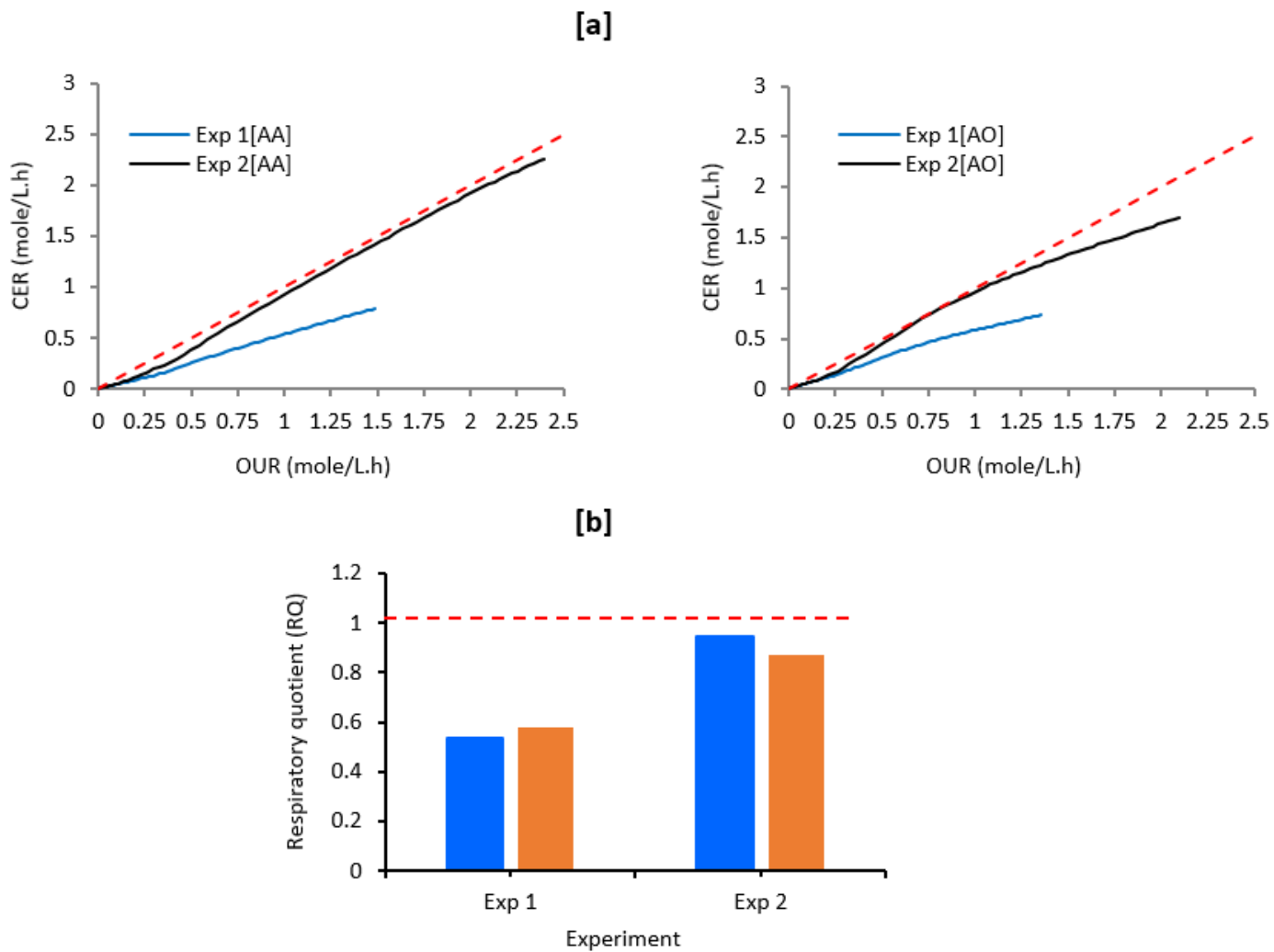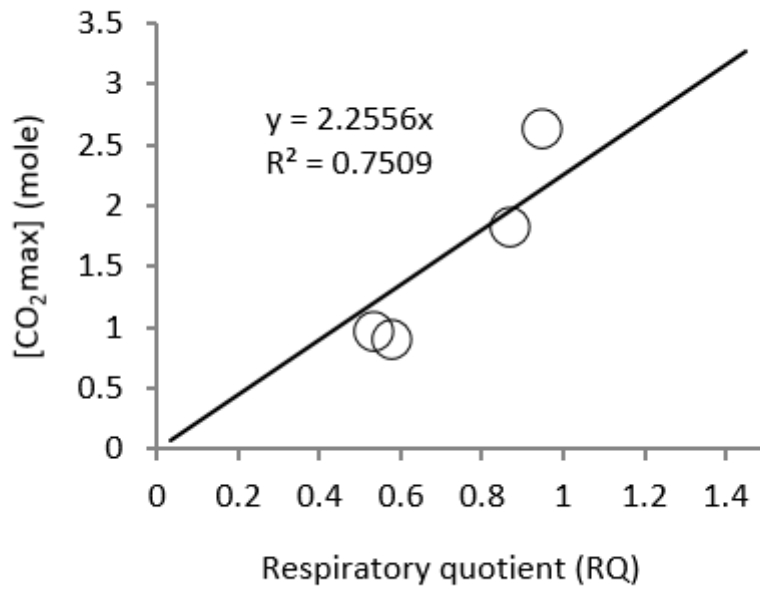"See the Supplemental Files section for the complete figure caption"
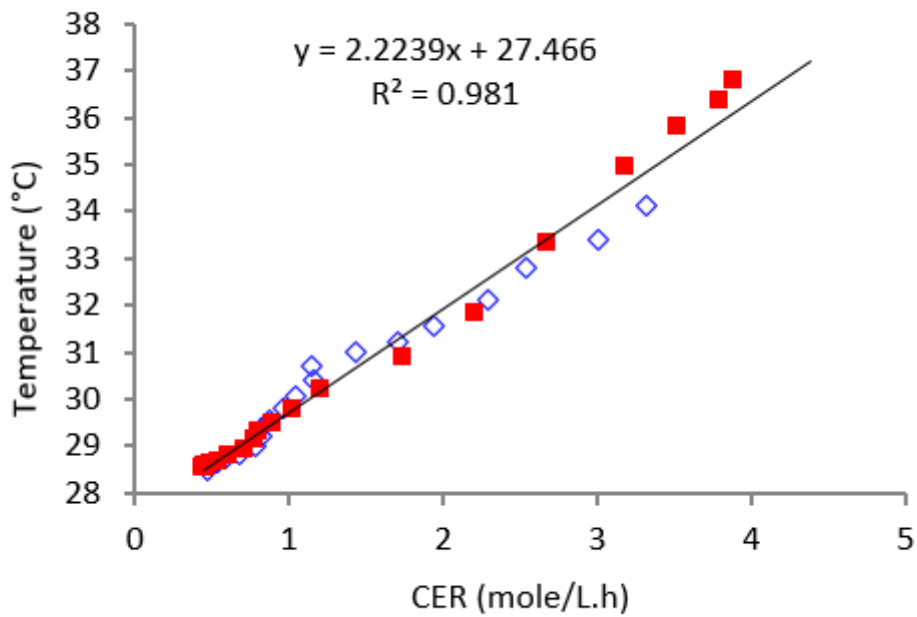
Figure 5

"See the Supplemental Files section for the complete figure caption"

Figure 6

"See the Supplemental Files section for the complete figure caption"

[a]

[b]

Figure 7

"See the Supplemental Files section for the complete figure caption"

$y = 2.2556x$

$R^2 = 0.7509$

Figure 8

"See the Supplemental Files section for the complete figure caption"

$y = 2.2239x + 27.466$

$R^2 = 0.981$

Figure 9

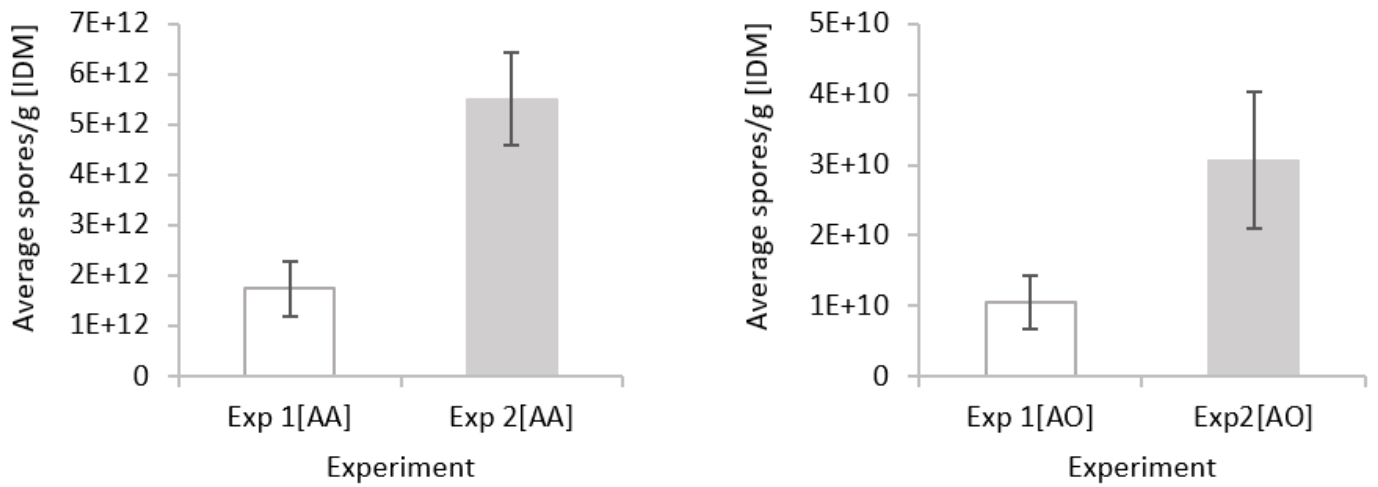"See the Supplemental Files section for the complete figure caption"

Figure 10

Fermentation summary of average spores' production from multi-layer tray SSB with two air arrangements. [AA] - A. awamori and [AO] - A. oryzae. The results are shown as an average from eight trays and mean ± standard deviation
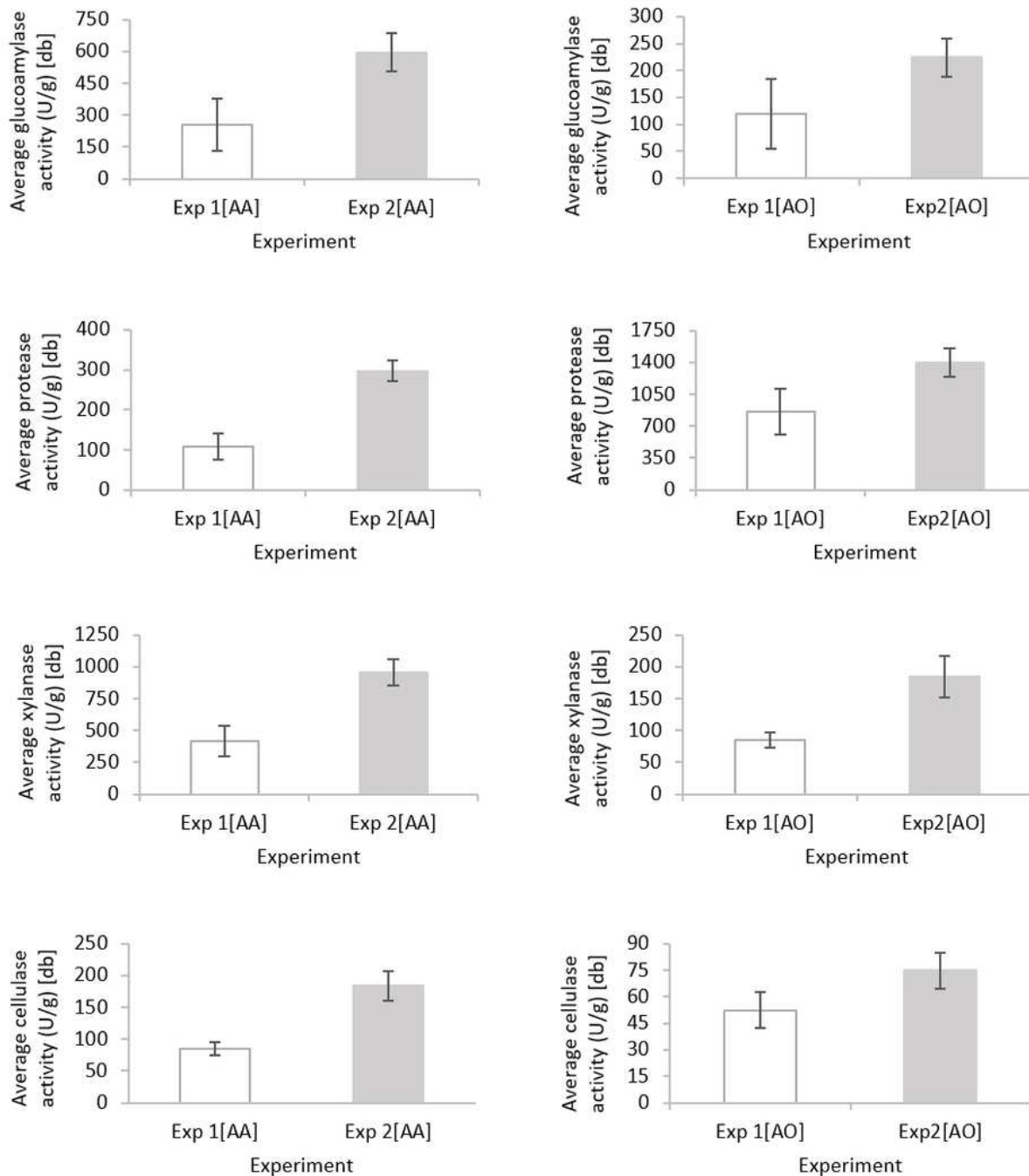
Figure 11

Fermentation summary of average enzymes production from multi-layer tray SSB with two air arrangements. [AA] - A. awamori and [AO] - A. oryzae. The results are shown as an average from eight trays and mean ± standard deviation

Supplementary Files

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