



**SDI Review Form 1.6**

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| Journal Name:            | <b><u>British Microbiology Research Journal</u></b>                                                                                                                                                                                  |
| Manuscript Number:       | <b>Ms_BMRJ_22959</b>                                                                                                                                                                                                                 |
| Title of the Manuscript: | <b>OVERPRODUCTION OF XYLANASE FROM MUTANTS OF <i>Bacillus subtilis</i> USING BARLEY HUSK AS THE PRIME CARBON SOURCE IN SUBMERGED FERMENTATION AFTER RANDOM MUTAGENESIS OF ETHYL METHANE SULFONATE (EMS) AND ACRIDINE ORANGE (AO)</b> |
| Type of the Article      | <b>Original research Articles</b>                                                                                                                                                                                                    |

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This journal's peer review policy states that **NO** manuscript should be rejected only on the basis of '**lack of Novelty**', provided the manuscript is scientifically robust and technically sound.

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### PART 1: Review Comments

|                                     | Reviewer's comment                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | Author's comment (if agreed with reviewer, correct the manuscript and highlight that part in the manuscript. It is mandatory that authors should write his/her feedback here)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
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| <b>Compulsory REVISION comments</b> | <p>1) Summing up, the work would strongly benefit if the authors included in the discussion of their results the relevant findings reported in [8]. On the other hand, the manuscript should only be considered if made clear that the microorganism evaluated in the present work differs from that in [8]. On this, the reviewer also calls "5. If a submitted study replicates or is very similar to previous work, authors must provide a sound scientific rationale for the submitted work and clearly reference and discuss the existing literature. Submissions that replicate or are derivative of existing work will likely be rejected if authors do not provide adequate justification, from <a href="http://www.sciencedomain.org/page/sdi-general-editorial-policy#Peer-Review-Guideline">http://www.sciencedomain.org/page/sdi-general-editorial-policy#Peer-Review-Guideline</a>, SDI General Editorial Policy. Moreover, the improvements in xylanase activity compared to the wild strain reported in the present work are only slightly better than those obtained in [8].</p> <p>2) The authors tested different incubation periods and concentrations of AO, yet for EMS induced mutation only one concentration and incubation time was tested, similar procedures were expected for either mutagenic agents. In the present state the work presents some lack of coherency, as the authors only established conditions for optimization of one of mutagenic agents.</p> <p>3) In 2.1, the authors report to [8] for the mutagenesis procedure. Although no extensive details should be presented, some concise information on the procedure should be provided, e.g, was it performed in a submerged fermentation, in which medium, incubation conditions...</p> <p>4) The authors state that "the pH of medium was adjusted to 6.5 before autoclaved at 121°C for 15 min", but did they check pH at the beginning of the fermentation? From Fig. 5, after 12 hours of fermentation pH was within 5.5 and 6. In the work described in [8] a similar procedure was used but the pH was adjusted to 7, yet the trend in pH was similar and the cell count was in the overall higher</p> <p>5) Fermentations are performed using 250 ml of medium in 500 ml Erlenmeyer flasks. This corresponds to a headspace of 50% whereas typical runs in shake flasks are carried out with headspaces of (or in excess) of 80%, as the larger the headspace, the larger the surface area exposed, thus the better is the aeration for the medium.. Under the</p> | <p>1) Reference [8] is in fact the groundwork for the current study with enhanced improvement on xylanase activity. Only top five selected better mutant strains on xylanase production compared to their parental wild type were chosen for the present study. Besides that, <math>2.0 \times 10^6</math> cells were used as the standard inoculum in the submerged fermentation for xylanase production by wild type and mutants of <i>B. subtilis</i> compared to only <math>1 \times 10^6</math> cells in [8]. In addition, pH of medium was adjusted to 6.5 before autoclaved rather than neutral pH in [8]. In this study, additional scientific data on confirmation of xylanase activity from mutants of <i>B. subtilis</i> was also carried out using plate hydrolysis assay. The significance of data on xylanase activity was also emphasized using statistical analysis where the xylanase production by wild type and mutants of <i>B. subtilis</i> in the submerged fermentation in this study was statistically evaluated using single factor analysis of variance (ANOVA) at a significance level of <math>p &lt; 0.05</math>. Most importantly, this current work also proved the top five mutants were able to retain their mutagenesis properties after many generations. All the improvements done in this study on the standard inoculum size and growth condition, confirmation of xylanase secretion using plate hydrolysis assay and analysis of xylanase activity using ANOVA have aimed to further improve the production of the enzyme by wild type and mutants compared to preliminary study in [8]. Thus, this paper showcased the improved results that found in [8]. Furthermore, this work is dreadfully important as it is the footing for the followed-up study on the detection of the mutagenesis sites of these five mutants.</p> <p>2) This study is the footing for the followed-up study on the detection of the mutagenesis sites of these five mutants. All the details on the comprehensive and comparison works among the UV, AO and EMS induced mutation with different concentrations and incubation time have been investigated and elucidated in [8]. This current work focused and emphasized the production of xylanase by the top five mutant strains in submerged fermentation generated from experiments in [8]. Hence, the conditions used for random mutagenesis of <i>B. subtilis</i> can be obtained from reference [8].</p> <p>3) All details of the mutagenesis were discussed thoroughly in reference [8]. Reference [8] focused on the mutagenesis protocols of creating mutants. Whereas, this current work focused on the overproduction of xylanase by several top five mutants in submerged fermentation.</p> |



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|  | <p>conditions used in the work, microbial growth might have become oxygen limited, can the authors comment on this? Or was it made purposely, as for instance xylanase production is favoured under such limitation (and is there evidence on this?)</p> <p>6) In 2.3 when stating “At the end of the experiments” the authors should rather provide the exact time frame of the experiments (1 day? Hours? Or overnight, as stated in results and discussion? In either case, such information should be in Materials &amp; Methods)</p> <p>7) The authors state that “Microbial enzymes production is usually growth-associated. The highest enzyme production from different microorganisms has been found to accelerate during stationary growth phase but deteriorate thereafter during the death phase [19].” In the manuscript presented as well as in [19] only two samples were collected for a time frame where biomass is increasing, the first corresponding to the early exponential phase and the second one eventually to the late exponential phase. However, in the absence of further sampling throughout the time frame of the fermentation, particularly during exponential growth, up to the stationary phase and early decline, the reader is not provided evidence that the sample at 24 h does really correspond to the late exponential phase or early stationary. Since there is evidence that production is growth associated more detailed information (more samples) should have been collected and assayed during the first 36 hours to more accurately pinpoint the exact period where enzyme activity production is maximized.</p> <p>8) In section 3.3 the authors provide several examples of other systems optimization of xylanase production using mutation approach or/and wild types. Can the authors clearly highlight the advantages of their approach, as in several of the examples activity of wild type strains (from <i>Bacillus</i> sp. sources) is higher than the ones presented in the work, and improvements due to mutation match or exceed those in the present work.</p> <p>9) The text from line 322 to 342 would be more adequate to the Introduction section. There it would provide the hint for the use of <i>B. subtilis</i> for the present study. On the other hand, this part of the text does not provide any contribution to the discussion of the results</p> <p>10) Conclusions section is too long, the authors should only consider the data in the manuscript, which is often not the case, as in the text between lines 629 and 637.</p> <p>11) Writing should be improved, possibly with the help of a native English speaker. Articles, in particular, are often left out of sentences.</p> | <p>4) In the previous study described in [8], the mutant MXB 1 produced 1.638 U/mL of the maximum xylanase activity by <math>3.6 \times 10^8</math> cells/mL at medium pH 6.7 at 36 h of fermentation while in this study, 1.64 U/mL was attained by <math>1.16 \times 10^8</math> cells/mL at medium pH of 6.2 at 24 h of fermentation. Both readings of xylanase activity were almost similar, nonetheless, the cell counts were differ, indicating that initial medium pH 6.5 was more optimised in producing same enzyme activity at fast rate.</p> <p>5) The whole fermentation was completed in 3 days, in fact, the maximum xylanase activity was achieved within 24 h. In order to proceed to scale up, the fermentation should be terminated when the maximum xylanase activity obtained. Therefore, microbial growth was not affected by the limitation of oxygen in the shake flask within the first 24 h.</p> <p>6) Section 2.3 has been revised. The time frame was stated in Materials and Methods.</p> <p>7) For the future study, the sampling would be conducted every 2 to 4 h. In the current work, the sampling was conducted every 12 h since this study was the preliminary and groundwork for the next study of scaling up.</p> <p>8) Many attempts have been performed for the improvement of the microbial xylanase through mutagenesis. Mutagenesis is a mutation process where the genetic information of an organism is changed in a stable manner. Mutagenesis is generally induced via ultraviolet (UV) irradiation, chemicals and other radioactive emitting substances. In the past, the construction of enzyme-producing industrial strains of <i>Bacillus</i> spp was generally based on the screening for desired properties, nevertheless, with the achievements of the last decade, basic genetic and biochemical information with the ability to manipulate genetic materials have helped us to further improve these strains by mutagenesis for the selection of highly productive variants [10]. Most of the strains used for enzymes production have been improved through mutagenesis by UV irradiation and chemical agents. These chemicals and UV irradiation are known as mutagenic agents. Mutation occurs any time when there is a change in the nucleotide sequence of DNA. When microbial culture simultaneously exposes to mutagenic agents, mutation increases in frequency, gene duplication that occurred enables it to increase the production of the inefficient enzymes. As a result, mutations may occur in the enzymes structural gene, enabling the former inefficient enzymes to metabolize their substrates more efficiently and effectively. Therefore, by employing combined approaches of strain improvement using random mutagenesis and economical source of substrate are potentially be cost effective in overproduction of xylanase for industrial perspective.</p> <p>9) Moreover, the paragraph discussed the use of microorganisms as the</p> |
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|  | <p>There are too many glitches to detail, but some examples of awkward sentences follow:<br/> “random mutagenesis of ethyl methane sulfonate”; “we targeted to verify and produce xylanase from mutant strains of <i>B. subtilis</i> ATCC 6633 as the potent bacterial xylanase producers”; “Enzymes are generally exhibit high catalytic activity”; “Notably, five mutants of <i>B. subtilis</i> ATCC 6633 were successfully showed...”<br/> 12) The authors state that “Enzymes [...] are highly biodegradable and economically feasible”, yet in reference [1] one finds “[...] no individual xylanase is capable of meeting all of the requirements of the feed and food industries. Moreover, as industrial applications require cheaper enzymes, the elevation of expression levels and the efficient secretion of xylanases are crucial to ensure the viability of the process; therefore, genetic engineering and recombinant DNA technology have an important role in the large-scale expression of xylanases in homologous or heterologous protein-expression hosts”; although in the same reference, one finds “second generation biofuels do not compete with food production and can provide environmental, economic, and strategic benefits for the production of fuels” and also “However, enzymatic hydrolysis is still a major cost factor in the conversion of lignocellulosic raw materials to ethanol”<br/> The point of this reviewer is that the claim that the use of enzymes is economically feasible presented in such a general manner is questionable.</p> | <p>producers of xylanase, in fact, compared among all the microorganisms, <i>B. subtilis</i> has been evaluated as a dominant xylanase producer with several reasons. Introduction would be too long if the paragraph is moved to the Introduction.<br/> 10) The first paragraph summarised the data from the current study. The second paragraph emphasized the significance of strain improvement using random mutagenesis on overproduction of xylanase for industrial perspective. N-methyl-N-nitro-N-nitroso guanidine (MNNG) would be one of the chemical used for the purpose. Furthermore, protein engineering, recombinant DNA technology and site directed mutagenesis also aid in improving the desirable traits of xylanase. Besides that, submerged fermentation is a preferred fermentation approach using carbon source from barley husk to increase xylanase productivity.<br/> 11) Corrections have been performed.<br/> 12) Notably, the cost of production medium in the industry also affects the economics of the fermentation process. Decrease in production cost could be achieved by using lower cost and easily available agricultural residual materials. Many lignocellulolytic substrates such as barley husks, corncobs, sugarcane bagasse and wheat bran have been studied and a good number of them have performed significantly better than isolated purified xylans with respect to the yields of xylanase in large-scale production processes. Therefore, barley husk produced as a by-product of barley is known to be rich in carbohydrates serving as a carrier in submerged fermentation, supporting the growth of microorganisms by providing nutrients in enhancing the production of enzymes. Thus, barley husk was used as economical substrate for hydrolysis of xylanase in this study. As the industrial applications require cheaper xylanase, recombinant technique made a significant contribution to the industrial enzymes productivity, random mutagenesis is preferable, it thus, frequently selected as the favorable choice due to the reliable strain development.</p> |
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| <p><u>Minor REVISION</u><br/>comments</p>   | <p>EC number (EC 3.2.1.8) of the enzyme should be added for further clarification. "Soliman et al. [6] where they reported that the maximum xylanase activity of 8.66 g/L was produced---", check either units or parameter evaluated.</p> <p>In the discussion, confirmation of xylanase activity from mutants of <i>B. subtilis</i> using plate hydrolysis assay, maybe some issues addressed in A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity, by of Meddeb-Mouelhi F, Moisan JK, Beauregard M. in Enzyme Microb Technol. 2014 Nov;66:16-9. doi: 10.1016/j.enzmictec.2014.07.004, could be added.</p> | <p>EC number (EC 3.2.1.8) of the enzyme was added in the manuscript. In this journal of Soliman et al. [6], <i>Aspergillus niger</i> and <i>Trichoderma viride</i> are used for xylanase (EC 3.2.1.8) production using some lignocellulosic substrates under Solid State Fermentation (SSF). The results indicate that barley bran is the most potential substrate for higher xylanase production compared with other used substrates. The influence of various culture conditions including; fermentation period, incubating temperature, initial pH, initial moisture level, nitrogen sources, mineral sources, inoculum size and different levels of barley bran were studied. Generally, <i>A. niger</i> is more active in xylanase production compared with <i>T. viride</i> when grown on 1.0 g barley bran with moisture levels of 1:3 (75%), at pH 5.5 and 35°C after 2.0 days incubation in absence of additive nitrogen source. Parametric optimization of xylanase yielded 11.1 and 6.9-fold increase on barely bran compared with rice straw as initial solid substrate by <i>A. niger</i> and <i>T. viride</i>, respectively. (Reference: Soliman HM, Sherief AA, Tanash BEL (2012) Production of xylanase by <i>Aspergillus niger</i> and <i>Thrichoderman viride</i> using some agriculture residues. International Journal of Agricultural Research 7: 46-57). Important information from Meddeb-Mouelhi et al. 2014 was discussed in the Results and Discussion.</p> |
| <p><u>Optional/General</u><br/>comments</p> | <p>The work presented in the manuscript is of the interest for the scientific community but is biased by several issues as detailed in Revisions that spread from the overall design of the work to the conclusion.</p>                                                                                                                                                                                                                                                                                                                                                                                                                                    | <p>The manuscript has been revised accordingly.</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |