Dear Editors and Reviewers:

Thank you for your letter and comments relating to our manuscript entitled “Characterization of an α-amylase from the honey bee chalk brood pathogen *Ascosphaera apis*” (ID: jof-2687324). The comments were very valuable in addition to being helpful for revising and improving our manuscript, as well as the important guiding significance to our research. We have read the comments carefully and made corrections accordingly. Revised parts are marked in blue in the manuscript. The main corrections in the paper and our responses to the reviewer’s comments are given below. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in *Journal of Fungi*.

Responses to the comments of the reviewers:

Reviewer 1

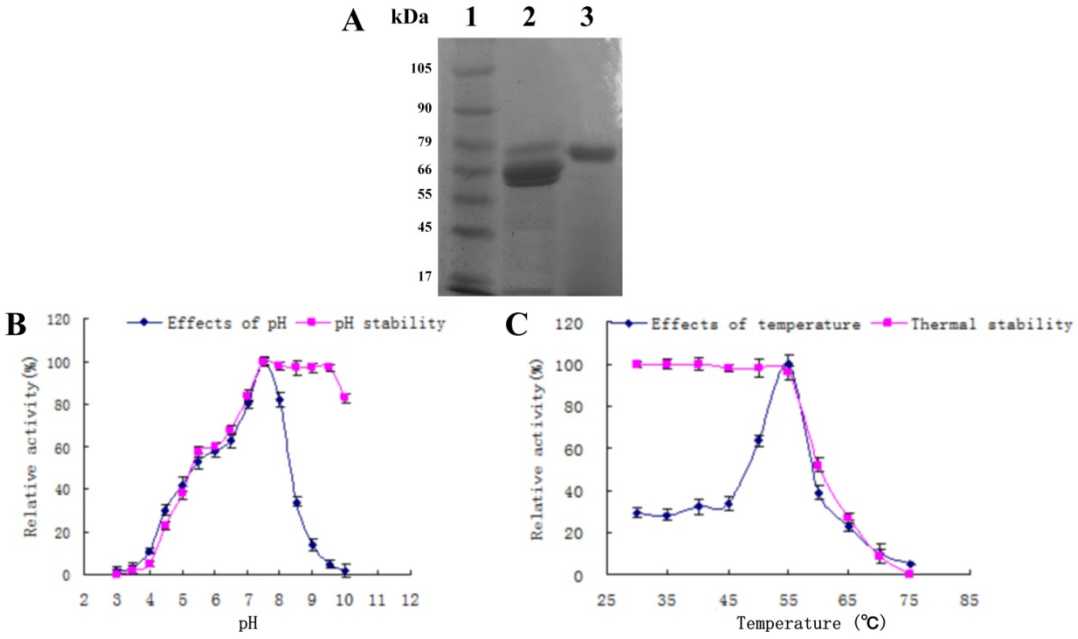
Comments 1: Figure 2: What are the corresponding purification steps providing the samples separated in the lanes 2 and 3 (denoted as “crude amylase”)? Please specify.

Response 1: We have changed it as shown in the following figure.

Figure 2 A. Results of SDS-PAGE of purified amylase from *A. apis*. Lane 1: molecular weight markers. Lane 2: crude amylase. Lane 3: purified amylase.

Comments 2: I do not see error bars in Figs. 2B and 2C.

Response 2: We have added error bars in Figs. 2B and 2C.



Comments 3: Table 3: Why lower concentrations of metal ions were not tested such as 100 μM or 1 mM for those ions, which completely inhibited at the level of 10 mM?

Response 3: As shown in Table 4, different metal ions demonstrated different degrees of inhibitory influences on enzyme activity at the concentration of 10 mM. However, different relative activities (21.31%, 33.48%, 37.65%) also could be respectively tested with the treatment of Cu^{2+}, Mn^{2+} and Ba^{2+} at the level of 1 mM.

Metal ion (10 mM)	Relative activity (%)	Organic solvent (10%)	Relative activity (%
None	100 (0.14)	None	100 (0.11)
Fe^{2+}	143.25 (2.12)	DMSO	99.14 (0.82)
K^{+}	91.73 (1.96)	isopropanol	96.98 (1.21)
Fe^{3+}	81.91 (0.73)	dichloromethane	95.31 (0.44)
Na+	77.46 (2.21)	acetone	91.97 (1.15)
Mg^{2+}	37.99 (0.65)	methanol	91.49 (1.53)
Ca^{2+}	37.99 (0.87)	n-butyl alcohol	86.26 (0.77)
Zn^{2+}	5.74 (0.38)	n-hexane	83.67 (1.49)
Cu^{2+}	0.00	ehanol	75.70 (0.68)
Mn^{2+}	0.00	chloroform	65.63 (0.93)
Ba^{2+}	0.00	-	-

Comments 4: Page 11, bottom sentence: I would like to see the Km a Vmax values provided in standard units, such as mmol/L and µmol/min, respectively.

Response 4: We have changed the sentence.

Under the optimal enzyme reaction conditions, K_m and V_{max} values of the purified *A. apis* amylase were calculated from Lineweaver–Burke double reciprocal plots to be 6.22 mmol/L and 4.21 µmol/mL·min, respectively.

Comments 5: In the discussion section the authors compare their results with previous data on amylases only as regards to the molecular mass of the enzyme. I would like to see an additional discussion particularly with respect to the kinetic data such as the determined pH and temperature optima or sensitivity to inhibitors.

Response 5: We have added some kinetic data and related references to the discussion section.

The molecular weight of amylases from numerous fungi is previously reported with the range of 42-70 kDa [38]. The *A. apis* amylase appears to be slightly larger with an approximate weight of 75 kDa. Moreover, the specific activity of the amylase was thermostable and in the broad pH range. The enzyme activity reached its maximum at 55 ºC, equal to the level of thermophilic *Anoxybacillus flavithermus* [40]. This enzyme was relatively insensitive to Fe^{2+} compared with other amylases [41], which still maintained 100% activity at the concentration of 10 mM Fe^{2+}. The K_m value (6.22 mmol/L) of the enzyme located in the middle of 1.02-34.06 mmol/L recorded for many fungal amylases [16, 17, 38]. The K_m and V_{max} values of the enriched α-amylase using soluble starch as the substrate here demonstrated that it possessed a moderately high affinity with soluble starch and reached V_{max} at a relatively low substrate concentration. Notably, the amylase from *A. apis* exhibited higher *in vitro* amylolytic activity on starch agar plates, which makes it potentially versatile for use in different industrial operations available.

40. Nam, Y.; Barnebey, A.; Kim, H.K.; Yannone, S.M.; Flint, S. Novel hyperthermoacidic archaeal enzymes for removal of thermophilic biofilms from stainless steel. *J. Appl. Microbiol.* **2023**. 10.1093/jambio/lxad106
41. Kim, J.K.; Lee, C.; Lim, S.W.; Adhikari, A.; Andring, J.T.; McKenna, R.; Ghim, C.M.; Kim, C.U. Elucidating the role of metal ions in carbonic anhydrase catalysis. *Nat. Commun.* **2020**, 11, 4557. 10.1038/s41467-020-18425-5

We tried our best to improve the manuscript and made some changes marked in blue in revised paper which will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly and hope the revision will meet with your approval. Once again, thank you very much for your comments and suggestions.

Kind regards,

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