Reviewer B:

Reviewer comments:

The literature review should include some more recent papers dedicated to the discussion of the feasibility of using immobilized enzymes for production scale use, viz. DiCosimo et al Chem. Soc. Rev., 2013, 42, 6437 (and eventually other papers published in Chem. Soc. Rev., 2013, 42)

*Accepted and done.*

The design of experiments is adequate and mostly standard. Still some issues should be looked into, out of concern for reproducibility. Missing is the quantification of protein immobilized, viz. through Bradford method (Anal Biochem. 1976 May 7;72:248-54.) or alike. This would also help to establish how much of the specific activity was actually retained upon immobilization.

*Accepted and done. The results, by Bradford method, of protein concentration in solutions after enzyme immobilization in negative, that’s why we wrote “immobilization was complete”.*

Also on this, the authors state that “The procedure was repeated until proteins could no longer be detected in the supernatant”. How did they access this? Please provide details.

*This state is written in section Enzyme isolation. Cell wall invertase, the enzyme we isolated and used in this paper, is not soluble enzyme, so it was necessary to remove all soluble protein from yeast cell. The enzyme was “washed” until Bradford protein test was negative.*

Also reference to the work of Miller (Anal Chem 1959;31:426-8) that a cornerstone of colorimetric quantification of reducing sugars should be made Line 81,

*Thank you for this observation, the reference for reducing sugar is missing in the paper. Instead of Miller modified method we use original method Bernfeld, P. Methods in Enzymology 1955, 1, 149 and the reference is added in section Enzyme activity assay.*

please provide details on centrifugation, viz. g force, temperature and time length of operation for reproducibility.

*Accepted and done.*

In line 87 the authors report of “invertase activity 9 U/mg”. Does “mg” refer to the defatted and acetone dried solid material resulting of cell wall processing?

*Yes*

Please clarify Line 88 “in a range of 0.25 – 2 g” does the mass report to the dried pellet? Please clarify. Also on this line the authors state “within 0.8% (w/v) cross-linker monomer N,N′-methylenebisacrylamide” within suggest a range so is 0.8% the lower or the upper limit? Please clarify

*6 different amount of CWI were immobilized 0.25; 0.50; 1.00; 1.50; 2.00; 3.00 and we thought it is not necessary to give all concentration in the paper because the concentration of CWI in the immobilizate is given in the x-axis Fig 1. The additional information about enzyme is added. The concentration of 0.8% (w/v) cross-linker monomer N,N′-methylenebisacrylamide was constant for all obtained immobilizate.*

Regarding the immobilization procedure, did the authors based their procedure in a known methodology as it seems evident (viz., see for instance Practical Enzymology by Hans Biisswanger) and in which case a reference should be added? Please clarify.

*The method has already been used for decades in our research group for polyacrylamide synthesis for electrophoresis, and now for the first time used for enzyme immobilization.*

Also on the procedure for immobilization, the authors state that the total volume was kept under 15 mL but if one adds up the volumes given they do not exceed 3 mL. Where does the remaining volume come from? Buffer solution? This would be adequate to ressuspend the dry pellets. Please clarify.

*Thank you for this observation. The buffer is missing in the text, and the Tris buffer was used. It is added in the text.*

Was polymerization performed at room temperature? How much time did the procedure require? Which device was used to cut the gel in discs of similar shape? Please clarify.

*The polymerization temperature and time is added in the text. A metal cylinder was used for cutting the gel.*

The “immobilized enzymes were stored at 4°C in 70% (w/w) invert sugar”. Was this solution prepared in buffer (which)? Otherwise? Please clarify.

*Acetate buffer was used for this solution, it is added in the text.*

Again, did the authors wash the disks with acetate buffer prior to testing the activity? Otherwise the invert sugar adsorbed into the gel could bias the results of the activity test as it would diffuse back into the reaction medium. Please clarify

*Yes, the disks were washed after testing and also as blank sample usually was used sample mixture after mixing all reactants in time zero. All the time we have this problem on the mind.*

The authors used different buffers to evaluate the effect of pH in enzyme activity, as each of the buffers had a limited range. Did they rule out the intrinsic effect of the buffer in enzyme activity, viz by determining the activity at the same pH but with different buffer and checking that the activity was the same? An alternative could have been the use of McIlvaine buffer, that has a pH range within 2.2 to 8.0. Please clarify

*Yes. We used acetate and phosphate buffers and we had tree same pH values under the pH 5.7 for activity checking. The activities were within the limits of experimental errors.*

Lines 209/210 , English should be improved and a reference added to support the claim for auto hydrolysis at low (< 3.0) pH values. Most curiously, after referring this, the authors discuss the effect of pH in stability. Why did they consider a pH of 2.0, where according to their own text, makes no sense accessing enzymatic hydrolysis? Why not check for pH 5.0 or 5.5?

*Accepted and done. Stability testing is intended to demonstrate the difference between free and immobilized enzymes under the extreme reaction conditions like low or high pH value and temperature. We believe that there is not necessary to test biocatalyst stability at pH higher than 4.5 because at this pH both free and immobilized CWI had no changes in activity during 7 days.*

Regarding thermal stability, since the authors determined half-life, why did they not determine the activation energy for enzyme inactivation for either enzyme formulation? This would further help to establish the advantages of immobilization.

*All data necessary to easy calculate the inactivation energy are given in the paper. We believe that it is not necessary extend the text with additional information.*

On the matter of continuous operations, the text between lines 281 and 283 should be improved, viz. bed volume, not bad volume. Also despite of the claim of constant invert sugar production, 30 % conversion is rather low and would not be attractive for production purposes, please comment

*Accepted and done. It is true, the conversion of 30% is low for invert sugar production, but the aim of this experiment was to test biocatalyst’s stability and possible mechanical changes in packed bad reactor under pressure, highly viscous solutions and high temperature. After all optimization of reactor we had more than 80% conversion, but we have not further tested this biocatalyst.*

Reviewer C:

Reviewer comments:

1- I suggest the title to be “Immobilization of cell wall invertase in polyacrylamide hydrogel for invert sugar production”.

*Accepted and done.*

16- abbreviation (km).

*Accepted and done.*

19- Put the abbreviation in the key words

*Accepted and done.*

21- Instead of operation, preferring process.

*Accepted and done.*

23- key words must be improved and related to the abstract i.e there is sacchrose.

*Accepted and done.*

53- Cheap ( I prefer ) to be easily purified or easily extracted.

*Accepted and done.*

63- Check the spelling of career.

*Accepted and done.*

209- Must change the sentence ( it does not have a sense to use invertase ).

*Accepted and done.*

228- Optimum temperature instead of temperature optimum.

*Accepted and done.*

259- ( kM) must be change in all paragraph to km .

*Accepted and done.*

Reviewer E:

ADDITIONAL COMMENTS

 Reject manuscript

REPORT:

 The authors have investigated the use of the immobilized cell wall invertase to produce high concentrated invert sugar in batch and packed bed reactor.

The results are of interest in biochemistry; however, the work lacks originality and novelty. In a previous work, described in reference 6: Food Chemistry 2007, 104, 81. Authors have already studied the Cell wall invertase immobilization within calcium alginate beads to produce invert sugars from sucrose. Thus, the authors haven't investigated any experiments in new applied fields. To improve the results they could try to produce others interesting bio-molecules such as the enzyme production of glyco-oligosaccharides or alkyl-oligosaccharides.

In my view, and also to enhance manuscript quality, authors could for example analyze the potential of the bioreactor to be reused.

Yet, authors should keep in mind that enzyme bioreactor conception for efficient biotechnological applications should be better optimized by a Response Surface Methodology Authors haven’t given any information about molecular properties of the enzyme.

The manuscript is poorly referenced: 13 references are cited before 2000 and no new references after 2012, only one in 2013.

For these reason, the Introduction paragraph should be rewritten and actualized with new and most recent references.

*Thank you for your comments. Response Surface Methodology is in used in our research at the moment and will be part of our future papers.*