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Long term enhanced solid-state fermentation: Inoculation strategies for amylase production from soy and bread wastes by *Thermomyces sp.* in a sequential batch operation

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Abstract

The effect of two thermophilic amylase producing strains was evaluated using different mixtures of soy and bread wastes. *Thermomyces* sp. was found to be better inoculum than *Geobacillus* sp. for a soy and bread waste mixture (90:10 w/w respectively) producing a maximum enzyme activity of $39.9 \cdot 10^3$ U g^{-1} dry substrate. Three strategies (a, b, c) were evaluated for solid-state fermentation (SSF) operation in sequential batches. Fermented solids from each batch were used to inoculate the following batch: a) solids at the moment of maximum biological activity; b) final solids (end of the process, maximum amylase production); c) final solids after enzymatic extraction. The evaluated strategies led to an increase in amylase production of 50, 500 and 98% for each strategy, respectively. This indicates the suitability of fermented solids to act as inoculum and the enhancement of amylase production compared to traditional batches. As one of the main challenges of SSF is the maintenance of a productive process along time, these results confirm SSF as an excellent option to produce amylases from organic wastes.

Keywords: solid-state fermentation (SSF); *Thermomyces;* inoculation strategies; amylase; sequential batch; waste valorisation.

1. Introduction

In the last decade solid-state fermentation (SSF) has received increased attention from researchers as a bio-based process. This technology allows for the valorisation of wastes for the production of valuable commodities such as enzymes [1-3].

SSF presents some advantages in comparison with conventional fermentations such as higher productivity or reduced energy requirements, and low wastewater output [3] However, important drawbacks are described concerning the SSF scale up largely due to heat transfer, sterilization costs and culture heterogeneity [4,5]. Abraham et al. [6] proposed to perform SSF as a thermophilic batch process under near-adiabatic conditions, similar to the composting process which is a robust, well known, easily scalable and low-cost process. This self-heating process does not involve heating/cooling costs since the temperature evolve due to the heat released in the biodegradation process. Based on this proposal, El-Bakry et al. [7] proved that thermophilic strains inoculated on non-sterile wastes were able to grow and compete with autochthonous microbiota and significantly increase the protease production. Still the setting up of a long term operation in continuous or semi-continuous regime remains a challenge and very few attempts have been reported [8-10].

Enzymatic products such as amylases have great potential in different biotechnological applications such as food, fermentation, detergent, textile and paper industries. Some thermophilic amylase-producers microorganisms have been found during the highest bio-oxidative activity stage during composting processes [11-13]. In addition, it has been well established that these microorganisms are able to produce substantial amounts of amylase in SSF systems [2,14-16].

Several attempts for the optimization of enzymatic production have been reported, such as nutritional supplementation for microorganism growth [16-18], use of engineered strains [16,19] or operational strategies such as batch or fed-batch [9,20,21]. However, most SSF for

the production of enzymatic compounds have been tested at lab scale and under sterile substrate conditions [10].

The first aim of this work was to develop a SSF process for the production of amylases using a thermophilic strain growing on non-sterile wastes in the lab scale (500-mL reactors) and bring it to the bench scale (10-L reactors), based on the proposal of Abraham et al. [6] and El-Bakry et al. [7]. Second goal was to perform a first approach to the assessment of the effect of inoculation strategies in sequential batch operation in order to develop a long term operation process in a solid-state fermentation configuration at the bench scale. SSF of organic wastes operated in sequential batches was evaluated for the production of amylases using thermophilic and amylase producer microorganisms (*Thermomyces* sp. and *Geobacillus* sp.).

2. Materials and methods

2.1 Microorganisms

Thermophilic and amylase producers *Geobacillus* sp. ATCC-31198 and *Thermomyces* sp. ATCC-200065 were incubated in Petri dishes on supplier´s recommended agar media: ATCC® Media 1207 and ATCC® Media 350 respectively.

Both strains were grown in a liquid medium containing the same formulation than that used for Petri dishes. Final pH was adjusted to 7.0 ± 0.2 and 100 mL were incubated in a 500 mL Erlenmeyer flask at 150 rpm and 55ºC for 16h. Final culture broth was used as inoculum in all lab scale fermentations and in the propagation reactor for sequential batch experiments in a 10% w/w ratio.

2.2 Wastes

Soy fibre residues were provided by Soy Nature (Barcelona, Spain). Bread waste (expired pre-cooked baguettes) was procured from a local market. Both wastes were obtained in a sufficient amount to do the entire experiments with the same material. Materials were used as received in 500 mL reactors and in the scale-up experiments. In the case of the inoculation strategies experiments, aliquots were frozen separately prior to their use. Characterization of both substrates is presented in Table 1. Moisture was initially adjusted to 70% using tap water in all the experiments.

2.3 Selection of inoculum and substrates ratio at lab scale

Solid state fermentations (lab scale) were carried out at 55°C for 7 days in 500 mL Erlenmeyer flasks with 100 g of wet substrate and at fixed airflow of 20 mL min^{-1} by means of a mass flow meter. Air was humidified prior entering the reactors. 10 g of wood sticks were added to the substrate to provide porosity. Oxygen content was measured continuously in the reactors gas output. Specific oxygen uptake rate (sOUR) was calculated with equation 1.

$$sOUR = F \cdot (0.209 - y_{O_2}) \cdot \frac{P \cdot 32 \cdot 60 \cdot 10^3}{R \cdot T \cdot DW \cdot 10^3} \qquad \text{Equation (1)}$$

where: sOUR is the Oxygen Uptake Rate (mg O_2 g^{-1} DM h^{-1}); *F*, airflow into the reactor (mL min^{-1}); y_{O2}, is the oxygen molar fraction in the exhaust gases (mol O_2 mol^{-1}); *P*, pressure of the system assumed constant at 101325 Pa; 32, oxygen molecular weight (g O_2 mol O_2^{-1}); *60*, conversion factor from minute to hour; 10^3, conversion factor from mL to L; *R*, ideal gas

constant (8310 Pa L K^{-1} mol^{-1}); *T*, temperature at which F is measured (K); DW, initial dry weight of solids in the reactor (g); 10^3, conversion factor from g to mg.

Samples were taken daily for amylase activity determination. Firstly, *Geobacillus* sp. and *Thermomyces* sp. were evaluated in the solid-state fermentation of soy and bread wastes for the production of amylases and the best inoculum was selected for further experiments. Secondly, mixtures of different soy:bread ratios (0:100, 10:90, 50:50, 90:10 and 100:0 (w/w)) were evaluated and the best mixture selected for following experiments in reactors. Experiments were carried out in triplicates.

2.4 Bench scale reactors operation

Experiments were performed in 4.5 L cylindrical reactors previously described by Abraham et al. [6] with 1.2 kg of wet substrate. Reactors were thermally isolated to operate under near-adiabatic conditions and with on-line temperature and oxygen monitoring. Continuous aeration was provided to the reactor by means of a mass flow meter, starting with a rate of 100 mL min^{-1} which was manually increased (maximum air flow 1000 mL min^{-1}) according to the oxygen content in the reactor ensuring aerobic conditions. The oxygen concentration in the exhaust gases was measured by means of an oxygen sensor (Crowcon's Xgard, United Kingdom). sOUR is the specific oxygen uptake rate and was calculated from airflow and oxygen content and expressed as mg of oxygen consumed per gram of initial dry mass in the reactor and per hour (Equation 1). Total oxygen consumed was calculated as the area below the sOUR curve by the trapezoidal rule and expressed as mg of oxygen consumed per gram of initial dry mass. Temperature was monitored by means of a Pt-100 probe located at the central point of the reactor.

All fermentations were carried out by using the inoculum and soy:bread mixture previously selected at the lab scale. Wood chips were added as bulking agent in 1:1 (v/v) ratio, in order to provide porosity to the mixture and improve oxygen transfer in the reactors.

2.4.1 Scaling-up of amylase production

The scaling-up effect on the process defined at lab scale was assessed by monitoring two 4.5 L reactors for 14 days in order to have a complete profile of all relevant parameters of fermentation. One reactor was operating without inoculation as control. A second reactor was inoculated directly with liquid *Thermomyces* sp. culture. Amylase activity was determined at days 0, 4, 7, 10 and 14 of operation.

2.4.2 Inoculation strategies for operation in sequential batches

One first batch was performed as a propagation reactor and it was inoculated directly with *Thermomyces* sp. liquid culture in a 10% w/w ratio according to El-Bakry et al. [7]. Samples were taken from this reactor in two different moments to act as inoculum for the following batches. Three sequential batches (SB) were performed and each batch was inoculated with solids from the previous batch. Inoculation size was 10% w/w (fermented solids / fresh solids in the reactor) in all cases. The three strategies assayed are described below:

a) Strategy MOUR: Using as inoculum the fermented solids obtained at the moment of maximum sOUR, that is, solids with *Thermomyces* sp. at maximum growth rate.

b) Strategy MAA: Using as inoculum the fermented solids obtained at the end of the process, that is, solids obtained at the moment of maximum amylase activity.

c) Strategy MAAE: Using as inoculum the solids obtained after amylase recovery when its maximum activity was reached at the end of the process.

2.5 Enzyme extraction and amylase activity assay

Fermented solids were mixed thoroughly on a magnetic stirrer during 30 min with 0.1M phosphate buffer, at a pH of 6.8 and ratio of 1:5 (w:v) respectively. The extract was separated by centrifugation at 10000 rpm during 10 min followed by a filtration with a 0.45 μm filter. The supernatant was used for the amylase activity determination assay.

The reaction mixture consisted of 0.2 mL enzyme extract, 0.25 mL of 0.1% soluble starch solution and 0.5 mL of phosphate buffer (0.1M, pH 6.8). The reaction was stopped by adding 0.25 mL of 0.1N HCl, followed by the addition of 0.25 mL of I/KI solution (2% KI in 0.2% I) [22]. The colour intensity developed was measured in a spectrophotometer at 690 nm. One unit of amylase activity (U) is defined as the amount of enzyme able to generate a reduction of 0.01% in the blue colour intensity of starch in iodine solution per minute. In this work, amylase activity is reported as U per gram of dry substrate, considering soy and/or bread wastes as substrate, without considering the wood sticks or the wood chips. All measurements of amylase activity were performed in triplicates.

2.6 Sampling and analytical methods

Sampling was performed on a composite sample of the reactor, after homogenizing all the reactor material.

Moisture content, total and volatile solids, pH and EC were determined according to the standard procedures of The US Department of Agriculture and The US Composting Council [23]. Soluble starch content was determined by a commercial assay kit (catalogue item STA20) purchased from Sigma-Aldrich (St. Louis, USA).

3. Results and Discussion

3.1 Selection of the inoculum and mixtures at lab scale

Fig. 1 shows sOUR and amylase activity profiles for experiments in 500 mL reactors for inoculum selection using soy fibre (Fig. 1a, 1b) and bread waste (Fig. 1c, 1d) as substrate. As observed in Fig. 1a and 1c, higher sOUR values were obtained in reactors inoculated with *Thermomyces* sp. and *Geobacillus* sp. strains than in non-inoculated reactors, when using soy fibre and bread waste as sole substrate. This increase was more obvious in the case of soy fibre, which presented a higher sOUR than bread waste confirming a higher biodegradability. sOUR values were maximum at around 24h in all the cases and slowly decreased after that. Similar results were obtained for amylase activity: inoculation showed a positive effect on amylase production for both substrates, and higher enzymatic yields were obtained for soy fibre than for bread wastes. Maximum activity was obtained 48 h after reaching the maximum value of sOUR and slightly decreased after that. For both substrates, reactors inoculated with *Thermomyces* sp. presented higher values of sOUR and amylase activity than reactors with *Geobacillus* sp. Best process performance was obtained using soy fibre and *Thermomyces* sp. with a maximum sOUR of 11.8 $mgO_2\ g^{-1}DM\ h^{-1}$ and a maximum amylase production of $34 \cdot 10^3$ U g^{-1}DS. Fungi were expected to be better inoculum due to their ability to produce all required enzymes to fully degrade polymeric compounds and also to be able to survive in extreme conditions [24]. Also, fungi play a key role in solid fermentations such as composting processes, where *Thermomyces* sp. has been detected during thermophilic and highest amylolytic activity production stage [12,25]. These authors reported the predominance of *Thermomyces* sp. in temperatures ranging 50-60ºC and alkaline pH, which is also the range of its optimum growth regarding temperature and pH.

In consequence, *Thermomyces* sp. was selected as the best strain. Published literature [26] reports the synergistic effect of different wastes used as substrates for amylase production by SSF. Accordingly, further experiments were performed with different mixtures of soy and bread wastes to assess this potential synergistic effect. Table 2 summarizes maximum sOUR and amylase activity for all evaluated mixtures using different ratios of soy:bread. It was found that sOUR and amylase production were improved at high soy fibre content due to its higher content and availability of easily biodegradable organic matter. Also the mineral content of soy fibre is higher than the bread waste content, especially in Mg, P, and K which are required for the growth of amylase producing microorganisms [27,28]. Starch contained on bread most probably acted as inducer for amylase production as reported by some researchers [18,21] and nutrients present on soy provided proper conditions for the proliferation of microorganisms. It has been reported that several cations present in great amounts on soy fibre such as Ca^{2+}, acted as stabilizing agent or co-factors for amylases [16,18], generating an amylase activity increase, which is in accordance with obtained results.

All the mixtures showed a maximum amylase production on day 4 of process. Maximum amylase activity was obtained at a 90:10 soy:bread ratio, with a value over $39.9 \cdot 10^3$ U g^{-1}DS and maximum sOUR of 11.5 mgO_2 g^{-1}DM h^{-1}. Lowest amylase production was obtained with bread waste as sole substrate, with a maximum activity of $10.9 \cdot 10^3$ U g^{-1}DS. As it is possible to observe in all enzymatic profiles, amylase production is not directly related to maximum biological activity or growth. This fact was expected as amylase production is regulated by starch inducible system and repressed in presence of glucose or mannose isomers [29]. Maximum amylase was observed 48h after maximum sOUR as also reported by Kunamneni et al. [15] and Petrova et al. [16].

3.2 Scale up effect on amylase production

The selected process (substrates mixture 90:10 w/w and *Thermomyces* sp) was evaluated in 4.5 L reactors and results are presented in Fig. 2. Maximum sOUR and temperature were obtained at day 2 of operation in both control and inoculated reactors. As observed at lab scale, maximum biological activity did not match with amylase maximum production, which was obtained 48h after the maximum sOUR (day 4) in both cases. There was a clear effect of inoculation in terms of biological activity and amylase activity production [7]. *Thermomyces* sp. inoculated reactor presented a temperature of 65ºC and a maximum sOUR of 11.1 mgO_2 $g^{-1}DM$ h^{-1} which represented an 8 and 47% increase respectively in comparison with non-inoculated reactors. Also, using *Thermomyces* sp. as inoculum presented a positive effect on amylase activity with a 36% increment in comparison with control reactor, reaching a maximum of $41.03 \cdot 10^3$ U $g^{-1}DS$.

Similar results were obtained at 4.5 L and 500 mL scales for maximum sOUR and amylase activity. This confirms the easy scalability of the self-heating SSF and its suitability to work with thermophilic strains with non-sterile substrates.

3.3 Inoculation strategies on amylase production by sequential batches

Process profile for the propagation reactor is presented in Fig. 3a, 4a and 5a. A lag phase of nearly 2 days was observed. This was due to the use of previously frozen substrates. As reported by Pognani et al. [30], freezing for less than one year does not affect the biodegradable organic matter content or the biological activity measured as sOUR, but a lag phase may appear. Thermophilic conditions were reached after that and remained nearly for 48h, achieving maximum sOUR of 23 mgO_2 $g^{-1}DM$ h^{-1} after sampling the reactor. Sampling

sOUR was 17 mgO_2 g^{-1}DM h^{-1} and obtained amylase activity of $31.7 \cdot 10^3$ U g^{-1}DS. Total oxygen consumed was 725 mgO_2 g^{-1}DM in 5 days. At the end of the fermentation (48h after maximum sOUR) amylase activity was 15% higher with a value of $36.6 \cdot 10^3$ U g^{-1}DS.

3.3.1 Strategy MOUR

Fig. 3 presents the process profiles for the propagation reactor plus the three sequential batches (SB1, SB2 and SB3) performed with inoculation using solids obtained at the moment of maximum sOUR (gap in temperature curve of SB2 was due to data acquisition failure). In all four cases, maximum sOUR was achieved after sampling, which could be of importance considering the quality of the inoculums for the sequential batches [7].

The three sequential batches presented maximum temperatures ranging between 53-61°C similar to the propagation reactor. As for sOUR profile, maximum values were obtained also after sampling, reaching values of 21, 23, 19 and 19 mgO_2 g^{-1}DM h^{-1} for the propagation reactor and the SB 1, 2 and 3 respectively (Fig. 3).

Amylase activity obtained during maximum sOUR were 31.7, 21.9, 7.1 and $43.0 \cdot 10^3$ U g^{-1}DS and during maximum amylase activity 36.6, 39.1, 47.2 and $29.3 \cdot 10^3$ U g^{-1}DS for the propagation reactor and the SB 1, 2 and 3 respectively.

In general terms, sOUR increase and lag phase reduction was only obtained during SB1. Latter inoculations resulted in lower or equal sOUR, obtained only after homogenization of reactors in the sampling process. In addition, each sequential inoculation produced an increase of 0.8, 0.9 and 15.1% in total oxygen consumption in comparison with propagation reactor.

Inoculation with solids from maximum sOUR period from the propagation reactor was expected to provide a reduction in lag phase and an increase in sOUR. This is based on the fact that fungi from the class Eurotiomycetes, such as *Thermomyces* sp. have been found

predominant in thermophillic stage during composting processes [11,12,31], indicating that a suitable environment for inoculated *Thermomyces* sp. is provided. This fact was observed through all sequential batches, where a slight increase in sOUR and total oxygen consumption was obtained.

3.3.2 Strategies MAA and MAAE

Fig. 4 presents process profiles for the propagation reactor and the SB 1, 2 and 3 for MAA strategy. Temperature profile was similar in all four processes, reaching values near 60°C. Maximum sOUR values were in the range of 21-25 mgO_2 g^{-1}DM h^{-1} except for the first sequential batch (Fig. 4b). However, total oxygen consumption presented a sustained increase of 1.9, 54.2 and 76.6% in the SB 1, 2 and 3 respectively in comparison with the the propagation reactor. Also lag phase was gradually reduced in each SB, and sOUR peaked in the mesophilic phase in SB 2 and 3 with values 8-9 mgO_2 g^{-1}DM h^{-1}.

During thermophilic period (temperature >45ºC), high amylase activity was produced in all batches. Highest amylase production of $228.9 \cdot 10^3$ U g^{-1}DS was obtained at the end of the MAA-SB2, which represents a 500% increase in comparison with the propagation reactor. In the MAA-SB3, amylase activity was $156.9 \cdot 10^3$ U g^{-1}DS, 329% higher than the propagation reactor.

Fig. 5 shows the process profile for the propagation reactor and the three sequential batches, using final solids after the extraction of soluble and enzymatic components as inoculums. This solid material would be the actual final output of the process after amylase recovery as targeted product.

Maximum sOUR values in the three sequential batches were lower than in the propagation reactor. A 16.6 % decrease in the total oxygen consumption on the first sequential batch SB1 was obtained but increased by 1.1 and 23.2 % in SB2 and SB3. Thermophilic phase was

longer in the three SB than in the propagation reactor. Also amylase activity was higher in the sequential batches than in propagation reactor, with an increase of 27, 98, 56% in SB 1, 2 and 3 respectively. Maximum activity of the sequential experiments was obtained at the end of batch 2, achieving a value of $72.4 \cdot 10^3$ U g^{-1}DS.

Regarding MAA and MAAE strategies, temperature profiles and total oxygen consumption presented a different trend than the propagation reactor. Temperature is known as a crucial parameter to describe microbial activity during aerobic fermentation of solid heterogeneous material. It has been stated that inoculation with different microorganisms to a solid state fermentation could lead to a rapid rate of temperature elevation and for a more prolonged time of high-temperature process [32].

In MAA strategy, inoculation with fermented solid inoculum with high enzymatic and soluble content in sequential batches may have generated a fast hydrolysis of available substrate for the consumption of all present microorganisms. On the other hand, an extraction of soluble and enzymatic compounds in the inoculums could have generated a reduction in sOUR values and in the oxygen consumption rate, due to a lack of hydrolytic capacity in comparison with propagation reactor and the other evaluated batches with different inoculation strategies.

A reduction in amylase activity in the SB3 in both MAA and MAAE strategies can be attributed to several factors such as the possible repression on enzymatic synthesis [14,18], presence of hydrolytic enzymes such as proteases [6], to a depletion of the amorphous form of the solid substrate [21] or even more, to a change in the production profile of amylases.

It is a fact that sugars and low polymerization degree polysaccharides are normally fast consumed by microflora present during fermentation. It is reasonable to assume that with high production of amylase activity, high amounts of sugars are released which could cause catabolic repression of amylase synthesis. On the other hand, researchers have presented that

catabolic repression at high reducing sugars content occurs even in metabolically engineered de-repressed strains [19].

The lack of literature about amylase production by inoculation strategies in sequential solid state fermentations and the many different methods of amylase activity determination made difficult to compare the obtained results with other reported researches. However, other enzymes have been produced in fed batch strategy [9], with results found in accordance to this study. In any case it is proved that the operation strategy is a key factor for the implementation of solid state fermentation at the commercial level. Further research shall focus on determining the number of sequential batches that can be performed following this strategy. Nevertheless, both MAA and MAAE strategies allowed a remarkable enhancement on amylase activity. In our opinion, both strategies are suitable for industrial operation: in strategy MAA, 10% of the solids would be diverted to inoculate the following batch prior to enzyme extraction; in strategy MAAE, 10% of the solids after extraction would be recycled to inoculate the next batch. To finally decide which the best strategy is, it would be necessary to perform a complete economic and environmental assessment.

4. Conclusions

Operation of SSF of soy and bread wastes in sequential batches has been proven as a suitable strategy for amylase production with *Thermomyces* sp. Additionally, it opens a new valorisation alternative for these wastes. A SSF process was operated for 19 days and the enhancement of amylase production was accomplished. In terms of amylase yield, the most suitable inoculation strategy was using fermented solids at the end of the batch to inoculate the following batch, obtaining a 500% increase in productivity and eliminating the need of producing fresh inoculum for each batch. Nonetheless this, the final strategy selection should

consider economic and environmental aspects. The development of these operational strategies could benefit process economics and provides the possibility of a continuous operation for amylases and potentially for other enzymes.

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Table 1. Characterization of raw materials used on SSF (average ± standard deviation)

Parameter*	Soy fibre	Bread waste
Dry mass (%)	17.7±1.3	62.8±0.8
Organic matter (%)	96.8±1.3	96.95±0.2
C (%)	67.5±1.8	48.1±2.1
N (%)	4.4±0.7	2.5±0.2
pH	7.0±0.7	6.1±0.1
EC	0.9±0.3	3.6±0.6
Starch (%)	3.1±0.1	75.7±0.1

* all percentages in dry basis except for dry mass, in wet basis.

Table 2. Maximum sOUR and amylase activity obtained in SSF using different soy:bread ratios

soy:bread (%w/w)	Maximum sOUR ($mgO_2\ g^{-1}DM\ h^{-1}$)	Maximum amylase activity ($10^3\ U\ g^{-1}DS$)
0:100	3.4	10.9 ± 0.5
10:90	4.1	14.7 ± 0.7
50:50	5.8	18.9 ± 0.9
90:10	11.5	39.9 ± 0.9
100:00	11	34.6 ± 1.2

Figure list

Figure 1. Solid-state fermentation at 500 mL scale using *Thermomyces* sp. and *Geobacillus* sp. as inoculums. Profiles of sOUR and amylase activity are presented using soy fibre a) and b) and bread wastes c) and d) respectively.

Figure 2. Solid-state fermentation at 4.5 L scale using soy:bread 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented using a) no inoculation, b) *Themomyces* sp. inoculation.

Figure 3. MOUR strategy in 4.5 L reactors using soy:bread 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented for the propagation reactor and the three sequential batches MOUR-SB1, MOUR-SB2 and MOUR-SB3.

Figure 4. MAA strategy in 4.5 L reactors using soy:bread 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented for the propagation reactor and three sequential batches MAA-SB1, MAA-SB2 and MAA-SB3.

Figure 5. MAAE strategy in 4.5 L reactors using soy:bread 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented for the propagation reactor and three sequential batches MAAE-SB1, MAAE-SB2 and MAAE-SB3.

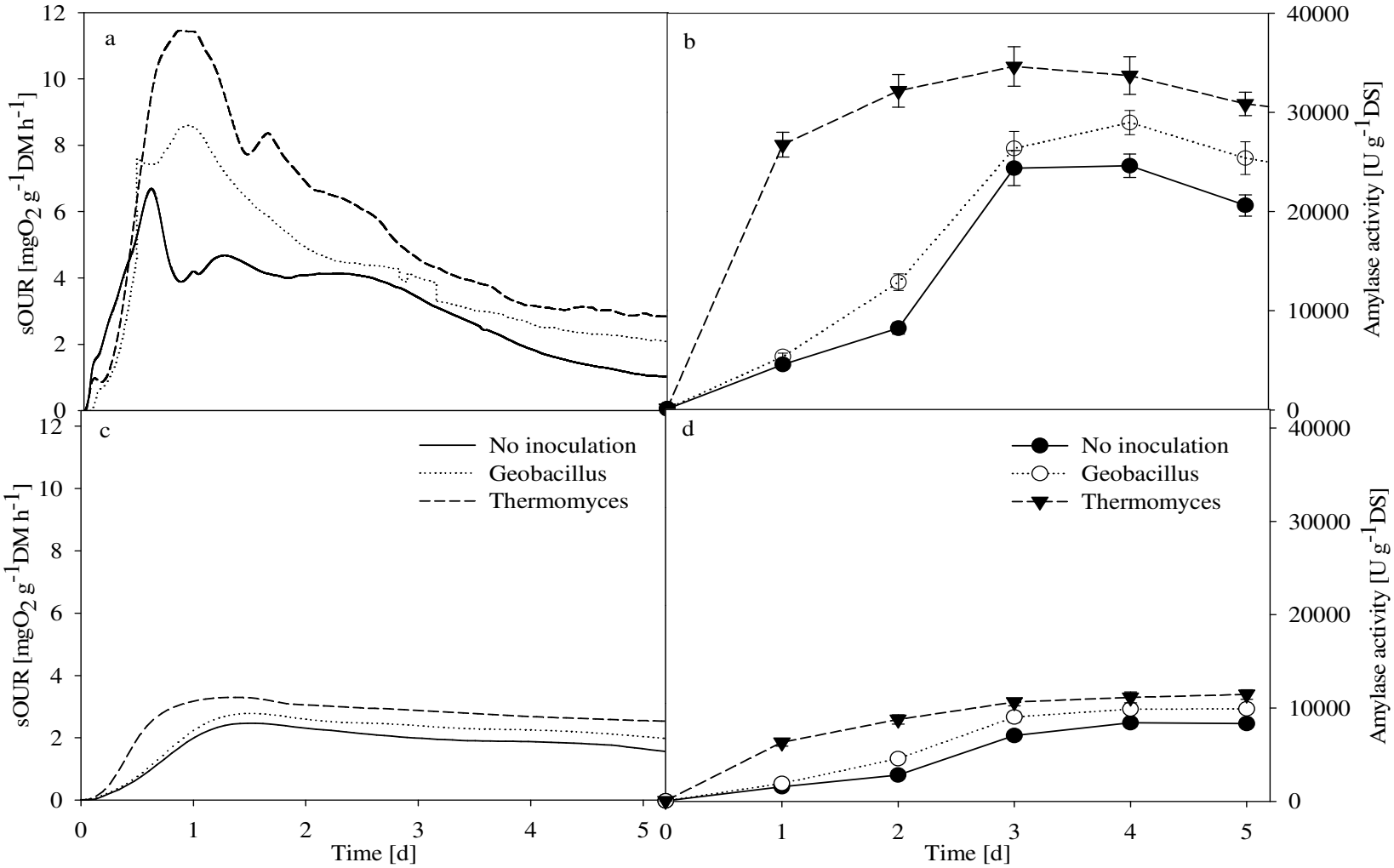
a
b
c
d
sOUR [mgO2 g-1DM h-1]
Amylase activity [U g-1DS]
Time [d]
No inoculation
Geobacillus
Thermomyces

Figure 1.

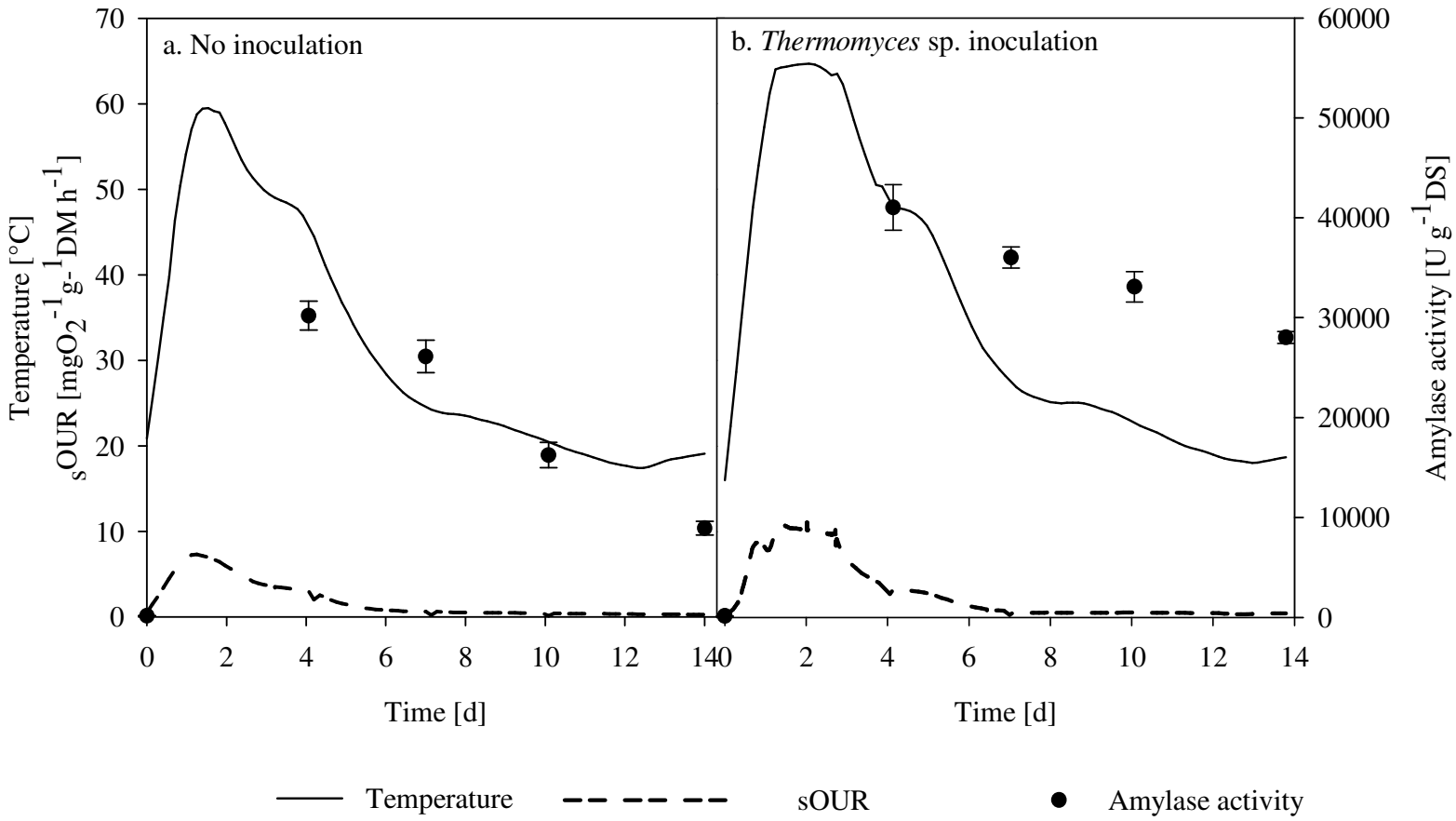
a. No inoculation
b. *Thermomyces* sp. inoculation
Temperature [°C]
sOUR [mgO2-1g-1DM h-1]
Amylase activity [U g-1DS]
Time [d]
Time [d]
Temperature
sOUR
Amylase activity

Figure 2.

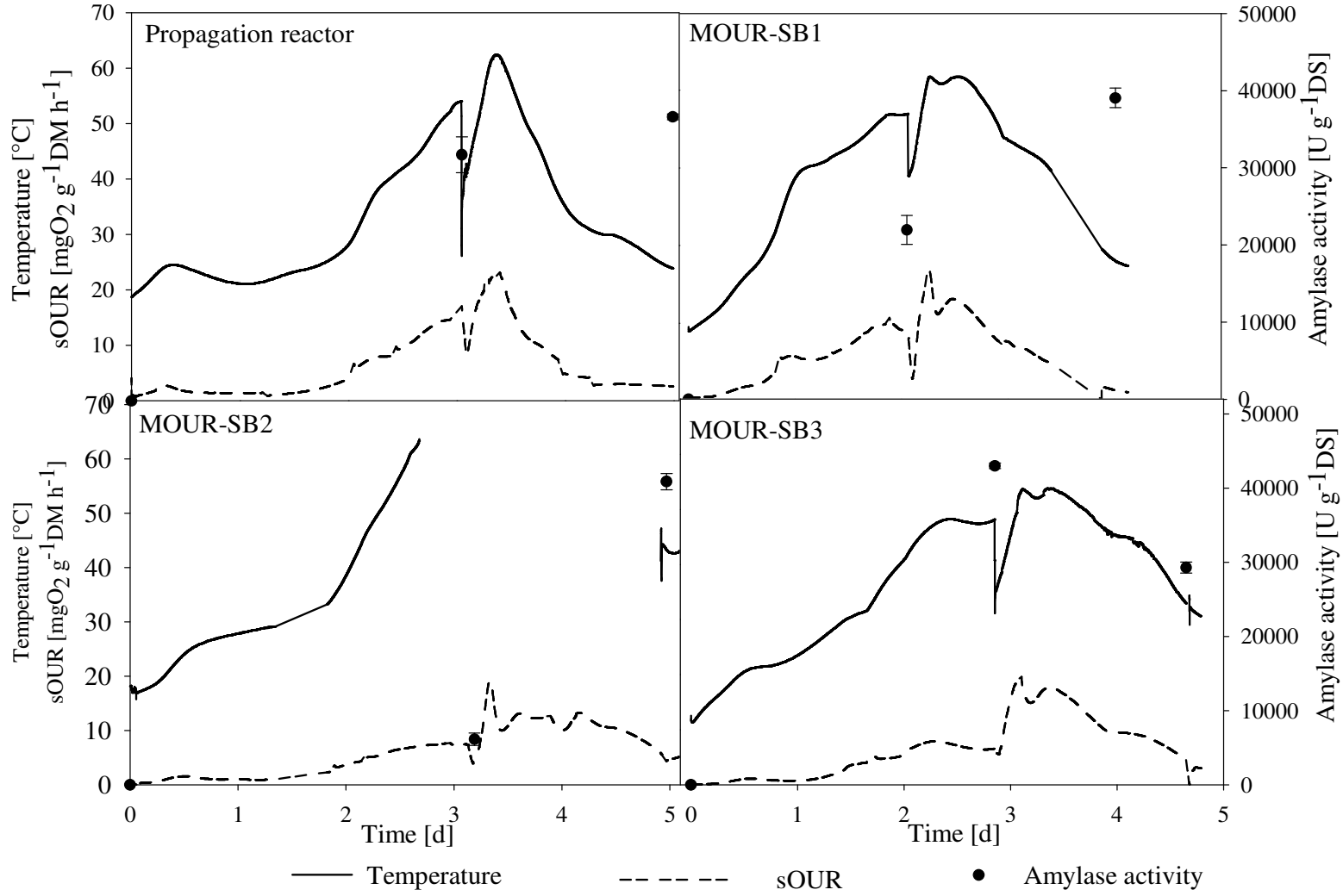
Propagation reactor
MOUR-SB1
MOUR-SB2
MOUR-SB3
Temperature [°C]
sOUR [mgO2 g-1DM h-1]
Amylase activity [U g-1DS]
Time [d]
Temperature
sOUR
Amylase activity

Figure 3.

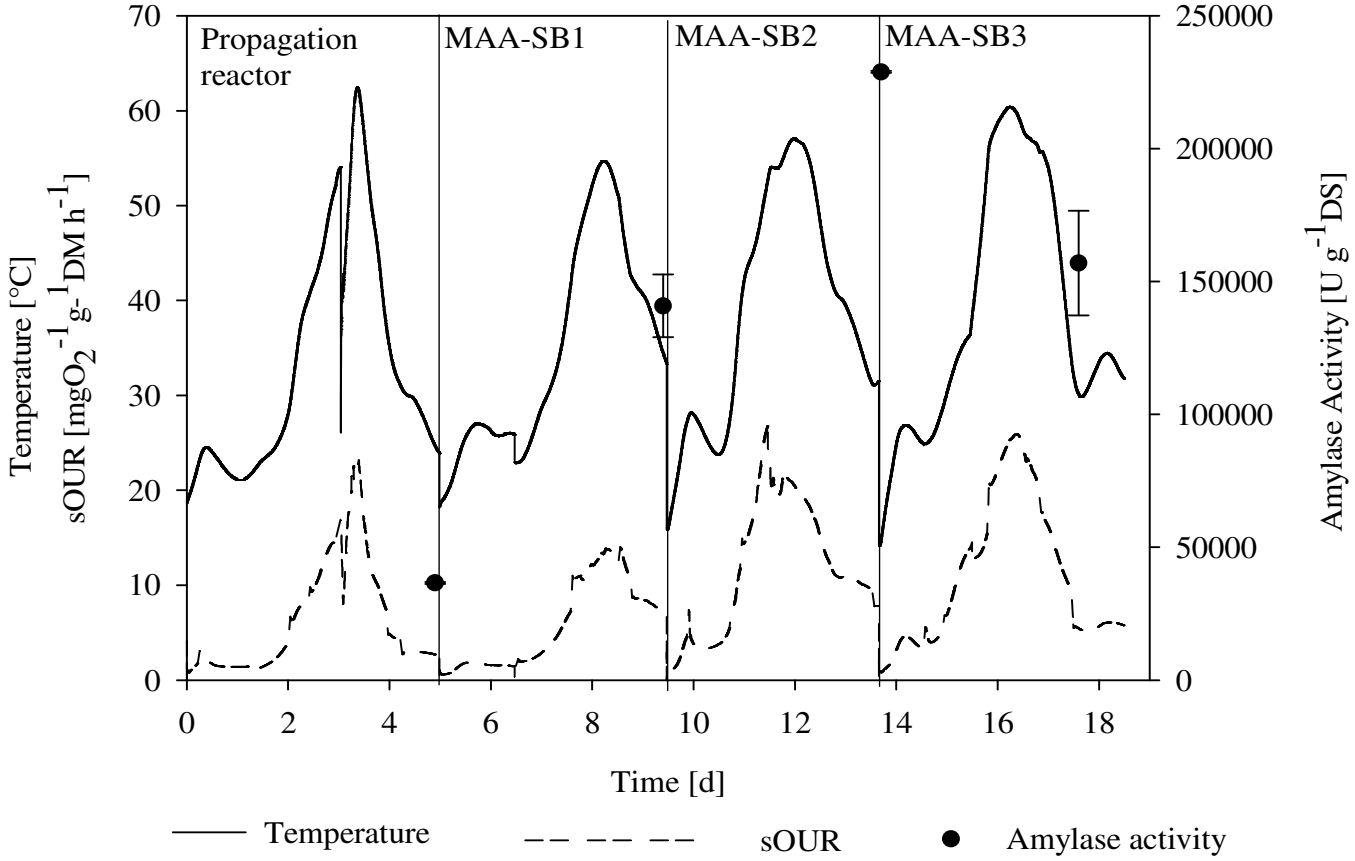
Propagation reactor
MAA-SB1
MAA-SB2
MAA-SB3
Temperature [°C]
sOUR [mgO2-1 g-1DM h-1]
Amylase Activity [U g-1DS]
Time [d]
Temperature
sOUR
Amylase activity

Figure 4.

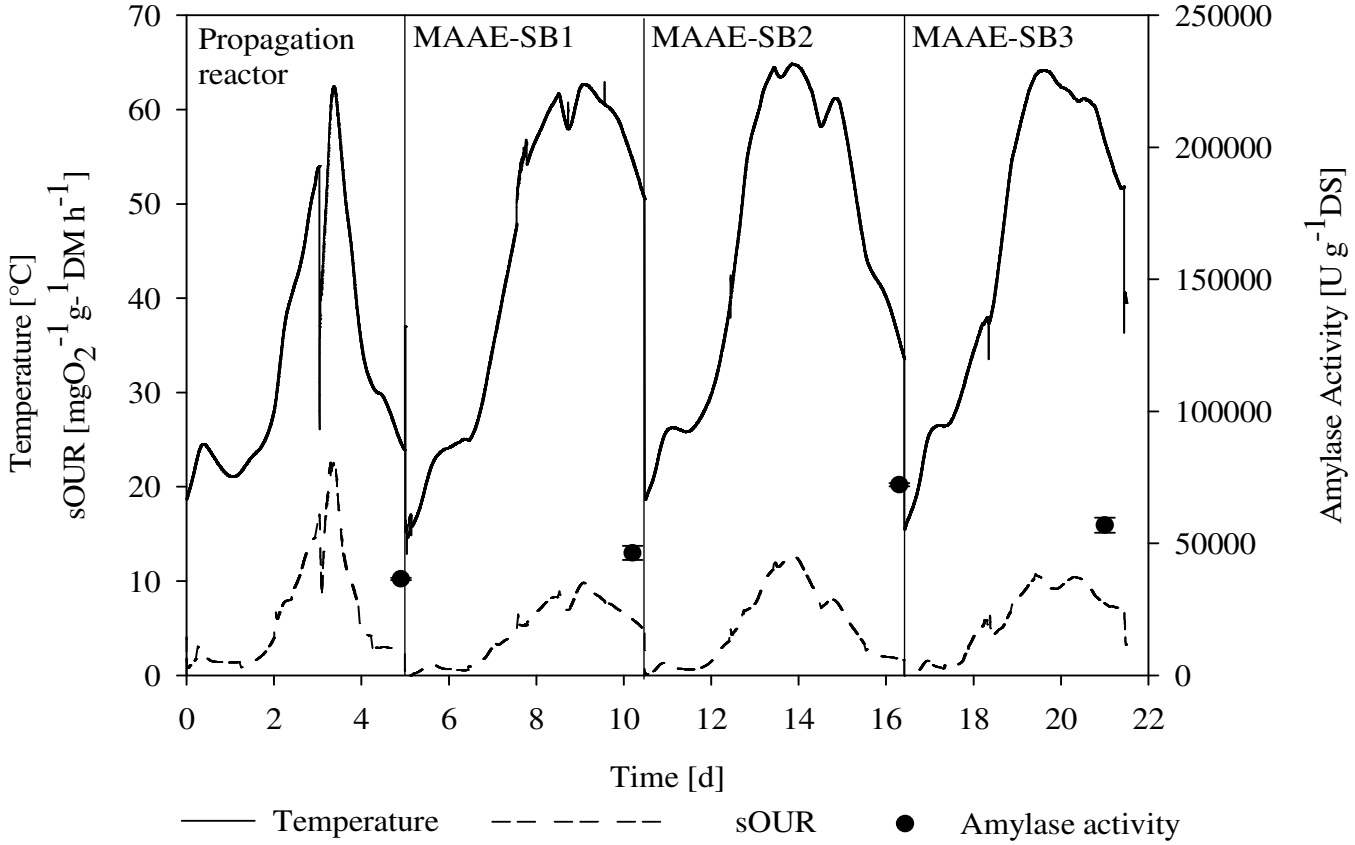
Propagation reactor
MAAE-SB1
MAAE-SB2
MAAE-SB3
Temperature [°C]
sOUR [mgO2 -1 g- 1DM h-1]
Amylase Activity [U g-1DS]
Time [d]
Temperature
sOUR
Amylase activity

Figure 5.