Closing the loop through the valorization of glycerol as a substrate in the production of hyperthermophilic β-glucosidase. A Life Cycle Perspective.

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Bioethanol production from lignocellulosic biomass requires cellulose attack, which can be carried out by enzymes, such as β-glucosidase. The production of the enzyme by *Yarrowia lipolytica* offers as a major sustainable advantage, as it can be produced from glycerol (Shaikh et al. 2021, Abdellaa et al. 2016, Rywińska et al. 2013). Thus, the use of a by-product of biofuel production would close the cycle within the framework of the circular economy. However, the integration of enzymatic processes for the biotransformation of biomass within the biorefinery framework requires to confirm whether the use of enzyme is conducted under environmental sustainability criteria.

The biotechnological process is divided into three sections: inoculum preparation, glycerol fermentation and downstream for enzyme purification. To ensure the better procedure and the highest productivity of the process, a surplus of C-source is required, in the form of glucose. Looking for sustainability approaches, different streams have been studied as glucose sources, including agro-industrial side-streams. With respect to the fermentation process, it would be based on a batch procedure, whereby the operating conditions were evaluated to ensure the highest enzyme titer. Regarding the downstream stage, further processing proceeds to biomass removal by centrifugation and microfiltration. The enzymatic crude stream must be purified for the purpose of removal of salts from the culture medium, as well as other extracellular proteins, fatty acids and polysaccharides. For this purpose, a sequence of several steps of chromatography must be conducted. After the lyophilization step, β-glucosidase is ready for commercialization (Abdellaa et al. 2016, Workman et al. 2013, Cybulski et al. 2018).

This study aims to evaluate the environmental profile associated with the production of enzymes, although being bio-based catalysts, the several stages involved in the process could incur to environmental impacts. In this regard, Life Cycle Assessment (LCA) methodology has been used for obtaining the profiles of the process, within a cradle-to-gate approach and using ReCiPe as calculation methodology. The results obtained showed that the fermentation stage, where enzyme production takes place, corresponds to the stage that contributes the most to the environmental impacts followed by the downstream separation scheme. The main hotspots identified are the ones of energy requirements and the use of certain chemicals. In this scope, de-fossilization of the biotechnological industry will be possible with thoroughly optimized bio-transformations, with carbon-based medium from residual resources, minimized use of chemicals and the implementation of energy integration measures (Abdellaa et al. 2016, Rywińska et al. 2013, Cybulski et al. 2018).

In order to evaluate production scenarios, once the main environmental hotspots have been identified, two alternatives have been considered. First, the use of alternative renewable feedstocks instead of glycerol, these being used cooking oil and olive mill wastewater, as both are assimilable by *Y. lipolytica* and have proven effective in the production of lipase enzymes (Gottardi et al. 2021, Lopes et al. 2021, Spagnuolo et al. 2018). Secondly, pursuing a biorefinery approach, the co-production of erythritol has also been evaluated, also using glycerol as C source (Rakicka-Pustułka et al. 2020). The commercial use of this co-product in the food industry is extended, which brings added value to the proposed biotechnological route, thus increasing its sustainability and circularity.

The environmental profiles of the scenarios proposed in this study could be considered as innovative and environmentally friendly alternatives for the formulation of culture media to produce enzymes. The concepts of sustainability and circular economy have been considered, seeking the valorization of secondary waste streams and proposing biorefinery approaches. However, certain conflictive points have been identified when applying the LCA methodology, with energy requirements and certain chemical products being those with the greatest environmental contribution. In this sense, further research would be necessary to obtain even better environmental profiles in the path of β-glucosidase production strategies. Thus, this study provides useful information for future research and stakeholders on where to focus.

Keywords: bioethanol; *Y. lipolytica*; biotechnological process; environmental assessment; circular economy.

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