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Solid State Fermentation Based Olive Pomace Using *Streptomyces* Strains: A Preliminary Study

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Authors' contributions

This work was carried out in collaboration between all authors. Author SR designed the study and wrote the protocol. Author LMH performed laboratory experiments and wrote the first draft of the manuscript. Authors SMA and VD managed the analyses of the study. Authors MK and FZ managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This study represents a preliminary investigation aimed to assess the possibility to recycle and valorize olive pomace by solid state fermentation (SSF) using *Streptomyces* strains. The olive pomace was collected from an olive pressing factory (super press system) during the olive fruit pressing season. The study was carried out at IMBE, University Aix Marseille-France, between April 2013 and June 2013, and at LMA, University of Bejaia-Algeria in September 2013. Three *Streptomyces* strains designated S1M3I, S1M3II and S1M3III were cultured on solid state fermentation based olive pomace at 30°C for 10 days, and subsequently, the lignocellulolytic enzyme activities (xylanase, CMCase and laccase), the viability of the microorganisms and the pH of the resulting substrates, were determined. The fermented substrate pH values remained significantly stable ($p < 0.05$) throughout the fermentation period for the three strains; they were fluctuated between 6.54 and 6.99. The viability of all *Streptomyces* strains studied, decreased significantly ($p < 0.05$) during the first four days of incubation, to reach up 0 cfu/mL of viability and 0 U/g enzymatic activities (xylanase, CMCase and laccase activities) were recorded for the three strains. *Streptomyces* strains, under the experimental conditions (30°C, pH 7 and 75% of moisture), were unable to grow and produce lignocellulolytic enzymes in solid state fermentation based olive pomace due to the mycelial morphology and *Streptomyces* developmental cycle, no neglect, the environmental factors. These preliminary results suggest that SSF – *Streptomyces* system is not suitable for conversion of solid waste from olive processing industry and to produce lignocellulolytic enzymes.

Keywords: Solid state fermentation; Streptomyces; olive pomace; enzyme.

1. INTRODUCTION

Cultivation of olive is an important agricultural activity in Algeria. According to the National Agency of Development of Investment (NADI, Algeria), 87 500 tons of olive pomace are produced each year, released directly to the environment. The problem for these industries is the management of these residues because of their pollution in some cases and the costs associated with the treatment necessary for their proper disposal [1]. Olive pomace is the solid residue obtained after olive oil extraction. It is one of the most abundant agro-industrial by-products in the Mediterranean area [2]. The olive pomace consists of lignocellulosic matrix with phenolic compounds, uronic acids and oily residues [1] and can represent an important alternative source for enzymatic process [3]. The recycling of this by-product, which is available in appreciable quantities, for the nutrition of ruminants, can be interesting [4]. The biological method to improve the quality of low-grade roughages is drawing much attention due to its potential advantages over chemical/physical treatments such as greater substrate and reaction specificity, lower energy requirements, lower pollution generation and higher yields of desired products [2]. The use of olive pomace as solid substrate in submerged fermentation and solid state fermentation processes is an attractive solution, since due to its lignocellulosic nature can be an inductor of lignocellulolytic enzymes. Solid-state fermentation (SSF) using fungus has been reported to be a relatively low-cost appropriate technology for upgrading - lignocellulosic materials in animal feeding [5]. Additionally, SSF, which is characterized by microbial growth on moist solids, has proven to be an efficient way to produce lignocellulolytic enzymes, since it provides the filamentous microorganism with environmental conditions similar to those of their natural habitat.

The Actinobacteria are a group of Gram-positive bacteria. Some form branching filaments, which somewhat resemble the mycelia of the unrelated fungi, among which they were originally classified as the Actinomycetes [6,7]. Actinobacteria play an important ecological role in recycling substances in the natural world using organic matter [8]. *Streptomyces* is the most important genus in this group, able to produce and excrete a large variety of enzymes, including those involved in the degradation of cellulose, hemicellulose and lignin [3]. Their enzymes are more attractive than enzymes from other sources because of their high stability and unusual substrate specificity [8].

In this work, we have extended our previous investigations of *Streptomyces* development on submerged culture based olive pomace to solid conditions using the same substrate [9,10].

Preliminary experiments were carried out to assess the performance of SSF using three *Streptomyces* strains designated S1M3I, S1M3II and S1M3III, in terms of the viability of the strains and lignocellulolytic enzyme (Xylanase, CMCase and laccase) production. To our knowledge, this is the first report on the evaluation of the potentiality of Actinobacteria-treated olive pomace in this sense.

2. MATERIALS AND METHODS

2.1 Lignocellulosic Substrate

The olive pomace (OP) was collected from an olive pressing factory (super press system) of Bejaia, Algeria during the olive fruit pressing season, dried at room temperature for three weeks, ground to a fine powder, than passed through sieves (ᴓ ≤ 1mm). Sugar cane bagasse used in this study was obtained previously prepared (washed with water to remove all residual sugar, dried and milled to 1–10 mm).

2.2 Microorganism

The *Streptomyces* strains designated S1M3I, S1M3II and S1M3III, used in this study, were taken from our previous studies [9,10].

2.3 Preparation of Spore Suspension

The inocula were prepared by growing *Streptomyces* strains in 100 mL flask of M3 agar medium containing: 8 g/L raw pomace powder (ᴓ = 75 µm) and a mineral solution containing: 2 g/L soluble starch; 0.3 g/L casein; 1 g/L glucose; 2 g/L KNO_3; 2 g/L K_2HPO_4; 2 g/L NaCl; 0.05 g/L $MgSO_4.7H_2O$; 0.02 g/L $CaCl_2$; 0.01 g/L $FeSO_4.7H_2O$; 18 g/L agar, The pH was adjusted to 7.2 with 0.1 N NaOH. The incubation was carried out at 30°C for 7 days. In a next step, 30 mL of sterilized distilled water were added. Finally, spores were counted with Malassez cell and suspensions were adjusted to about 10^7 spores/mL [11].

2.4 Culture Conditions and Inoculation

At the first observation OP already formed a paste with 50% (v/w) of moisture which limits the aeration. For a large area of exchange between air and substrate, the OP was mixed with the sugar cane bagasse (Sugar removed). The SSF has been carried out at 30°C in 250-mL flasks containing 10 g of lignocellulosic substrate (5 g of olive pomace and 5 g of sugar cane bagasse) moistened to reach 50% with the mineral solution (MS) containing: 3 g/L KH_2PO_4; 6 g/L K_2HPO_4; 3 g/L $(NH_4)_2SO_4$; 1.2 g/L $NaNO_3$; 0.2 g/L $MgSO_4.7H_2O$; 0.05 g/L $CaCl_2$; 0.01 g/L $MnSO_4.7H_2O$; 0.001 g/L $FeSO_4.7H_2O$; 0.05 g/L yeast extract. The pH was adjusted to 7.2 with 0.1 N NaOH. These flasks were sterilized by autoclaving for 1 hour at 110°C. The inoculation of the sterilized solid medium was carried out with a suspension of 10^7 spores/mL, ultimately achieving 75% of moisture. A control for each strain was also run with the same conditions above, taken at T_0. The mineral salts will provide the minerals necessary for the growth of the microorganism but also have a buffering effect in order to obtain a regulation of the culture medium [12]. The calculation of the moisture was carried out as follows, according to Bettache [13]:

> 50% humidification = (10 g x 100)/50 = 20 g (wm)
>
> For 50% of moisture, a quantity Y of mineral solution is required, Y = 20 g (wm) – 10 g

Where *10 g*, weight of the lignocellulosic substrate used; *wm*, wet material humidified with mineral solution for 50% of moisture and *Y*, quantity of mineral solution required for 50% of moisture (g).

75% humidification = (10 g x 100)/25 = 40g (wm’)

For 75% of moisture, a quantity Y’ of mineral solution is required,

Y’ = 40 g (wm’) – 20 g (wm)

Y’ = 20 g ≃ 20 mL = 3 mL of spore suspension + 17 mL mineral solution.

Where *10 g*, weight of the lignocellulosic substrate used; *wm’,* wet material humidified with mineral solution for 75% of moisture and *Y’*, quantity of mineral solution required for 75% of moisture (g).

2.5 Treatment of Samples Obtained in SSF

Three Erlenmeyer flasks for each strain were taken daily during 10 days of the incubation period and their contents were treated as follows, 1 g of the fermented must was taken under sterile conditions then suspended in 9 mL of sterile physiological water, for viability analysis. The enzymes are extracted into the solid medium by adding 50 mL of distilled water to the whole of

the fermented mixture. Grinding was carried out using an ultra thorax until complete homogenization. The pH values of the fermented substrate were measured in a suspension (0.1 g / mL distilled water) using a HANNA pH meter.

2.6 Viability Assay

Viability assay was used as an indicator or measure of the amount of living microbial biomass in SSF. It was determined according to the method described by Gassara et al. [14], which consists of taking a sample of 1 g of fermented substrate and diluted it in 9 mL sterile physiological water. Adequate dilutions were made for these samples. At least five different dilutions per sample were used for the analysis. Each dilution (20 µl) was aseptically pippetted on an agar plate at six different spots. Three plates per dilution were seeded. The plates were incubated for 3 days, after which the growing spots on the plates were calculated. While calculating the results, the first dilution, where all the pippetted spots did not grow, and the last dilution, where at least one pippetted spot grew, and all the samples between these, were taken into account. The results in colony forming units (cfu) were calculated according to the following formula:

$$K = P/(Nn \times Nk)^{0.5}$$

Where *K,* number of live spores in a dry sample; *P,* number of positives in all the accounted series; *Nn*, amount of sample in the negative parallels (g); *Nk*, amount of sample in all the accounted series (g). In the screening studies, the growth was detected only visually.

2.7 Measurement of CMCase, Xylanase and Laccase Activities

Xylanase and CMCase activities were determined by measuring the release of reducing sugar according to Tuncer et al. [15]. Laccase activity was assayed according to Criquet et al. [16]. The results were expressed in units (U), where U is defined as the amount of enzyme required to liberate 1 µmol of xylose, glucose and quinone per min.

2.8 Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyze data and multiple pair-wise comparisons were performed by the Tukey test using Xlstat® software to analyze the differences between the treated and the control. The results considered statistically significant at ($p < 0.05$). All analysis were conducted in triplicates and expressed as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1 Viability of Microorganism and Enzyme Activities

In this study, the living mycelium as a relative measure of active biomass was used. The viability of the all *Streptomyces* strains, decreased significantly ($p < 0.05$) during the first four days of incubation, to reach up 0 cfu/mL of viability (Fig. 1). Additionally, all the strains studied appear to no grow on OP under SSF. An absence of mycelial growth was observed throughout the 10 days of incubation at 30°.

Extracts from cultures were assayed to determine the CMCase, xylanase and laccase activities. The results were shown that, all *Streptomyces* strains studied did not exhibit any enzymatic activities (0 U/g for xylanase, CMCase and laccase activities). The negative results of the viability indicate cell death, inducing absence of secondary metabolites (enzymes), which occurred from the first days of fermentation. These results indicate that the production of enzymes involved in lignocellulose degradation was wholly growth associated in cultures of *Streptomyces* strains on OP. This is in accordance with previous studies of Tuncer et al. [15] and Adhi et al. [17], who showed that the production of lignocellulolytic enzyme was growth associated in Actinobacteria cultures.

We demonstrated in our previous studies that these *Streptomyces* strains tested were able to grow in submerged fermentation based OP and exhibit enzyme activities, comprised between 1.44-0.57 U/mL for CMCase, 6.65-2.97 U/mL for xylanase and 5.63×10^{-3}-2.15×10^{-3} U/Ml for laccase [9]. At the present time, as far as we are concerned, there is no citation in the literature describing the use of OP for the production of lignocellulolytic enzymes by the Actinobacteria, especially the *Streptomyces.* However, several studies focusing on the production of lignocellulolytic enzymes by SSF of fungi using OP as substrate have been reported earlier [2,4, 18,19].

The decrease in viability and cell death observed with SSF was, probably, due to the mycelial morphology and *Streptomyces* developmental cycle. Indeed, it has been unanimously accepted

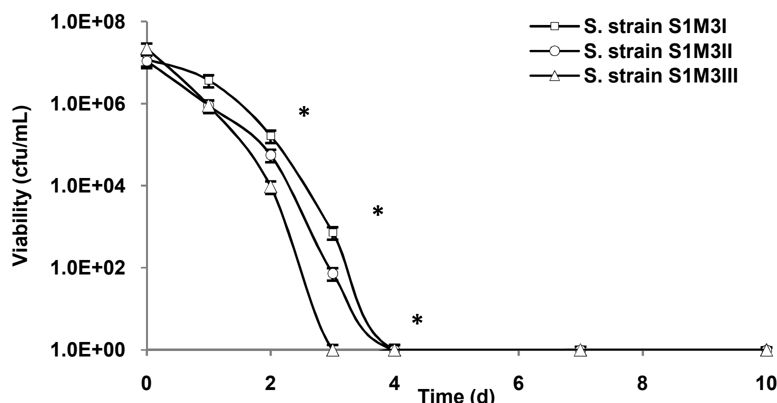

Fig. 1. Viability of *Streptomyces* strains: S1M3I, S1M3II and S1M3III, during the incubation period in SSF based OP at 30°C

Results are means ± SD of triplicate measurements. "" indicates a significant difference in viability between control (T_0) and SSF treatment (T_n) for the three strains studied (ANOVA, $p < 0.05$). Key: S = Streptomyces*

that mycelial morphology in liquid fermentation was correlated with the growth and the production of secondary metabolites [20]. On the other hand, there are important differences between solid and submerged development of *Streptomyces* [21], which reside in the absence of a second death round and sporulation in the case of liquid cultures. However, as in solid cultures, there is a first, compartmentalized, mycelium (first mycelium) which begins to group into pellets at early time points and which starts to die from the center outward. There is also a transient growth arrest which precedes the emergence of a multinucleated mycelium (second mycelium) that grows from the remaining viable segments of the first, compartmentalized, mycelium [20,22,23].

It is important to know that, *Streptomyces* is considered as a "multicellular" prokaryotic model that includes programmed cell death and sporulation in solid cultures [20]. Several studies reported that, the growth arrest prior to the appearance of the second mycelium has been detected in solid cultures [23–25], conditions under which nutritional stresses have been postulated to activate the formation of aerial mycelium, secondary metabolism, and even lysis of the mycelium [21,26,27]. It is possible that it was the case of the *Streptomyces* strains, in the present study.

On the other hand, environmental factors (Temperature and heat transfer, water content and water activity, aeration, pH and substrate) may be the cause of cell death and, consequently, the absence of enzymatic production [28–30].

The temperature of the substrate is very critical in SSF as it ultimately affects the growth of the microorganism, spore formation and germination, and product formation [28,29,31–33]. The typical effect of temperature during SSF is heat accumulation [34]. The temperature at the center of the fermenting solid medium could be higher up to 20°C than the incubation temperature [31]. An increase in temperature promotes the higher evaporation of water from the fermentation, which causes cell death or an absence of spore germination [35]. Compared to liquid state fermentation, the problem of heat transfer makes it difficult to reach the optimum temperature, as the metabolic heat production for the unit volume of solid medium is much higher than that of liquid medium [35,36].

Water content is one of the key factors in a successful SSF [37]. Therefore, changes of water content in the medium will affect growth and the metabolic capability of cells during SSF [36]. The water content will also affect a substance's physical characteristics, the diffusion and utilization of nutrients, the exchange of oxygen and carbon dioxide, and the process of heat and mass transfer [36,38]. High moistures results in decreased substrate porosity, which in turn prevents oxygen penetration [39]. This may help bacterial contamination [40]. On the other hand, low moisture content may lead to poor accessibility of nutrients and oxygen resulting in poor microbial growth [31]. Knowing that, oxygen

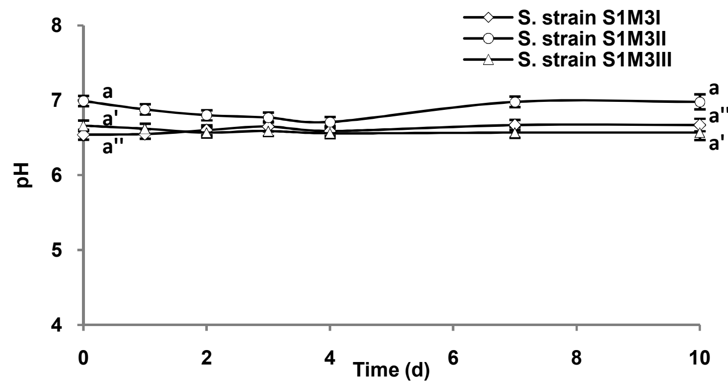

Fig. 2. Evolution of pH of the fermented substrate of *Streptomyces* strains: S1M3I, S1M3II and S1M3III, during the incubation period in SSF based OP at 30°C

Results are means ± SD of triplicate measurements. Same label (a, a' or a") above the graph for the same strain indicates that no significant difference exists between control (T_0) and SSF treatment (T_n) throughout the fermentation period, for the three strains studied (ANOVA, $p < 0.05$). Key: S = Streptomyces"

transfer is undoubtedly the most important phenomenon to sustain microbial activity [41]. The rate of oxygen transfer to the cells is often the limiting factor that determines the rate of biological conversion. An insufficient oxygen transfer leads to a decrease in the microbial growth and product formation [42].

Olive pomace, used as solid substrate, is rich in phenolic compounds, uronic acids and oily residues [1]. The presence of free fatty acids in the fermentation medium can cause an inhibitory effect and an antimicrobial action [43]. According to Sado Amdem et al. [44], this antimicrobial action is due to the penetration of acid in the lipid membrane of the bacterial cell. The corresponding cellular acidic pH leads cell death by suppressing cytoplasmic enzymes and nutritional transport systems as well as uncoupling ATP driven pumps [45]. Similarly, Polti et al. [46] indicated that high amount of fatty acid content plays important role in broadening the antimicrobial spectrum modified oils. We noted in our previous study [10] that the saturation of the culture medium by polyphenols released during the incubation period is at the origin of cell death and absence of viability. Additionally, several studies demonstrate that polyphenols are responsible for the antimicrobial activity [47–49].

3.2 Evolution of pH

The fermented substrate pH values remained significantly stable ($p < 0.05$) throughout the fermentation period, they were fluctuated between 6.54 and 6.99 (Fig. 2). These pH values are in the optimum range reported in the literature for growth and enzyme production of *Streptomyces* species [3,15]. In this study the fermentation medium was supplemented with 3 g/L KH_2PO_4 and 6 g/L K_2HPO_4 for the maintenance of a fermentation pH around 7.0.

During a fermentation, that takes place normally, the metabolic activity of the microorganisms causes a drop in the pH of the culture medium [50], due to acid formation [51], affecting the growth and enzyme production of the microorganism [51]. The use of buffers could be solving this problem [51,52]. At the same time solid cellulosic substrate is a good buffer for pH change during SSF [28]. It was reported by Banks et al. [53] that lignocellulosic substrate offered little natural pH buffering capacity for the medium during fermentation. But in our case there is no production of enzymes, that can destabilize the pH of the culture medium and this may be explain the stability of pH throughout the fermentation period, in addition to buffering capacity of the medium.

4. CONCLUSION

The results of this experiment show that *Streptomyces* strains, under the experimental conditions (30°C, pH 7 and 75% of moisture), were unable to grow and produce lignocellulolytic enzymes in solid state fermentation based OP due to the mycelial morphology and *Streptomyces* developmental cycle, no neglect, the environmental factors. These preliminary results suggest that SSF – *Streptomyces* system is not suitable for conversion of solid waste from

olive processing industry and to produce lignocellulolytic enzymes. However, SSF allows the recovery of large quantities of OP compared to the submerged fermentation that is why, further research work in these areas is necessary to verify our conclusion and find solutions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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