

A PROCESS FOR THE PREPARATION OF DERIVATIVES OF RUSCUS
ACULEATUS STEROID GLYCOSIDES

The present invention relates to a process for the preparation of derivatives of *Ruscus Aculeatus* steroid glycosides (ruscosaponins).

More particularly, the invention relates to the preparation of desglucodesrhamnoruscin by hydrolysis of ruscoside and/or desglucoruscoside through the fermentation route.

Desglucorhamnoruscin and the corresponding free aglycons, ruscogenin and neoruscogenin, which can be easily obtained from desglucodesrhamnoruscin by acid hydrolysis, are valuable pharmaceutical active principles having antiinflammatory and connective-protecting activities.

The chemical preparation of said active principles starting from ruscoside or desglucoruscoside is however problematic, since it requires drastic conditions, such as hydrolysis with strong acids, and complex operative steps, which yield a very heterogeneous mixture of intermediates and products.

It would therefore be highly desirable to provide a process for the preparation of desglucodesrhamnoruscin, which overcomes the drawbacks mentioned above connected with the known chemical processes.

The present invention meets such need, by providing a process for the preparation of desglucodesrhamnoruscin which comprises the hydrolysis of *Ruscus aculeatus* steroid glycosides (ruscosaponins) through fermentation of a substrate containing said glycosides by means of fungi of the *Aspergillus niger* species.

A culture broth is typically used as a nutrient complex substrate.

Fermentation is generally carried out at a temperature

of 25-30°C, preferably 27°C, under stirring and aeration so as to attain a pO_2 higher than or equal to 50%.

The concentration of the starting steroid glycosides usually ranges from 5 to 15% w/v, preferably from 8 to 10% w/v and the pH of the culture broth ranges from 4 to 6, preferably 4.5-5.5.

The biotechnological process of the present invention allows to carry out the whole reactions sequence in a single fermentation step, in that the microorganism, selected with suitable microbiological techniques, is capable of expressing the necessary enzymatic activities for operating all the transformation sequential reactions, from the starting complex heteroglycoside to the monoglycoside or the final aglycone. Said hydrolase transformations comprise in fact a sequence of β -glucosidase, α -rhamnosidase reactions on the intermediates which are successively released during the process. A further α -arabinosidase reaction allows to obtain the free aglycone (ruscogenin).

Said approach is quite novel, in that no applicative examples of said procedure for the preparation of said products can be found in literature.

The microorganisms suitable for carrying out the transformations involved are obtained by selection on synthetic or semi-synthetic media, added with the same substrates to be transformed, in addition to or in place of the conventional carbon sources (glucose, saccharose, and the like). The concerned substrates (ruscoside, desglucoruscoside) can be added in this case in even high concentrations, e.g. 90-100 g/L. The agarized isolation media comprise the usual formulations for microbiology, such as Malt Agar and Czapek Agar, or similar formulations, wherein the nitrogen source is represented by peptones, urea, ammonium nitrate, and the like, whereas the

conventional carbon source (glucose, saccharose) has been substituted or supplemented by ruscoides or desglucoruscoides. Said media can be further added with mineral salts of potassium, magnesium, manganese, zinc etc., such as phosphates, sulfates and/or chlorides. The pH of the isolation media can range from 4 to 6, preferably from 4.5 to 5.5.

The microorganisms suitable to the required biotransformations are recovered by scalar dilution and plating of aqueous suspensions of samples of soil, humus, vegetable extracts and other similar organic sources.

The microbial cultures selected as described above are isolated in microbiology test-tubes containing the same culture media and used for the biotransformation of ruscoides and desglucoruscoides, added in high concentrations (to 100 g/L) to liquid culture media containing the same nitrogen sources as used in the isolation media, such as urea or peptone, with the addition of phosphates and other mineral salts, as described above, at pH ranging from 4 to 6, preferably 4.5 + 5.5.

Following the procedures described it has been found that selected cultures of *Aspergillus niger* are capable of transforming ruscoides and desglucoruscoides into desglucodesrhamnoruscoides, a direct precursor of ruscogenins, by a sequence of enzymatic β -glucosidase and α -rhamnosidase reactions.

A subsequent α -arabinosidase reaction provides the saponin in the aglyconic form (ruscogenin - neoruscogenin).

The selected culture is capable of operating said transformations growing in controlled (thermostatic) conditions, at optimal temperatures ranging from 25°C to 30°C, under stirring on a rotatory shaker (200 + 300 rpm). Said fermentation can also be carried out in a suitable bioreactor, at different scale levels, for the industrial

production of the desired saponin derivatives.

The microorganisms used for said biotransformation are capable of steadily maintaining the catalytic activity, even for repeated fermentation cycles, in batch or continuous processes.

The present process provides important advantages, such as less complex the steps for the separation and recovery of the product, and is also easy to carry out as well as cost-saving.

The selected microorganisms can be frozen for the to long-term storage, in suspensions enriched with cryopreservatives, such as glycerol, peptone and the like, at temperatures ranging from -80°C to -196°C (in liquid nitrogen), or subjected to freeze-drying treatments.

The progress of the bioconversion can be monitored by TLC and HPLC analysis on the culture broth, using the following analytical methods:

TLC Analysis

- Silica gel plates 60 F250 Merck
- Eluents:
 - A) Ethyl acetate - Methanol 9:1
 - B) Ethyl acetate - Methanol - Water 100:15:10.
- Detection: reaction with 10% sulfuric acid and heating to 120°C for 5 minutes, then visible and UV detection.

HPLC Analysis

- Column: Supelcosil LC18, 250 x 4,6 mm, 5 µm
- Eluent: acetonitrile - water 60:40
- Wavelength: 200 nm
- Injection volume: 10 µl
- Flow: 1 mL/min.

The final biotransformation products, such as desglucodesrhamnoruscin, can be recovered by extraction of the culture broth with n-butanol, subsequent purification

steps with chlorinated solvents (such as trichloroethane) and silica gel chromatography. Finally, the product can be crystallized from different solvents, such as isopropanol, ethyl acetate, chloroform, acetone, methanol. In addition to the main product, desglucodesrhamnoruscins esterified at C-2', for example with 2-hydroxy-3-methylpentanoic acid, can be obtained.

The saponins in the aglyconic form (ruscogenins) are obtained by acid hydrolysis of the fermentation products described above.

The following examples disclose the invention in greater detail.

Example 1

2 Flasks of culture medium (Malt Broth, 250 mL per flask) are inoculated with spores from a culture of *Aspergillus niger* on Malt Agar, obtained by selection on Modified Agar malt medium (added with 2% ruscocide). The flasks are incubated for 48 hours at +27°C, on an orbital stirrer at 250 rpm. After incubation, the preculture is transferred into the bioreactor, containing about 7 L of sterile production broth R090, having the following composition (values referred to one litre of deionized water): Ruscocide Dry extract (g 90), Urea (g 1), Peptone (g 1), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (g 5), KCl (g 1,5), KH_2PO_4 (g 1), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (g 0.2), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (g 0.1) antifoam P2000 (mL 1,5), at about pH 5.

The fermentation is carried out on the basis of the dissolved oxygen percentage (pO_2), progressively increasing the stirring rate and the air flow, to obtain a pO_2 value higher than 50%. The progress of the bioconversion is monitored by HPLC and TLC analysis. After 5 day incubation at +27°C the fermentation is over. TLC and HPLC analysis of the broth show that ruscocide has disappeared while desglucodesrhamnoruscin is present as main product, with

ruscogenins traces.

The culture broth is exhaustively extracted with n-butanol. The butanol extract is concentrated to dryness under vacuum at +60°C, redissolved in 70% methanol and
5 back-extracted with trichloroethane. The chlorinated solution is concentrated to a solid under vacuum. After redissolution in a chloroform-methanol mixture, the product is purified by column chromatography (Kieselgel, Merck), with ethyl acetate-chloroform 9:1 as eluent. The fractions
10 are checked by TLC or HPLC analysis. The purified product fraction is concentrated under vacuum, then redissolved in acetone and crystallized. A further crystallization from methanol yields about 7 g of product, identified by spectroscopic analysis as desglucodesrhamnoruscin. In
15 addition to the main product, a desglucodesrhamnoruscin esterified at C-2' with 2-hydroxy-3-methylpentanoic acid is obtained in lower amount (about 800 mg).

Acid hydrolysis of the fermentation products described above yields the saponins in the aglyconic form
20 (ruscogenins).

Example 2

Operating in a fermenter as described in example 1, a first biotransformation cycle is carried out, after that 90% of the culture broth is taken to be extracted for
25 obtaining the product; the remaining 10% fermentation broth is added in the fermenter with fresh R090 medium to a final volume of about 7 L. This second fermentation cycle is carried out with the same parameters and analytical controls as described above. After about 5 day incubation
30 at +27°C the fermentation is completed.

The culture broths from the two fermentation cycles are treated as described in example 1, for the extraction and recovery of the product. At the end of the final step, about 14 g of desglucodesrhamnoruscin are obtained.

CLAIMS

1. A process for the preparation of desglucodesrhamnoruscin which comprises the hydrolysis of
5 *Ruscus aculeatus* steroid glycosides (ruscosaponins) through fermentation of a substrate containing said glycosides by means of fungi of the *Aspergillus niger* species.
2. A process as claimed in claim 1, wherein said substrate is a culture broth.
- 10 3. A process as claimed in claim 2, wherein said fermentation is carried out at a temperature of 25-30°C, under stirring and air bubbling so as to reach a pO₂ higher than or equal to 50%.
4. A process as claimed in claim 3, wherein temperature
15 is 27°C.
5. A process as claimed in claim 3 wherein the starting concentration of said steroid glycosides ranges from 5 to 15% w/v.
6. A process as claimed in claim 5, wherein said
20 concentration ranges from 8 to 10% w/v.
7. A process as claimed in any one of claims 2-6, wherein the pH of the culture broth ranges from 4 to 6.
8. A process as claimed in claim 7, wherein the pH of the
25 culture broth ranges from 4.5 to 5.5.