Response to examiners comments following viva voce examination for the thesis:

The joint report states that the external and internal examiner's comments must be addressed as well as the joint report comments: *In addition to the corrections outlined above by the external examiner, the candidate must explicitly address comments contained in the Independent Reports when completing corrections.* Therefore the present document presents responses to all comments made in both individual and joint reports

Contents

1 External examiner individual report

External examiner comment	Response

2 Internal examiner individual report

Internal examiner comment	Response
1.1 **Although the magnitude of the problem is clearly stated, it isn't clear that what's been proposed or investigated is a solution to the problem.**	"The chemical and mechanical recycling methods are viable solution however, with limitation of economics and the low rate of recycling indicates that majority of plastic waste ends up in the environment where plastic and chemicals within the plastic contaminates the environment. To minimize the harm done to the environment, it is crucial to identify and figure out a method to not only remove plastic waste from the environment but to reduce the harm done to the environment. Whilst most research have been done on degradation of commercial plastics with well-known strains such as *Bacillus sp, Pseudomonas* sp., *Streptococcus* sp., and even animals such as mealworms and silk worms (Bombelli et al., 2017, Kumar et al., 2007, Satheeshkumar. S. et al., 2016). It is crucial to identify and isolate potential microorganisms capable of degradation of polymeric structures not only the plasticizers and other contaminants found in the commercial plastics." Has been added as a proposed solution.
1.2	Explanation was added to describe how plastic is being currently treated and how it is important to find an alternate method to degrade plastic as described on chapter 1.2 it

The introduction outlines a global problem, but then does not proceed to define a global solution. If the thesis isn't aiming to solve the millions of tons of plastic in the ocean or buried in landfill, this is not the context or background against which the thesis should be cast.	does more harm to the environment at the end. Also, chemical recycling may allow partial recovery of small percentage of plastic collected, it does not solve the issue where discarded plastics will end up in the environment not only polluting but altering the microbiome. Hence it is crucial to identify novel microorganisms and potential enzymes capable of degrading plastic to solve ever growing plastic pollution.
1.3 **It also isn't clear why enzymatic degradation would be worth exploring compared to other next best alternatives. What are the advantages and disadvantages and in light of this, why is this study of note?**	First there are only few enzymes known to be capable of degrading synthetic polymers such as PETase, cutinase, cellulase, and lipases. Because only few enzymes have been identified, it is crucial to screen and search for novel enzymes that may be able to degrade synthetic polymer. Also, I have expanded on chapter 1.2 on comparison between alternative method of degradation/recycling.
1.4 **Chapter 1 should have a more comprehensive literature review on microbial degradation of polymers. What is the current state of the art?**	Additional chapters comparing different recycling methods and advancement in microbial degradation have been added.
1.5 **An aim is a statement of the vision you have, i.e. the overall purpose of your research, i.e. why are you undertaking the research. What is it that you hope to achieve or contribute to the filed? Objectives are the measurable steps you will take to achieve your aim. The thesis has no clearly stated aim, e.g. are you aiming to create an enzyme degradation technology that can complete with e.g. chemical depolymerisation in plastic waste treatment?**	The aim and Objectives have been re-written to be more appropriate

1.6 **Need to illustrate the biodegradation pathway of both TPA into central metabolism with co-factors. This is necessary to show that the carbon flux from TPA to PCA is reducing equivalent neutral, i.e. there's no cost to the cell in converting TPA to PCA at a faster rate than is the case for PCA metabolism.**	Included Figure 2. this diagram states how TPA is converted into PCA which in turns is broken down into smaller components like acetyl CoA and succinate which is easily taken up into the central metabolism
2.1 **The Materials and Methods summarises in Table 2 the isolates from this study, which should be included in the results section given the stated objectives, i.e. this Table is obtain as a consequence of the methods outlined in the material and methods section.**	The list of the isolated were not explicitly stated again on the results section as successful strains were listed in ch 4.3.1 "Strains CV 2,3,4,5,11,13, and 16 showed potential signs of growth indicated by the colour change."
2.2 **The systematic enrichment and isolation of microorganisms from environmental samples is not sufficiently clear. A process flow diagram of the overall procedure with reference to the text would improve the clarity. Enrichment is done using the plastic as sole energy and carbon source, followed by isolation of viable microorganisms onto selective plates, followed by identification using API or 16S rRNA.**	Process flow diagram was included as figure 5

2.3 **Why is there a chitin assay or a cellulose assay? There's no mention of this as an objective in the introduction, nor is there any background to support why these methods are included in the Materials and Methods section.**	The characterisation of novel Streptomycete strains includes the assessment of the strains on a variety of different carbon sources. This is standard practice in microbiology and widely referenced in the literature (Law et al., 2019, Wei et al., 2020.) Also, as to current knowledge, enzymes that have capability of degrading synthetic polymer have high resemblance to cellulase, this was done. The reasoning on cellulase assay is added on 1.3
2.4 **Given storage of the plastics in ethanol, how can you be sure that the isolates are not growing on ethanol? 100 [%] (v/v) ethanol is not an effective disinfectant, whereas 70 [%] (v/v) ethanol is given that the water content is required.**	Both 100% and 70% ethanol are routinely utilised to clean plastic in the laboratory, the plastic is air dried to remove any residual ethanol and controls are always utilised to confirm that the bacterial growth is purely down to plastic/TPA and not ethanol.
2.5 **The Materials and Methods section needs reorganising into a more logical order that demonstrates it is a robust response to the stated objectives and a clear methodology against which to assess the results that have been obtained.**	The methodology is written in a categorical manner as two separate experiments happened before and after covid lockdown. The methodology starts with general information such as 16s primer, strains, media used, then explores Plastic degradation, then identification of the novel strain.
2.6 **It isn't clear that the study was geared towards isolating and identifying microorganisms other than *Pseudomonas* or *Streptomyces* for the degradation of plastic. The methodology does not appear to be generalised towards enrichment, isolation (dilution and streak**	The initial study was geared towards plastic degradation by pseudomonas strains provided by Dr.Nagamani Bora, however, upon further experimentation into the strain, it was unsuccessful in degrading both PET and PS hence, isolation of novel plastic degrading microorganisms was done. Other strains were isolated and these were frozen in the -80 for further characterisation at a later date. The focus on Streptomycetes was due the interesting observation that this genus can degrade plastic.

plating) and identification (API, 16S rRNA) in a general sense.	
3.1 **The phylogenetic bioinformatics analysis is not described in the Materials and Methods section.**	Chapter 2.9.9 was added to explain the phylogenetic neighbour-joining tree method.
3.2 **The results in chapter 3 present details that should be outlined methodically in the materials and methods section and referenced accordingly.**	Because the frame of the work shifted from pseudomonas to isolation of novel microorganisms, some aspects of the methods were not used for pseudomonas.
3.3 **ALE isn't the correct description of the successive serial transfers, i.e. unless evidence is presented that the genome has mutated as a result.**	Yes, I do agree the ALE was changed to selective enrichment.
3.4 **Robust science would require the verification that the *Pseudomonas* strains grow on plasticizer, dye, and unreacted monomers that may be found in the respective polymers. An inference is not sufficiently robust.**	The growth on plasticizers, dye and other contaminants from the plastic were not tested as they seemed not valuable for the aim of the project as the aim is to degrade synthetic polymer not the plasticizers or dye. Also, many plasticizers and dyes were shown many times to be degraded by *Pseudomonas.* Making it not novel findings. The Pseudomonas strains which were utilised in this study were originally tested on commercial plastic (Dr Bora), hence they were able to grow on plasticizers etc, this study evaluated their growth on virgin plastic. None of the strains provided by Dr Bora were able to grow on virgin plastic.

3.5 **Also, a demonstration of growth on the monomeric constituents of the PS and PET would have led to more robust conclusions.**	Yes I do agree however, as there were no visible growth on PET nor PS it did not seem crucial as degradation of Styrene, Ethlyene glycol and TPA by Pseudonomas was recorded many times making it not a novel finding. The literature has numerous citations of *Pseudomonas* sp. able to grow on TPA, EG and styrene, therefore this would not have been a novel discovery and hence once the strains had failed to grow on the virgin plastic, they were excluded from further research. The discovery that Streptomyces can grow on TPA is novel and will form the basis of a paper currently in prep.
3.6 **There's mention of genome sequencing, but no further mention of bioinformatics analysis to e.g. explain the PLC zone clearing, but no growth on PET. Could C2 grow on PLC as sole energy and carbon source?**	The Pseudomonas strain was sequenced by Dr Bora, however the sequence was not available and would not have been relevant given the strain cannot grow on plastic. *Pseudonomas* sp. cannot grow on agar (without any carbon source) so it could indicate that it may be able to utilize PCL as sole carbon source however, liquid culture was not done due to the strain lacking in degradation capabilities towards PET and PS.
3.7 **Referencing to previously BSc work is problematic, since this is not apparently published work (not included in the references section). May be best to include this BSc work as an appendix to the MRes.**	The BSc paper is added in appendix
4.1 **The focus on *Streptomyces* from the outset is unclear, i.e. why was the process of enrichment, isolation and identification not extended to other microorganisms? If the aim of the thesis is to identify highly active enzymes involved in plastic degradation to enable a new depolymerisation technology**	The aim was to isolate bacterial strains capable of the degradation of PET and PS. A section has now been added to the literature review to clarify why these plastics were chosen. The Streptomyces strains were selected based purely on the fact that after the very long enrichment experiments, they were the only strains remaining. The aim was not to identify highly active enzymes but rather identify new enzyme candidates with capability of plastic degradation.

	Other microorganisms were done however, after 5 months of covid 19 lockdown the only surviving strains were *Streptomyces* sp and as they are known to be able to product high value and have wide metabolic capabilities, there were selected.
4.2 **Similar to Chapter 3, the manner in which the thesis is structured implies that all Materials and Methods are contained in chapter 2. Nevertheless, chapter 4 contains sections that should be included in a methodical manner within chapter 2.**	The parts of the chapters that more methodology appropriate were removed and added to methods.
4.3 **What is the evidence that ALE has occurred rather than simply serial enrichment? Has sequence analysis demonstrated that the genome has been mutated?**	It was not ALE rather Serial enrichment to promote expression of various enzymes the strain may have that may degrade PET
4.4 **The error bars in Figure 17 would suggest that there is no evidence for growth on PET film. Similarly, Figure 18 suggests the same. This should be concluded in each of the figure captions as the principal observation.**	The comments were added into the figure captions
4.5 **It's not clear how dried cell mass was used as a means of assessing growth given a biofilm is likely to form on the polymer film. There needs to be explicit reference that no biofilm was observed as in Figure 21.**	"To remove as much biofilm as possible, the culture was vortexed for 30 minutes prior to pipetting and centrifuging"

4.6 **How was possible growth on ethanol excluded in the experimental design?**	The growth on ethanol was done but not included as the PET films were placed in the beaker and was left in 55C overnight for ethanol to dry off. Also, as the flasks were not sealed, the aeration and the temperature would have promoted evaporation and was not able to show signs of growth. KJ just state that no growth was observed with ethanol.
4.7 **Though figure 20 shows more growth with TPA, why was this plate observation not confirmed in liquid media with and without TPA, particularly given the growth on M9 agar?**	Liquid media with TPA and without TPA was done and the information was added on 4.4.3 TPA degradation as well as figure 23 was included
5.1 **Given the TPA degradation pathway presented in the Introduction chapter, this chapter should include a bioinformatics assessment of the genes that may be involved in the TPA degradation. The sequencing of HST would have been appropriate and why this wasn't undertaken should be outlined – Covid-19 does not seem a limiting reason. The presence or absence of *pcaGH* would also have been instructive.**	Agreed The figure 25 was added to reiterate the currently know pathway by R*hodococcus.*
5.2 **It's unclear why the pH range of HST is important to the objectives of the study**	This is part of the microbiological characterisation of an organism and is commonly utilised this has now been clarified in the text. (Abony et al., 2018, Wei et al., 2020).
5.3 **It's unclear why the substrate versatility is important to the objectives of the study, i.e.**	As above this is a common technique widely utilised to characterise microorganisms and is now highlighted in the relevant text. (Shirling and Gottlieb, 1966).

cellulose and chitin seem peripheral to objectives outlined in chapter 1.	
5.4 **Figure 24 – No evidence is presented that a *chitinase* is being excreted. The phenotype suggests that chitin is degraded, but there's no evidence that the extracellular enzyme(s) involved is classed as a *chitinase***	A zone of clearing on the chitin plate indicates that an enzyme has been excreted into the extracellular media and given that chitinases are used to degrade chitin, it is a logical to assume that this is the enzyme being excreted. These assays are commonly utilised to assess chitin degradation and widely cited in the literature. Kumkum and Garg, 2019 explained that zone of clearing is caused by extracellular chitinases hydrolysing chitin polymer into monosaccharides and disacchrides which the iodine stain does not bind with.
5.5 **Figure 24 – No evidence is presented that a *cellulase* is being excreted. The phenotype suggests that chitin is degraded, but there's no evidence that the extracellular enzyme(s) involved is classed as a *cellulase*.**	Please see above as the same logic applies and the cellulase assays are widely utilised and cited in the literature, this has now been clarified in the text. (Gupta et al., 2012) also explained that zone of clearing shown after the iodine staining indicates that extracellular cellulase was secreted to hydrolyse cellulose into cellbiose and glucose which iodine does not bind to. "Another potential group of enzymes responsible for the degradation of synthetic polymers are esterases, cutinases, and lipase(Krakor et al., 2021, Kaushal et al., 2021, Austin et al., 2018). More specifically serine hydrolases like cutinases (EC.3.1.1.74) have shown effective degradation of PET to TPA and Ethlyene glycol. All above mentioned group of enzymes are naturally found do hydrolyze large macromolecules. Large polymer degrading enzymes such as cellulases and chitinases were screened as a part of characterization as well. The

	screening was done with emulsion plate with emulsified cellulose and chitin.(Gupta et al., 2012, Kumkum and Garg, 2019). " has been added to further explain the reason why Chitinase and Cellulase assay was done.
5.6 **The experiment in Figure 26A should have had the time course for TPA (decreasing) and PCA (increasing) to prove that PCA is not metabolised or slowly metabolised relative to TPA. Figure 26B may only demonstrate that 10mM PCA is inhibitory to cell growth at this concentration and as shown in Table 12, at low concentration PCA is metabolised. This would be consistent with Figure 20.**	I appreciate this but due to the Covid impact on my project I was unable to complete these experiments and despite trying I was unable to pick up any increase in PCA on the HPLC, however, Dr Edward Spence has done further work to show that in presence of TPA, the PCA degradation does happen while without the TPA, PCA degradation does how happen. Potentially indicating that it is crucial for HST to have TPA when degrading PCA. This will form the basis of the paper currently in prep.
5.7 **The PCA analysis needs to explicitly state which percentage of the variance is explained by the two latent variables that have been plotted.**	The PCA analysis was qualitative and the percentage variance is inaccurate as the biomass was difficult to control due to the morphology of the strain in liquid media.
5.8 **Figure 29 – As no genome sequencing for HST was undertaken, the pathway analysis using the genome from another organism (presumably *E. coli*) does not seem entirely appropriate**	I agree. Genome sequencing was done on the HST strain and has now been included in the relevant chapter. However, because of IP I am not able to provide any further information other than the basics on genome size, GC etc. I have now included the genes in the TPA pathway.

6.1 **The pre-ample in section 6.1 seems inappropriate with respect to 6.1.1 which speaks to polymer degradation in *Pseudomonas*.**	The chapter 6.1 is summary of the achievement of this research and subchapters of 6.1 goes into more detailed descriptions of the different works I have done such as work done with *Pseudomonas sp.,* isolation, and characterization work.
6.2 **For 6.1.1, the speculation that the degradation pathway genes may have been silenced given the growth conditions is not supported by any results. What is missing here is a control with the Pseudomonas strains using the commercial polymer, which would have verified the assertion that the difference between virgin and commercial polymer is the reason for the discrepancy between this study and previous unpublished work. Not sure why styrene vapour would need to be used, i.e. styrene is a liquid at room temperature. The H&S reasoning seems precarious, given styrene can be safely used within COSHH constraints. Styrene wasn't the only consideration for these *Pseudomonas* strains, i.e. PET was also a consideration, yet no attempt was made to confirm monomer (PE, TPA) degradation alongside the commercial polymer control.**	1. It is true that it is mere a speculation that gene may have been silenced however, I was told by Dr. Bora that there were potential signs of plastic degradation on commercial plastics. However, when they were tested on virgin plasticizer free PET and PS, they did not show any signs of degradations and as biodegradation of plasticizers by *Pseudomonas* sp. is widely known, it was not a novel research. 2. Styrene is vapour in room temperature however while being agitated and in an incubator will release a lot of styrene vapour into the lab. And I was told by Stephen Hall that I cannot use styrene and try to avoid it at all costs. 3. Ethlyene glycol and TPA was not tested for degradation as the degradation by *Pseudomonas* is common and not novel .
6.3 **Recommendations for future work are interspersed within the conclusion sections. Recommendations for future work should be presented in a separate section after the conclusions.**	Agreed. The future work has been removed from conclusion chapter and turned into a new CH7

7.1 **Though a reasonable references section, it does not attest of wide reading which is reflected in the narrow background section presented in chapter 1 as a literature review of the state of the art.**	The additional references were added and the introductions were expanded to cover the internal examinar's requests

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