Dear Editor, dear Olgica Nedic,

We thank for handling our manuscript, for recognizing its potential and for giving us the opportunity to submit the revised version. We have carefully considered all comments and suggestions raised by the reviewer, made the changes accordingly and provided feedback, as detailed above.

We trust that the manuscript is now improved and wehonestly hope that in this current is acceptable for publication in Journal of the Serbian Chemical Society.

Best regards, also on behalf or other authors,

Dr. Katarina Mihajlovski

REPORT:

The manuscript entitled “Effective valorization of barley bran for simultaneous cellulase and β-amylase production by *Paenibacillus* CKS1: Statistical optimization and enzymes application” by Mihajlovski et al., describes the optimization of media components through

the statistical approach for the synthesis of cellulases and beta-amylase bythe organism. Before it is published it needs minor revision mainly inanswering above listed questions:

1. Please correct the following sentence from the Introduction:

Barley bran is a complex material which is the major by-product of thebarley milling process. Due to its lignocellulosic-starch structure, it ismainly used as substrate for microorganisms fermentations and for theof enzymes laccase, xylanase and amylase but not for cellulase

production.

This is not quite true, moreover in the Discussion section authors arepapers which deals with the cellulase production from barleybran.

REPLY 1:We thank the Reviewer for his comments and indication of the points that need correction.In the Manuscript we made some changes referring to the production of cellulose on barley bran and on barley stalk. Please see page 3, section Introduction, red marked sentence.

2. The authors should include the preliminary statistical experiment inwhich the parameters range was determined.in this work. We need to see why the authors decided to use YE at all. First of all, theresult with the barley bran alone would be more significant, since it wouldjustify the statement that this kind of residue can be valorised as an inexpensive raw material. Secondly, it is quite obvious from the surface response plots that loweringquantity of YE might enhance the activities of all enzymes. CCD was goodmodel however there is no plateau obtained in the 3D plots indicating thathigher activities could be obtained with higher quantity of barley bran orlower YE, or both. Also from the quadratic equation we can see thatβ22 is negative also indicating that YE has negative effect on enzymeproduction. Table 1 is also confusing coding factors which are in bracketsshould be uniformly distributed. What is also confusing is why the authorsused casein hydrolysate and YE for fermentation.

REPLY 2:

*Paneibacillus chitinolyticus* CKS1, a new species, was isolated from soil sample, which are had taken from aconiferous forest, from a foot of the Austrian Alps by members of our laboratory. The strain was not ordered from any culture collection,and it was necessary to find an adequate nutrient medium (broth) suitable for optimal growth of the microorganism. The most suitable liquid medium for growing the starin CKS1 was the ISP1(International Streptomyces Project 1) broth containing 3 g/l yeast extract and 5 g/l casein hydrolysate.

In microbilogy, yeast extract, as additional supplement, rich in nitrogen, amino acids and vitamins is standardized for bacteriological use and cell cultures, and is an excellent stimulator of bacterial growth. Yeast extract is generally employed in the concentration of 3 – 5 g/l.Casein hydrolysate is an excellent source of free amino acids and short peptide fragments, which are required by microorganisms for growth. Also, it contains trace of minerals and ions that could enhance the enzyme secretion.

In this paper, barley bran was used as carbon source for cellulase and amylase induction. During his growth in IPS1 broth, the strain CKS1 does not produce cellulase and amylase. Using yeast extract in a medium with barley bran, positive influence on microorganism growth and enzymes production were reached. This was the reason why the yeast extract was selected as process variable. Although in the CCD model yeast extract has a negative influence on enzymes production, addition of a little concentration leads to the higher enzymes activities thus the production costs were reduced.

The content of Table I is shown as Table I and Table II. Table I refers to the experimental ranges of the independent variables in the CCD, while Table II presents results enzymes activities. Please see page 4 of Table I and Table II page 6.

3. How it is possible to perform activity assay for Cellulase activity that

was measured by reduction of 3,5-dinitrosalicylic acid in the presence ofglucose released by enzymatic hydrolysis of cellulose according to themethod of Müller, as explained in Material and methods and to know the activities of avicelase and CMCase?

REPLY 3:Cellulase systems consist of endoglucanases, exoglucanase, and β-glucosidase and the synergy of all three enzymes enables to hydrolyse cellulose to glucose.The highly soluble cellulose CMC is widely used as a substrate to test endoglucanase activity while microcrystalline cellulose (Avicel) is used to measure exoglucanase activity. In our previous study, we showed that the novel isolate *P.chitinolyticus* CKS1 could hydrolyse both amorphous (CMC) and microcrystalline cellulose (Avicel) on different optimal temperatures. Optimal temperature for CMC ase activity was 50 °C, while for Avicelase activity was 80 °C.

4. Please remove this sentence from Discussion:

The possible explanation in low cellulase activity is that the strain CKS1during his growth on barley bran could produce both CMCase and Avicelase while Geobacillus sp. produced only CMCase.

This is too speculative whit no proofs.

REPLY 4:This was corrected. Page 12.

5. Please remove this sentence from Discussion:

Higher β-amylase activity, in comparison with cellulase (CMCase andAvicelase) activities obtained in this study may be a consequence of thedifferent substrate consumption.

Those two activities especially because defined with different substratescannot be compared that way.

REPLY 5:This was corrected. Page 13.

6. Since the activities of cellulase and amylase obtained in this study arelower than previously published, some information about the activities ofpreviously produced individual enzymes with this substrate is missing. Thiscould help in understanding is simultaneous production effort worthy.

REPLY 6:In our prevoious papers different substrates were used for the production of only cellulase or only amylase. In this paper,for the first time, we showed that barley bran, as waste substrate, could be used for simultaneous production of two enzymeswhich would reduce the cost of its production.

In our previous paper, the strain CKS1 was used for cellulase (CMCase and Avicelase) production using medicinal herbs waste and produced only cellulase. CMCase activity and Avicelase activities were 0.203U/ml and1.94U/ml, respectively. Molasses and sugar beet pulp was inducers only for beta amylase with activity of 2.237 U/ml.

It should be kept in mind, that during the production of cellulase on medicinal plants waste, only cellulasewas induced, therefore no amylase activity was detected. Similar was mentioned in our previous work, when only amylases were produced on molasses and sugar beet pulp.

7. According to the HPLC analysis the authors calculated the grams ofobtained glucose form cotton fabrics and maltose from barley bran. Why notcalculate the glucose from bran as well?

REPLY 7:We fully agree with the reviewer’s opinion.The main product after cotton hydrolysis is glucose,so we calculated the grams of obtained glucose. During barley bran hydrolysis with cellulase and β-amylase the main product was maltose with a little glucose, amylases were dominant thus we wanted to point this and calculated only maltose.

8. The authors cite reference 16 as a reference for beta amylase activityassay, however there is no amylase activity assay in reference 16. If the authors meant reference 17 they need to recheck all reference as to beproperly cited.

REPLY 8:Thank you for pointing this. We apologize for incorrectly cited references. This was corrected. Please see page 18.