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Research Article

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Potential Use of Lipid Waste Industrial Residues for Lipase, Phospholipase and Bio surfactant Production Using a Newly Isolated Bacillus Safensis Strain

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Abstract

The aim of this work was to produce lipases by a newly bacterial strain using as substrate, reach lipid waste by products from oil refining or commercially soap industry. For the fermentation tests, two substrates were investigated to produce lipases: soap stock (solid lipid waste from an oil refining industry) and glycerin (liquid lipid waste from soap industry). The higher level lipases production was obtained with the soap stock as the sole carbon source. Different parameters such as pH, temperature of the medium and incubation time were optimized. A correlation was also obtained between detected lipolytic activity and reduction of surface tension in the culture medium. The surface tension decreased from 50 to 25.7 mN/m indicating that biosurfactants were produced in the culture medium. As soap stock contains phospholipids molecules, this by product also enhances phospholipases production by the newly Bacillus safensis strain.

Keywords: Bacillus safensis, Soap stock, Glycerin, Lipases, Optimization, Biosurfactants, Phospholipases

Introduction

Despite the great interests in the application of lipases in various industries, their high costs of production often restrict their use as biocatalysts [1]. One of the research areas involving lipases currently focuses on the use different microorganisms, supplements and substrates to find the best combinations to obtain high value lipases using operational conditions that facilitate the reduction of the production costs at industrial scale [2]. This can be achieved through the use of low cost culture media especially residues from agro-industry so that production can become economically viable [3]. Lipases have been found in many species of animals, plants, and microorganisms. However, the enzymes from microbial sources are currently receiving more attention because of their interesting characteristics, such as action under mild conditions, stability in organic solvents, high substrate specificity and region- and enantio-selectivity [4, 5]. Bioconversion of agricultural residues for lipase production as well as other value added products would hold a prominent position in future biotechnologies, mainly because of its eco friendliness and flexibility to both developing and developed countries. Many microorganisms secrete lipases during growth on organic residues. This is because industrial waste, agro-related and complex organic residues constitute a significant source of residual nutrients that can serve as growth media for microorganisms that are able to produce lipases. Thus lipase production depends largely on microorganisms, culture practices, medium composition and bioreactor design. Among the industrial waste residues used for lipase production, by-products of oil extraction from seeds and oil refining process. The aim of this work, was to produce lipolytic enzymes and biosurfactants using a lipid waste (soap stock) as the sole carbon source using a newly Bacillus safensis strain.

Materials and Methods

Screening of lipolytic microorganisms

Initial screening of lipolytic microorganisms from various Tunisian biotopes was carried out using a plate assay in a medium containing triacylglcerols and the fluorescent dye Rhodamine B [6]. The solid medium contains 1‰ olive oil, 1% nutrient broth, 1% NaCl, 1.5 g agar and 1‰ Rhodamine B. The culture plates were incubated at 37°C, and colonies giving orange fluorescence halos around them,

upon UV irradiation, were regarded as putative lipase producers 6). After extensive screening of lipase producers, only one bacterial colony, isolated from olive-oil contaminated soil continued to give positive signal when commercial detergent (1%) was added to the solid medium described above.

Industrial by-product

The first by-product from sunflower oil refining, commercially codified as soap stock, was kindly provided by an oil refining industry (Agrimed, Sfax, Tunisia). The soap stock result from the neutralization process, was mainly composed by water, soaps and triacylglycerols. The second by-product a liquid lipid waste codified as glycerin, was kindly provided by a soap industry (Zwila, Mahdia, Tunisia). The glycerin result from saponification process was mainly composed by glycerol and fatty acids.

Microorganism identification

The identification of the bacterial strain has been previously determined [6]. The methods used for 16S rRNA gene amplification and sequencing have been previously reported [7]. Sequence data were imported using the ABI PRISM, 3100. The full sequence was aligned using BLAST program. The phylogenetic tree was constructed using the neighbor-joining method by MEGA 4.D [8]. Strain was affiliated to Bacillus genus and designed as Bacillus safensis strain.

Media and culture conditions

Bacillus safensis strain was grown overnight at 30 °C and 160 rpm in a liquid medium autoclaved at 121°C for 20 min. containing per liter: 5 g yeast extract, 10 g NaCl, 10 g peptone, 1% sunflower oil; pH 7.0. Fourteen hour Bacillus safensis culture was used as inocula for lipase production.. A 24 h culture of Bacillus safensis was used as inocula added in amounts of 2-3% v/v of the medium volume. During cultivation, pH of the culture liquid was controled. After the end of cultivation, the culture was centrifuged at 12 000 rpm for 15 min at 4 °C, and the cell-free supernatants were used for measuring lipolytic activities. Moreover, others experiments were realized using culture media composed only by soap stock or by glycerin.

Cell growth determination

Biomass concentration was measured via turbidimetry at 600 nm and the obtained values were converted to concentration by using a previously determinated calibration curve.

Lipolytic activity assay

The lipase activity in the culture liquid and the preparations was assayed by measuring the free fatty acids released from mechanically stirred emulsions of triacylglycerols, using 0.1 N NaOH with a pH-Stat (Metrohm, Switzerland). The kinetic assay was performed, in optimal conditions (pH 8.5 and 37°C) using 0.25 ml TC4 (Sigma) in (30 ml 2.5 mM Tris-HCl, 150 mM NaCl and 0.5 mM Sodium deoxycholate (NaDC)) or in olive oil emulsion obtained by mixing (3×30 s in a Waring blender), 10 ml of olive oil (Sfax-huile, Tunisia) in 90 ml of 10% GA (Gum Arabic). One lipase unit corresponds to 1 µmol of fatty acid released per minute [9].

Phospholipase activity determination and qualitative analysis of reaction products

The phospholipase activity was measured titrimetrically at pH 8.0 and at 37°C with a pH-stat, under the standard assay conditions described previously10, using PC or egg yolk emulsions as substrate in the presence of 3 mM NaTDC and 7 mM $CaCl_2$. One unit of phospholipase activity was defined as 1µmol of fatty acid liberated under standard conditions. The composition of the hydrolysis product was investigated by thin-layer chromatography (TLC) on silica 60 F 254 previously activated at 60°C for 30 min. The developing solvent was a mixture of chloroform/methanol/ammoniac (32%) (65:25:5,v/v/v). The lipid spots were visualized with iodine vapor.

Biosurfactant production determination

Surface tension measurement was used to evaluate biosurfactant production when soapstock was used as culture medium. Samples of the culture media were centrifuged at 8000 x g for 20 min. Surface tension (ST) of the supernatant of the culture was measured according to the De Nouy methodology using a tensiometer TD1 (Landa-Konigs hofen, Germany). The measured was performed in triplicate.

Results and Discussion

Effects of alternative substrates on lipase production

Effluents produced from edible oil refinery, slaughterhouses and dairy products industry contains high concentrations (100 mg/ml) of lipids. Effluents such as olive mill waste water and palm oil mill effluents showed potentials to be used for lipase production [10]. In addition, others used olive mill waste water as a growth medium for lipase production in which C. Cylindracea NRRL-Y-17506 showed the highest lipase activity of 9 U/ml in the medium supplemented with ammonium chloride and olive oil among the twelve microbial species tested [11]. In addition, these others, assessed lipase production in bench-top reactor using the olive mill waste water medium and found a maximum production of 20 U/ml. Based on these studies, effluents from oil related industries can be used as valuable liquid growth media for production of microbial lipases. In order to verify the effect of lipid waste substrates on lipase production, soap stock and glycerin were used as the sole carbon source to produce lipase by Bacillus safensis strain. Lipase activities observed after 24 hours fermentation in 500 ml Erlenmeyer flasks can be seen in (Fig. 1). A comparison of the results of lipolytic activities obtained with two lipids industrial waste (soap stock and glycerin) showed that the maximum activity (18 U/ml) was obtained when using soap stock as an inducer substrate. On the contrary the maximum activity reached 5 U/ml when used glycerin as an inducer. This decrease in the lipolytic activity can occur due to oil acidity of the second lipid waste substrate (presence of fatty acids). Based on these results, soap stock was selected as the best and the main inducer for lipase production by Bacillus safensis strain.

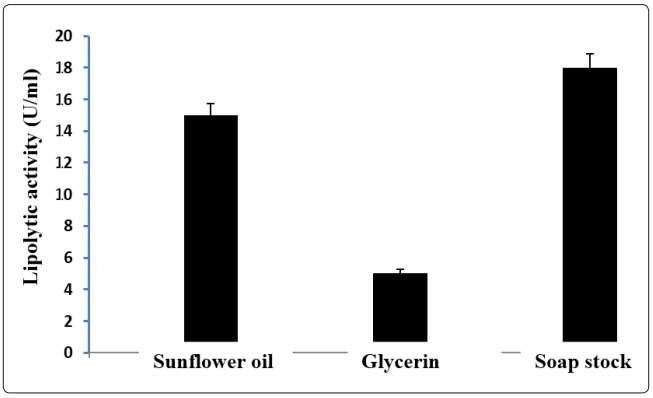

Figure 1: Lipase activity determination after 24 h of incubation using different lipids as the sole carbon source. Values are means ± SD (n = 3).

Effect of pH on enzyme production using soap stock as an the sole carbon source

The production of microbial lipases has been shown to be influenced by several factors. The pH of the culture medium has been described as an a determinant factor that increase the production of enzymes with lipolytic activity [12]. Also in the same cases it is essential for lipolytic activity to be detected. Last, the engineering of culture conditions has also been shown to be an effective mode to achieve enzyme preparations enriched in selected isoenzymes which are effective for particular biotechnological applications 13]. As pH is one from important parameters required for growth of bacterial culture in respective media, the effect of pH on the enzyme production was assessed in a wide range (5-10). The obtained clearly indicates that there is a strong influence of pH on lipase production. Thus the maximum activity was reported at pH 9, mentioned in (Fig. 2). For pH values outside this range, enzyme production seems to be completely inhibited a fact which reveals the importance of studying this factor in culture and of controlling the pH variations during its cultivation. This fact, that has also been found in our previous works, indicates that the determination of medium optimum pH value represents an adequate operating strategy that can favors the increase of enzyme level in the medium and facilitate its recovery [9].

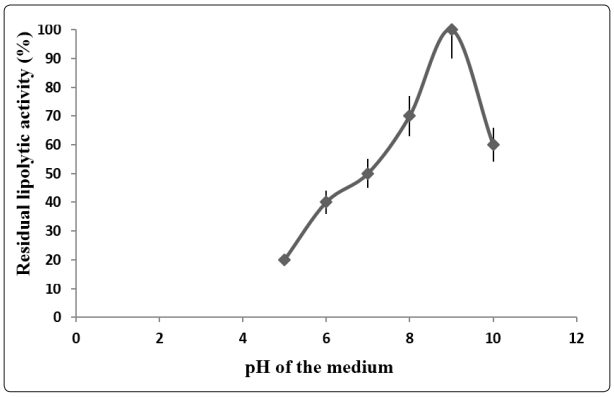

Figure 2: Effect of pH on lipase activity production when soap stock was used as the sole carbon source

Temperature and incubation time modulate cell growth and lipolytic activity

The studied strain was grown in shake flasks using soap stock as a substrate (the sole carbon source, within a wide range of temperature (from 30 to 55°C). The increase in temperature seemed to have a negative effect on biomass production and also lipolytic activity. Biomass production and total lipolytic activity reached their maximum, in flask culture incubated at 30°C after 24 h of incubation. For flask, cultures incubated at temperature higher than 30°C, biomass production and total lipolytic activity were negligible after the same incubation time. The maximum biomass production and the total lipolytic activity levels were measured for 40 and 55°C at 48 h and 72 h incubation, respectively (Fig. 3). One can say that in all cases, the highest final values were obtained when operating at a wide temperature range from 30 to 55°C.

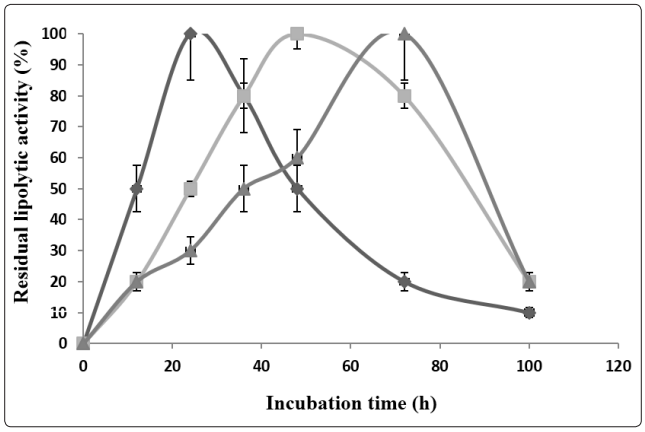

Figure 3: Effect of temperature and incubation time on lipase activity production

Simultaneous production of lipolytic activity and biosurfactant when using soap stock as the sole carbon source

Lipases and biosurfactants are compounds produced by microorganisms generally involved in the metabolization of oil substrates. However, the relationship between the production of lipases and biosurfactants has not been established yet. Therefore, this study aimed to evaluate the correlation between production of lipases and biosurfactants using Bacillus safensis strain which was isolated from olive oil-contaminated soil. When Bacillus safensis strain was cultivated on soap stock, the surface tension of the culture dropped rapidly until the 24 h of the incubation to reach after several hours its lowest point which was about 25.7 mN/m (Fig. 4). The diameter of the clear zone obtained by oil displacement test method was more than 8 cm (data not shown). The reduction of the surface tension of the culture indicated that biosurfactants were produced. Simultaneously, Bacillus safensis stain produces enzymes essentially lipases with a maximum reaching 18 U/ml.

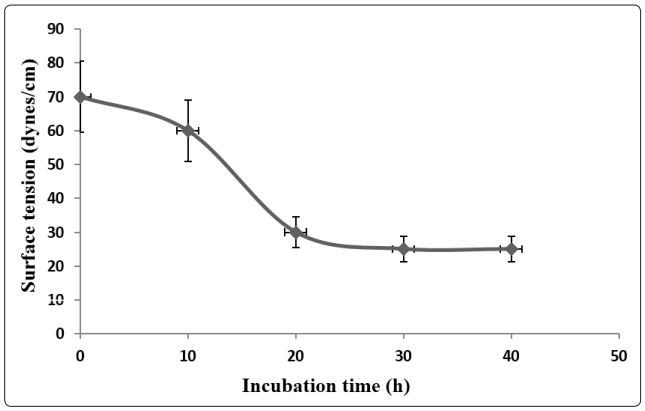

Figure 4: Kinetic of biosurfactants production by Bacillus safensis using soap stock by measuring the increase of the surface tension

Detection of a phospholipase activity when using soap stock as the sole carbon source

It is well known that soap stock was mainly composed by lipids molecules such as triacylglycerol and fatty acids, but it contains also a little percentage of phospholipids. Considering that the presence of phospholipids compounds in the culture medium generally favours the production of phospholipases, we are interesting to test the presence of a phospholipase activity using pH-stat method when using soap stock as the sole carbon source. Our results showed that this novel Bacillus safensis strain produced phospholipase activity simultaneously with lipolytic activity and biosurfactants when soap stock was used as the sole carbon source. The presence of phospholipase activity was confirmed by the analysis of the lipolysis product of TLC. An accumulation of fatty acids was observed after 30 min of soybean PC hydrolysis using Bacillus safensis supernatant culture (Fig. 5).

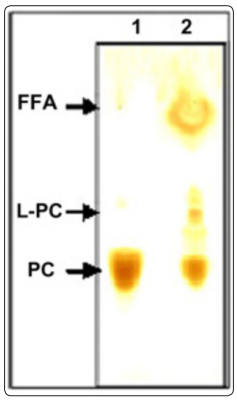

Figure 5: TLC analysis of the hydrolysis of soybean PC by Bacillus safensis supernatant culture. Lane 1, soybean PC before hydrolysis, lane 2, soybean PC after hydrolysis

Conclusion

In conclusion, our investigation indicated that soap stock can be considered as a growth medium for microorganisms providing a satisfactory supply of nutrients for growth for lipolytic enzymes and biosurfactants production. This study has proved that the optimization of growth parameters in a suitable medium has significant effect on improved production. Thus, efficient enzymes and metabolites production from industrial waste can add value and ensure overall reduction in the enzyme's final production cost.

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