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Abscisic Acid Production: Biotechnology Process Development and Scale-up

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In the last years it's noted a growing interest in the use of microorganisms, and their metabolites, in agriculture. Microorganisms studied are those naturally present in the soils. Attention was focused on microorganisms producing abscissic acid (ABA). Their possible uses are in pre- and in post- harvest practices. ABA production was studied on laboratory scale, then costs were estimated for ABA industrial production that were compared with those of the products, containing ABA, on the market. The maximum ABA production rate (calculated by Monod model) is the one corresponding to *B. cinerea* in YD, at 298K, no stirring and sunlight, equal to 1.08E-8 kg m^{-3} s^{-1}. In order to produce ABA with a cost lower than 20,000 €, it's estimated that is necessary to have a bioreactor size between 15 - 20 m^3. Relative investments costs estimates are about 1,400,000 €. To maintain supernatant, stream ABA rich, production cost below 1 €/kg, size bioreactor must be about 10 m^3, its relative investment costs are estimated about 1,300,000 €.

1. Introduction

Traditional agricultural practices have the disadvantage of not being able to provide inexpensive products in large quantities. A part of this traditional farming now takes the name of organic farming (BIO), which represents a sizeably niche market with medium-high prices; anyway this practice cannot avoid the use of chemical additives for fertilization, for the control of pathogens, for the maintenance of the sensory characteristics of the products and to ensure quality standards comparable to non-BIO products. Today these productions are very popular. This scenario is compatible with an increase in the use of additives with biological criteria. In this context, biotechnology can offer alternative solutions based on products of natural origin.

ABA was identified and characterized in 1963 by Frederick Addicott and his staff during a study of the substances responsible for abscission of the fruits of the cotton plant. Two substances were isolated, abscissina I and II, now called ABA, ubiquitous substance in the vascular plants.

The first real demonstration of the production of ABA by a fungus was reported by Assante et al. (1977) who discovered a fungus, *Cercospora rosicola*, causing the appearance of brown spots on the leaves of rose, able to produce in liquid culture ABA (0,06 kg m^{-3}). Since then, it has been found that other species were able to produce ABA in vitro: most are plant pathogenic fungi. This suggests that ABA production by fungi may have a possible role in phyto-pathogenesis. There are no data regarding ABA production in plants during the process of fungal infection. ABA is responsible for multiple effects and currently the commercial formulations containing this hormone are used in agriculture for different applications, in particular to:

- improve plant tolerance to various types of environmental stress (especially to water stress);
- delay the rate of growth of plants;
- treat the fruit in pre-harvest.

ABA has also insecticidal capacity. Microorganisms used in this work for ABA production were *Botrytis cinerea* and *Cercospora rosicola*. Data obtained in the laboratory scale tests were used to estimates industrial production costs of ABA.

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2. Methods

Cercospora rosicola (CBS n° 138.35) was purchased from CBS and *Botrytis cinerea* (ATCC n° 90870) was purchased from ATCC. The two strains, delivered in frozen form in vials coated by dry ice, were revitalized: the vials were placed in a water bath at a temperature of 25 to 30 ± 0.1 ° C until completely thawed (about 5 minutes). Following thawing, vials were cleaned with 70% ethanol. 50 µl of material were aseptically transferred in the reference medium: on plate and in broth. The mediums were used, respectively, PDA and PD; the plates and the flasks were incubated in a thermostat at 24 ± 0.1 ° C; the growth of the strains was checked regularly during incubation and after 7-10 days was observed the presence of viable colonies both on the plate and in broth. For the maintenance of these strains we were performed every two weeks on the ground skim PDA and the plates were incubated in a thermostat at 25 ± 0.1 ° C for 4-5 days.
To estimates industrial production costs SuperPro Designer® Software was used.

2.1 Medium Composition

Medium composition for strains growth were: YPD (Yeast extract 50 kg/m^3, Y, peptone 50 kg/m^3, P, Dextrose 200 kg/m^3, D) with addition of starch; YD (yeast extract 50 kg/m^3, Y, Dextrose 200 kg/m^3, D) with calcium carbonate ($CaCO_3$ 100 kg/m^{3}YDC) or without; PDA (infused potatoes 4kg/m^3, P, Dextrose 20 kg/m^3, D, Agar 15 kg/m^3, A); PD (infused potatoes 4 kg/m^3, P, Dextrose 20 kg/m^3, D).

2.2 Analytical methods

ABA determination was performed using the "Phytodetek® ABA Test Kit" (Agdia) as reported in Annex procedure. Method Enzyme-Linked Immunosorbent Assay (ELISA) of competitive type for ABA determination was used. The concentration of ABA in the unknown samples was obtained using a straight line of from 0 to 6E-5 kg/kg calibration.

3. Experimental plan

Table 4 shows the experimental plan carried out. Types of medium, temperature, different exposure to sunlight and agitation speed were varied. In each test as inoculum (0.05 m^3 m^{-3}) a microorganism culture in exponential phase (spores 51 days aged) was used.
Each of the test was carried out in three replicates. *Botrytis cinerea* was used in the tests from 1 to 10; *Cercospora rosicola* in the tests from 11 to 20 (Table 1).

*Table 1: Operating conditions of tests carried out. Tests from 1 to 10 refer to B. cinerea, those from 11 to 20 C. Rosicola. * Exposure at natural sun light*

Test	1-11	2-12	3-13	4-14	5-15	6-16	7-17	8-18	9-19	10-20
Medium	YPD+starch	YPD+starch	YPD+starch	YPD+starch	YD	YDC	YD	YDC	YD	YDC
Length (h)	480	480	480	480	528	528	1,008	1,008	1,872	1,872
Temperature (K)	298±0.1	298±0.1	298±0.1	301±0.1	298±0.1	298±0.1	298±0.1	298±0.1	298±0.1	298±0.1
Llight exposure*	No	Yes	No	NO	Yes	Yes	Yes	Yes	Yes	Yes
Stirring (rad s^{-1})	0	0	10.47	0	0	0	0	0	0	0

In each flask sterile gauze pads were introduced that act as inert material and allow fungus to adhere and produce spores to improve fungal growth, morphology, and production of secondary metabolites bioactive as described by (Bigelis et al., 2006). More particularly, the immobilization on an inert support is able to protect the biomass, support the growth of the mycelium and improve the fungal activity (Rodriguez-Couto et al., 2009). It was observed that in some cases the biomass immobilized on a support shows a higher enzyme production, approximately 2÷25 times, compared to the one observed when the cells are free to move (Gao et al., 2010). On this basis we hypothesized that supporting *Cercospora rosicola*, and *Botrytis cinerea* on solid material it's possible to obtain a large quantitative of hormone such as ABA. To confirm this, tests were planned with only sterile gauze pads as inert material. In addition, the calcium carbonate (CaCO3) was added to the minimal medium YD (YDC), to have a more tenacious adhesion of the biomass to the inert matrix (gauze).
Samples were taken for any test, at regular time intervals, to determine ABA production. The mycelium was recovered at the end of the test and was rinsed with distilled water 3-4 times to remove traces of medium. Then it was dried in a vacuum oven at 353K for 4h to obtain also dried weight.
The differences in production of ABA and biomass between the two fungal strains at different times and at different conditions were evaluated using ANOVA (Systat 13).

4. Results and discussion

4.1 Experimental data

In Figure 1 ABA production data obtained in the better tests (Test 9, Test 10, Test 19 and Test 20 as described in Table 1) are reported.

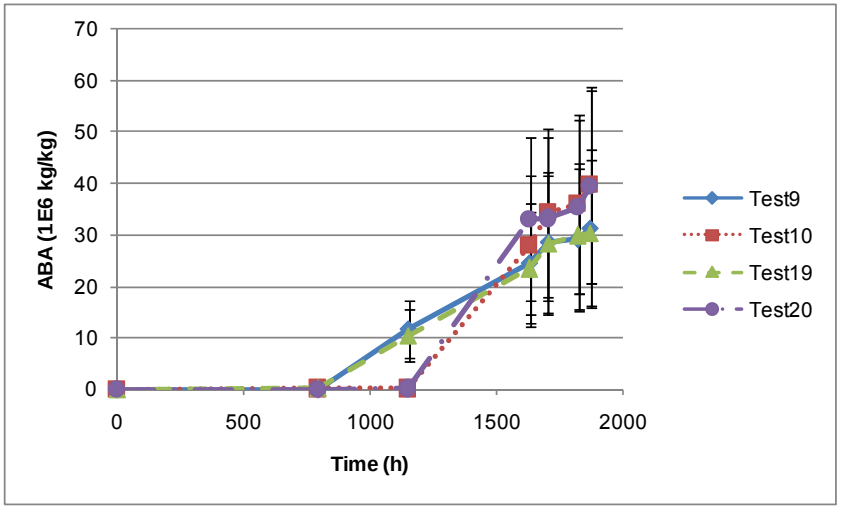

Figure 1: ABA production in better tests (T9, T10, T19, T20)

The maximum ABA production rate (calculated by Monod model Eq(1) and Eq(2)) is the one corresponding to *B. cinerea* in Test 9 – YD, 298K, no stirring, sunlight – equal to 1.08E-8 kg m^{-3} s^{-1}.

$$\mu = \mu_{max} \cdot \frac{S}{k+S} \quad (1)$$

where:
S: initial concentration of reference substrate (kg m^{-3})
μ_{max}: maximum production or growth rate (kg m^{-3} s^{-1}); calculated as reported in Eq(2) in exponential phase
K: Monod constant

$$\frac{dX}{dt} = \mu \cdot X \quad (2)$$

where:
X: ABA or cells concentration (kg m^{-3})
μ: maximum production or growth rate (kg m^{-3} s^{-1}).
For Test9 the biomass production rate was equal to 4.4E-7 kg $m^{-3}s^{-1}$. This data were used in the estimation of ABA industrial production costs.

4.2 Estimation of ABA industrial production costs

To estimate ABA industrial production costs the SuperPro Designer® software was used. SuperPro Designer is a valuable tool in process development, process engineering, and manufacturing. SuperPro provides under a single umbrella modeling of manufacturing and end-of-pipe treatment processes, project economic evaluation, and environmental impact assessment.
The results obtained showed that the different sets of physical conditions (light, temperature and stirring) did not affect the production of ABA. Due to this result, estimation were done in the most economical conditions to reduce the energetic cost of the process. In particular it was hypothesized that the strains growth is done at natural light, at room temperature (25±0,1 °C), without stirring.
Block diagram of process simulated for industrial ABA production is reported in Figure 2.
Medium component are mixed (3/MX-101) than stored (2/V-101). Medium is feed at bioreactor (1/F-101).
To simulate bioreaction hypotheses were: continuous process, temperature 298K, residence time to simulated exponential production, and growth, rate, air flow 2.5E-4 m^3 m^{-3} s^{-1}, reaction stoichiometry as reported in Eq. (1)

150 Glucose + 5 Air + 25 Yeast Extract→ 160 Biomass + 0.1 Debris + 10 ABA + 9.9 H2O (1)

Broth is stored (4/V-102) before centrifugation (5/BC-101). Centrifuge performance are those reported in Table 2. Sediment is stream rich in biomass instead supernatant is ABA rich.

Table 2: Operating conditions of industrial centrifuge simulated

Component	Biomass	Debris	Glucose	ABA	Protein	Water
Recovery in sediment (kg/kg)	0.98	0.6413	0	0	1	0

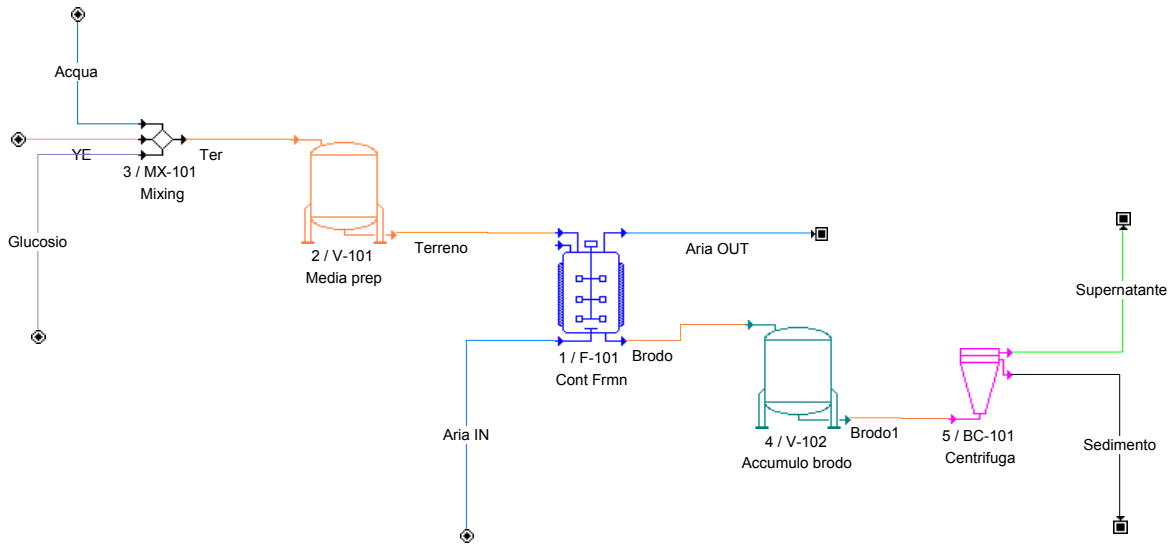

Figure 2: Block diagram of simulated process for industrial ABA production

Supernatant is the stream of interest (ABA rich). Yields, investment costs, operative costs and ABA production costs were calculated as below described. In Table 3 industrial process yields for 3 m^3 bioreactor are reported; in Table 4 characteristics and costs of major equipment are described.

Table 3: Estimated process yields. 3 m^3 bioreactor

	Sediment	Supernatant
t/y	623.6	1.9

Table 4: Characteristics and costs of major equipment 3 m^3 bioreactor (Figure 2)

Equipment	V-101 (0.11m^3)	F-101 (1.4m^3)	V-102 (0.165m^3)	BC-101	Auxiliary equipment	TOTAL
Cost (€)	15,000	250,000	20,000	120,000	101,250	506,250

Other hypotheses are: 250 operating day for year; 3 consecutive eight-hour shifts for 2 people per shift.
Purchase cost for industrial plant was estimated equal to 506,250 €. In Table 5 investment costs for industrial plant are reported.

Table 5: Investment costs for industrial plant 3 m^3 bioreactor (Figure 2)

Equipment (€)	Design, construction management, authorizations (€)	Civil works, structures (€)	Engineering, contingenties (€)	TOTAL
506,250	24,300	74,925	32,400	637,875

In Table 6 costs items for industrial plant are reported.
It was hypothesized to sell sediment at 1.50 €/kg in order to have other revenue (935,000 € for industrial plant with 3 m^3 bioreactor).
ABA production cost was estimated equal to 27,750 €/kg for 3 m^3 bioreactor; this cost is compatible with ABA commercial price.

Table 6: Production costs of ABA for industrial plant 3 m^3 bioreactor (Figure 2)

Depreciation (€/y)	Medium (€/y)	Staff salaries (€/y)	Utilities (€/y)	Maintenance (€/y)	Operating costs (€/y)	Revenue sediment (€/y)	Supernatant production costs (€/kg)	ABA production costs (€/kg)
63,787.50	100,000	180,000	565,840	31,893.75	941,521.25	935,000	3.44	27,750

It's possible to estimate supernatant (stream ABA rich) production cost equal to 3.44 €/kg. Supernatant production cost was estimated because some results, not reported, demonstrated that supernatant is useful in pre-harvest to speed up the ripening process of some fruits and its cost it's very competitive.
Same iter was used to estimate ABA production costs vs. different bioreactor size (Figure 4); this is useful to define plant size in order to have profit. In Figure 3 supernatant production costs v. bioreactor size are reported.
In order to produce ABA with a cost lower than 20,000 €, it's possible to estimate that is necessary to have a bioreactor size between 15 - 20 m^3. Relative investments costs estimates are about 1,400,000 €.
To maintain supernatant production cost below 1 €/kg, size bioreactor must be about 10 m^3, its relative investment costs are estimated about 1,300,000 €.

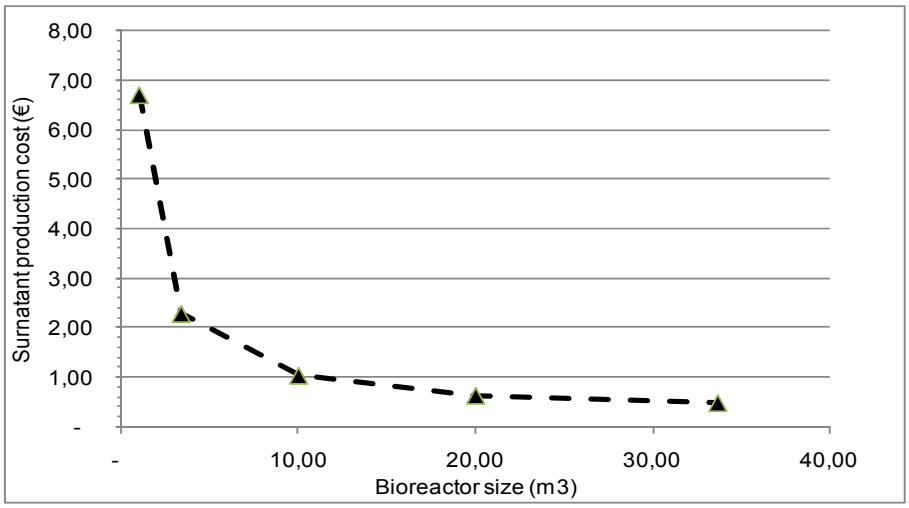

Figure 3: Surnatant production cost vs Bioreactor size

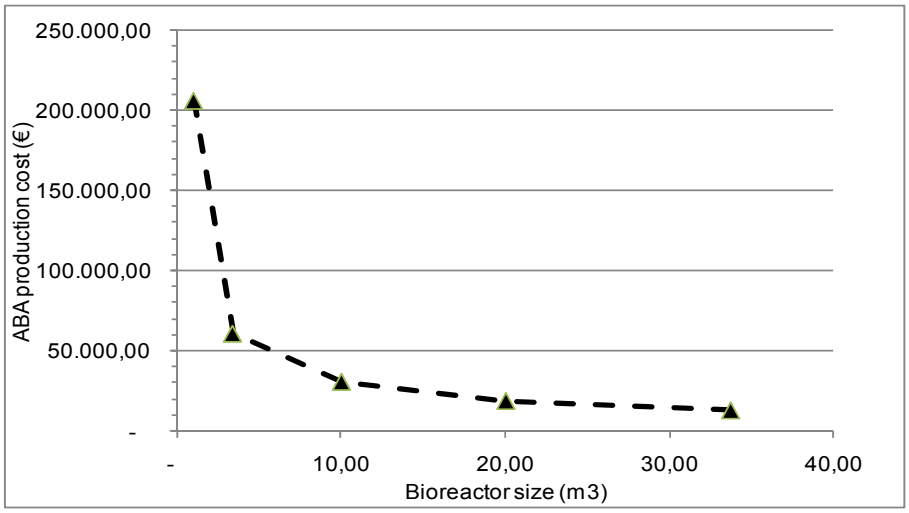

Figure 4: ABA production cost vs Bioreactor size

5. Conclusion

The results obtained showed that both the selected microbial strains are able to produce ABA. Some of the different sets of physical choices conditions tested (light, temperature) did not affect the ABA production. It is instead necessary to use gauze to promote adhesion and, consequently, the growth of the mycelium and production of ABA.
The maximum ABA production rate (calculated by Monod model) is the one corresponding to *B. cinerea* in YD, at 298K, no stirring and sunlight, equal to 1.08E-8 kg m^{-3} s^{-1}.
Using the SuperPro Designer® software it was possible to simulate, with data obtained in laboratory scale, an industrial process (Figure 2) for ABA production. This software was useful to estimate investment cost, operative costs and ABA production cost.

Acknowledgments

This work is dedicated to Stefano Bianchini, died prematurely, who started the experiments on abscisic acid production in CRAB Laboratories.

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