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Investigation into a Potential DNA Polymerase of Thermomyces lanuginosus

Kameron J. Kruger
University of Northern Iowa

INVESTIGATION INTO A POTENTIAL DNA POLYMERASE OF THERMOMYCES LANUGINOSUS

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Kameron J. Kruger

University of Northern Iowa

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Approved by:

Dr. Ira Simet, Honors Thesis Advisor

Dr. Jessica Moon Asa, Director, University Honors Program

Abstract

Thermomyces lanuginosus is a species of fungus with ever-expanding applications in the world of industrial chemistry. Primarily, this fungus is known for producing enzymes with a wide variety of industrial uses. These enzymes include xylanases, which are produced by *T. lanuginosus* in greater amounts than any other fungus (Mchunu et al., 2013). Xylanase is a degradative enzyme that breaks down hemicellulose, a key component of plant cell walls (Singh et al., 2003). Due to its stability over broad temperature and pH ranges, this enzyme serves many industrial purposes, primarily as a food additive and an alternative to chlorine-containing compounds in pulp bleaching (Singh et al., 2003). *T. lanuginosus* is also a large producer of chitinases, another class of enzymes with widespread industrial applications. Chitinases are widely used in agriculture as an alternative to chemical insecticides. These enzymes protect plants from chitin-containing pathogens, and unlike typical insecticides, they are not compromised by resistance over time (Khan et al., 2015). These industrial applications, along with many others, show the influence that *T. lanuginosus* currently holds in a variety of fields.

The production of xylanases and chitinases, among other key enzymes, has recently caught the attention of the scientific community. As a result, much of the current literature surrounding *T. lanuginosus* has focused on these industrial uses. However, much is still unknown about this fungus at the genetic level, as the genome was not fully sequenced until just a decade ago (Mchunu et al., 2013). An ongoing area of research seeks to investigate the genome of *T. lanuginosus*, which was the primary purpose of this study. Specifically, this research attempted to identify a small region of DNA polymerase alpha (Pol α), a gene that is essential to DNA replication. To this end, primers were designed to amplify regions of the genome where the target gene was expected to be. Polymerase chain reaction (PCR) was used

to stimulate this amplification process and the results were then visualized using agarose gel electrophoresis. Ultimately, these methods were unsuccessful at amplifying the expected regions of the *T. lanuginosus* genome. Variations in primer design and PCR conditions were not able to achieve the expected results. There is potential for further research into the Pol α gene, which is necessary to improve our understanding of this fungus at the genetic level.

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I. Introduction

The relevant techniques to this research are PCR and agarose gel electrophoresis. The fundamental concepts of these techniques are explained in this section, along with their relation to the overall goal of the study. The purpose of PCR is to amplify, or copy, a specific portion of DNA from within a larger sequence. This sequence is referred to as the template, and can be used to produce millions of DNA copies within a relatively short time. The copies are produced through DNA replication, which is a complicated biochemical process in its own right. When replication occurs naturally in the cell, this process begins with genomic DNA, in which the cell's entire collection of genes is stored. This massive DNA sequence functions as the template. The genomic DNA then undergoes denaturation, in which the two strands are partially separated so that each may be used to create new DNA sequences. Normally, the cell has many regulatory techniques to control precisely when and where replication occurs throughout its genome (Brody, 2023). In PCR, however, replication is regulated through the use of primers. Primers are short DNA sequences (18 to 30 bp) that only bind to the template at specific annealing sites. They achieve this by mimicking one of the template strands, which allows them to bind to their respective portions of the complementary strand. The main function of a primer is to act as the starting point of replication (Smith, 2023).

PCR utilizes a pair of primers that are designed to copy a target segment of template DNA. The primers “bracket” this segment at either end, which allows for selective amplification of the entire DNA sequence between them. New strands of DNA are then synthesized from the primers, which is the final step of replication (Smith, 2023). Various enzymes are involved with this elongation, but PCR utilizes a specific type of polymerase enzyme known as Taq. The thermostability of this polymerase makes it particularly useful for

PCR experiments, which stimulate the replication process using rapid temperature changes (Coleman & Tsongalis 2017).

The entire process of DNA replication involves three stages: denaturation, annealing, and elongation. The template DNA is first denatured at the appropriate site, primers anneal to the newly separated strands, and those primers are then extended until the target sequence has been completely copied. In a PCR experiment, each of these stages are conducted at different temperatures for maximum efficiency. For example, denaturation must occur from 94-96 ^{o}C in order to completely separate the two strands of template DNA. From here, the PCR mixture must be cooled to a range of 50-60 ^{o}C depending on the qualities of the primers. The ideal annealing temperature is that at which both primers will bind to the template at the proper positions, as any colder will result in non-specific annealing elsewhere along the template. Elongation occurs at 72 ^{o}C, at which Taq polymerase has optimal activity (Borah, 2011). The purpose of a PCR machine, or thermocycler, is to cycle through the stages of replication by repeating this three-stage temperature pattern. After one cycle, the number of amplicons in the reaction mixture has effectively doubled. After many cycles, each of which takes a matter of minutes, a successful PCR experiment will yield millions of amplicons (Smith, 2023). This is the power of exponential growth; it allows us to amplify a microscopic fragment of DNA to a scale that we can then visualize through other lab techniques.

A drawback of this type of experiment is that the results of PCR are not immediately apparent. Even after a successful run, any new DNA in the reaction mixture will be invisible to the naked eye. Fortunately, agarose gel electrophoresis is capable of visualizing the DNA and other nucleic acids in a PCR product. This technique places samples of DNA into a porous gel through which an electric field is applied. As DNA molecules have a net negative charge, the electric field causes them to migrate through the gel. The rate of migration is inversely proportional to the size of the molecule, as smaller molecules will encounter less

resistance as they move through the gel. As a result, this technique can be used to estimate the size of various DNA fragments in a sample (Lee et al., 2012). In relation to this study, agarose gel electrophoresis was used to verify the presence of the expected amplicon, therefore determining the success of each PCR experiment.

DNA and other nucleic acids are visualized through this technique using a fluorescent dye known as ethidium bromide. This dye intercalates between the individual bases of nucleic acids as they migrate, which has several implications for the way that electrophoresis results are analyzed (Lee et al., 2012). First, larger fragments will naturally show stronger fluorescence than smaller fragments, as they can have a greater number of interactions with the dye. This also poses issues of contamination from other nucleic acids. Ideally, the template DNA used in a PCR experiment should be purified of all other nucleic acids, which will also interact with the ethidium bromide in the gel. This includes RNA from the original source and any nucleic acids from outside sources. Fortunately, this type of contamination is fairly easy to diagnose. In this research, each PCR product was expected to yield a single band of DNA representing the amplicon. As each amplicon has a known length, any bands appearing outside of that range would indicate contamination.

In summary, the purpose of agarose gel electrophoresis is to determine the success of a PCR experiment. If PCR was found to be successful, this result would prompt further research into the sequence of the amplicon. By improving our understanding of the Pol α gene, such as its sequence or location within the *T. lanuginosus* genome, this research attempts to learn more about how this fungus relates to other organisms. In addition, this gene is expected to encode a heat-stable DNA polymerase enzyme that could have further applications in biochemical research.

II. Methods

In preparation for this research project, multiple sets of primers were designed to amplify specific regions of the *T. lanuginosus* genome. The genomic regions of interest were determined by comparing the fungal DNA to that of related organisms (*Ustilago maydis* and *Cordyceps militaris*). Both of these fungi have known DNA sequences for the catalytic subunit of the Pol α gene, and these sequences are available to the public through the National Center for Biotechnology Information. Fungi were chosen for comparison because related organisms tend to have more genetic overlap. Therefore, if the Pol α gene is truly contained within the *T. lanuginosus* genome, then it should have significant similarities to the exact same gene in other fungi. The genetic similarities between species were determined using Clustal Omega, a DNA sequence alignment program. This program allows the user to input two or more DNA sequences to find the best possible match between them. As shown in Figure 1, asterisks are used to indicate matches between the two DNA sequences. A large cluster of stars indicates a region that should be tested, as this region shows similarities to the gene of interest. In short, the *T. lanuginosus* genome was compared to other fungi in order to identify the most likely locations of the Pol α gene. This information was then used in designing primers for the eventual PCR experiments.

Each set of primers was designed to amplify a region of genomic DNA between 300 and 800 nucleotides long. The catalytic subunit of Pol α is expected to be several thousand nucleotides long, but amplifying a shorter length of DNA tends to be more reliable. There are many qualities that make a primer "good" or "bad" in terms of a PCR experiment. For one, a pair of primers should have similar melting points to ensure that both are maximally functional at a single annealing temperature. They should also not form dimer or hairpin structures, as this can significantly decrease their efficiency (Borah, 2011). The quality of a primer can be assessed using the OligoAnalyzer™ Tool from Integrated DNA Technologies

(IDT). All primers designed for this research were assessed using this tool. A total of seven sets of primers were created and the most promising three were ordered through IDT for use in PCR experiments. This decision was made based on the qualities of the primers themselves and the amount of genetic overlap found by the Clustal Omega program. Table 1 lists the base sequences of each primer, while Table 2 summarizes their key characteristics.

Figure 1

Sample comparison of T. lanuginosus and C. militaris genetic sequences

Table 1

Base sequences of PCR primers

Primer	Base sequence
KKLeft5	GCA CAT CTG AAT CGC TCG ATC TGG
KKRight5	GCA ACT TCA GCG AAC GCC TCT TC
KKLeft6	CTT AGT CCC CTG GCG GAT TCG TC
KKRight6	CAT CAT CGT CGT CTT CGG TGG TC
KKLeft7	GAT GCT GGA GCT GAC GGG CAA G
KKRight7	GAC ACT CTG CAC GTC CTT GTC AC

Table 2

Predicted characteristics of PCR primers

Primer	T_m (ºC)	% GC	Min. hairpin ΔG	Min. self-dimer ΔG	Min. hetero-dimer ΔG
KKLeft5	59.8	54.2	-0.90 kcal/mol	-6.76 kcal/mol	-9.92 kcal/mol
KKRight5	60.8	56.5	-0.84 kcal/mol	-6.75 kcal/mol	
KKLeft6	61.4	60.9	-0.78 kcal/mol	-4.64 kcal/mol	-3.61 kcal/mol
KKRight6	59.5	56.5	-0.86 kcal/mol	-3.61 kcal/mol	
KKLeft7	62.8	63.6	-1.18 kcal/mol	-6.34 kcal/mol	-6.53 kcal/mol
KKRight7	59.9	56.5	-0.78 kcal/mol	-7.05 kcal/mol	

The template DNA for this research was purified from *T. lanuginosus* cells using the E.Z.N.A.® Soil DNA Kit from Omega Bio-tek. A fungal DNA kit is also available through this company, but research by previous undergraduate students has found this kit to be less effective. DNA purification is a fairly standard procedure that lyses a sample of cells and isolates their DNA from other cell components. The initial attempts at this process were done using frozen *T. lanuginosus* cells. The effectiveness of purification was determined using a

NanoDrop™ 2000 Spectrophotometer to measure the absorbance of nucleic acids in a sample of purified DNA. The instrument showed that these initial attempts did not yield sufficient concentrations of template DNA, which made them useless for PCR experiments. It was concluded that the frozen cells were no longer viable due to a freezer malfunction that had recently occurred (I. Simet, personal communication, 2023). In response, fresh colonies of *T. lanuginosus* were grown in a sterile environment at 55 ^{o}C. DNA was then extracted from these colonies and purified. This iteration was much more successful, yielding DNA at a sufficient concentration for this research (55.8 ng/μL). This sample of purified DNA was then used in all PCR experiments.

The final step of this research process was to test the quality of the primers through PCR and agarose gel electrophoresis. In preparation for PCR, a master mix was created from the following ingredients: template DNA, primers, dNTPs, and Taq polymerase. A 1x magnesium buffer was also added to each master mix in order to facilitate DNA replication. As PCR is typically run in triplicate, three master mixes were created for each set of primers with varying magnesium concentrations (1 mM, 2 mM, and 4 mM). PCR experimentation was performed using the Veriti™ 96-Well Fast Thermal Cycler from Applied Biosystems™, which allows for the selection of multiple annealing temperatures. These temperatures were chosen by subtracting 2 ^{o}C, 5 ^{o}C, and 8 ^{o}C from the lowest melting point of the corresponding primers. This ensured the best possible chance for specific annealing. Figure 2 shows the typical run method that was used, indicating all three steps of the replication process. Aliquots of each master mix were run at each annealing temperature, creating a total of nine unique PCR conditions for each pair of primers. This methodology is summarized in Table 3.

Figure 2

Run method for PCR experimentation

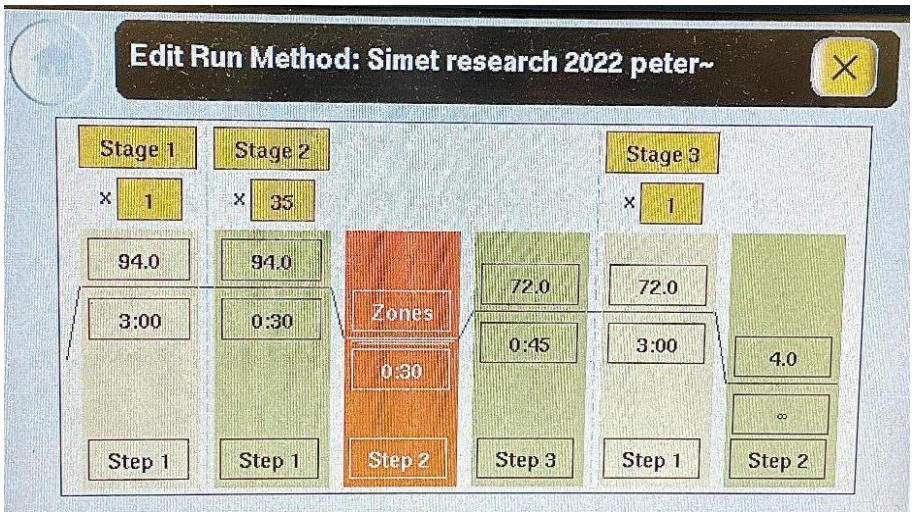


Table 3

Summary of PCR conditions

	Trial 1 master mix	**Trial 2 master mix**	**Trial 3 master mix**
Primers used	KKLeft5/KKRight5	KKLeft6/KKRight6	KKLeft7/KKRight7
Primer volume	4 μL each	8 μL each	8 μL each
DNA volume	2 μL	2 μL	2 μL
dNTP volume	2 μL each	2 μL each	2 μL each
Taq polymerase volume	2 μL	2 μL	2 μL
1x Buffer volume	10 μL	10 μL	10 μL
Annealing temp. (^{o}C)	46/49/52	46/49/52	49/52/55

Note. Distilled water was added to each master mix to a total volume of 100 μL.

III. Results and Discussion

The results of agarose gel electrophoresis are shown in the following three figures. As a point of reference, 2-log and lambda-*Hin*dIII DNA ladders were run alongside the PCR products, denoted as "L" and "λ" respectively. These ladders contain linear DNA fragments of known size, which allows for the size estimation of any unknown fragments in the PCR products. For example, a faint band can be seen at the bottom of each "product" lane in Figure 5. The 2-log ladder indicates that these fragments are smaller than 100 bp. As the expected amplicon for these primers should have been around 500 bp in size, these bands must belong to the primers themselves. The same bands can also be seen in the following two figures, which confirms that all of these bands correspond to primers. No other bands were observed in any of the gel images. These results suggest that the primers were unsuccessful at amplifying a portion of the template DNA, as these amplicons would have appeared as a sharp band of high intensity around 500 bp. To reiterate, such bands were not observed.

Figure 3

Results of PCR with KKLeft5 and KKRight5 primers

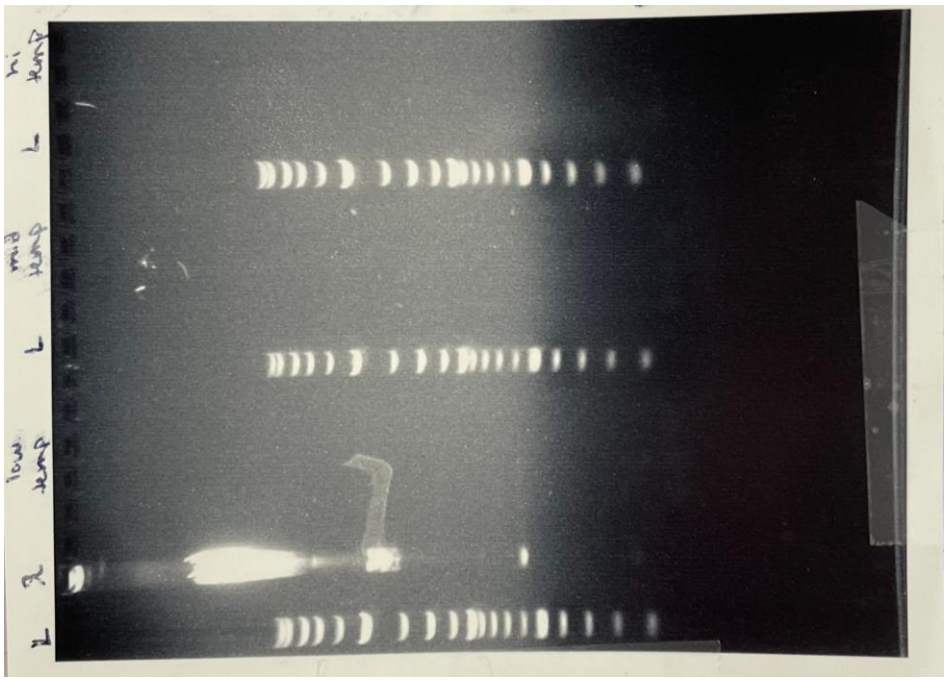


Figure 4

Results of PCR with KKLeft6 and KKRight6 primers

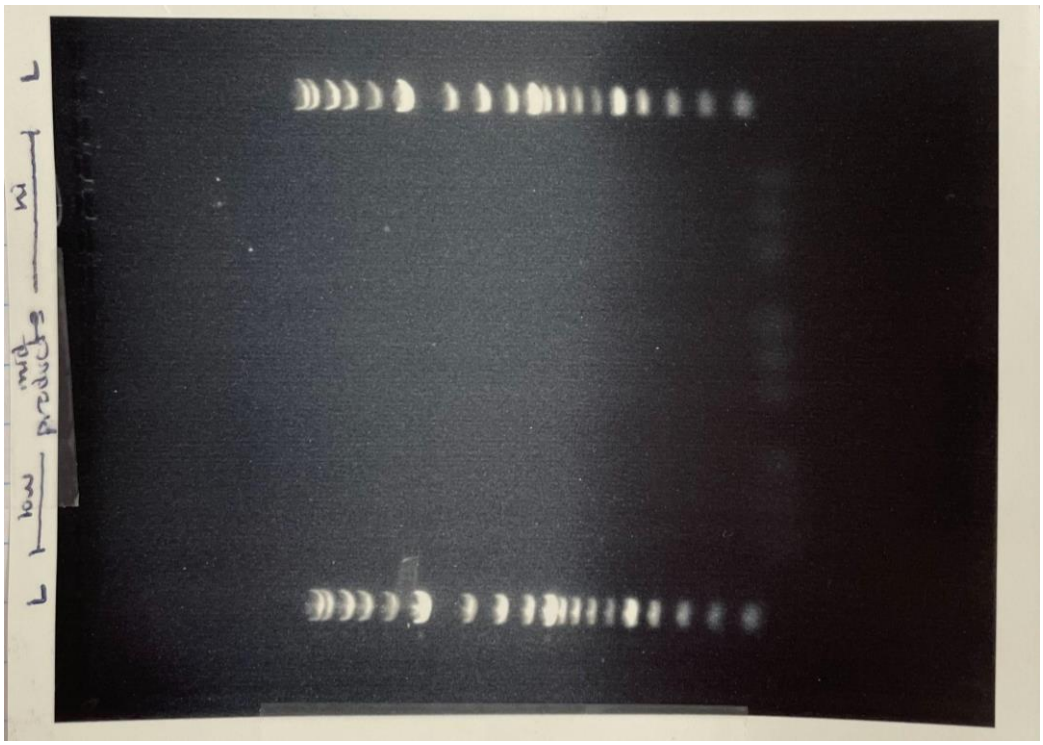
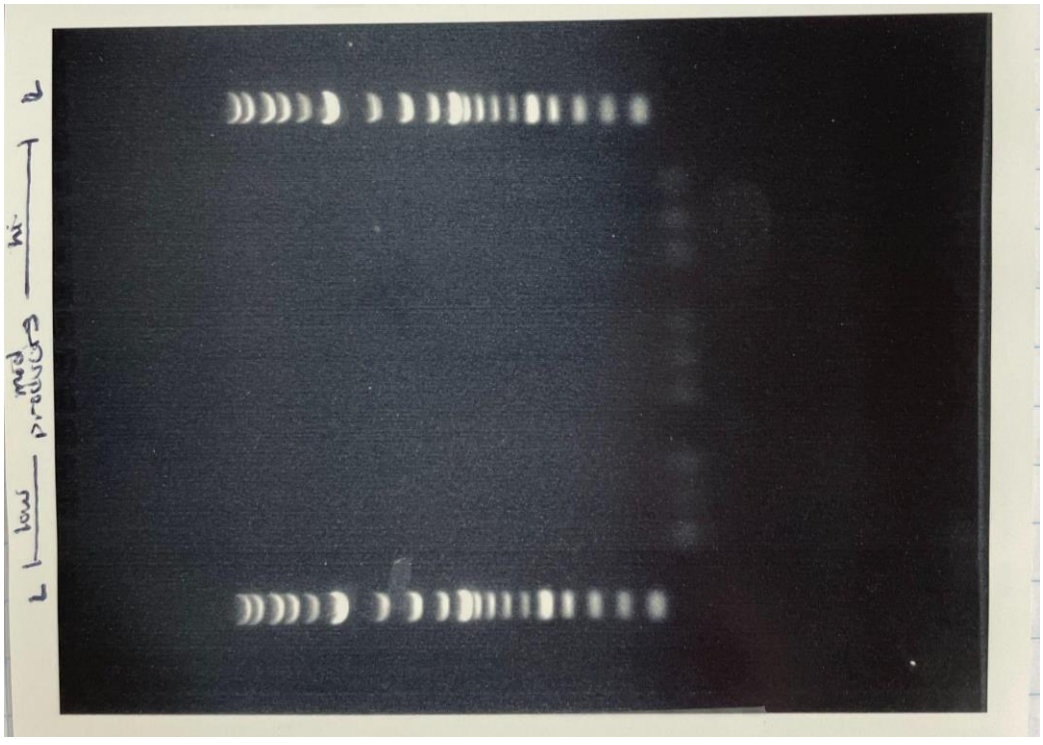


Figure 5

Results of PCR with KKLeft7 and KKRight7 primers



The downside of a PCR experiment is the sheer number of "moving parts"; if an experiment is unsuccessful, it can be difficult to pinpoint exactly which variable is at fault. In an attempt to circumvent this issue, many variables were changed over the course of this research (refer back to Table 3). Manipulating primer identity, primer concentration, magnesium concentration, annealing temperatures, and dNTP solutions did not result in a successful PCR reaction. These results mainly raise concerns with primer design. In previous undergraduate research, before the *T. lanuginosus* genome had been released, students had to design primers based on the genetic sequences of other organisms. This process is called shotgunning, and is likely to produce ineffective primers that do not anneal to the *T. lanuginosus* genome. However, the primers used in this research were designed to be an exact match for the species of interest. It was expected for these primers to anneal and produce amplicons, even if they had not targeted the correct gene. The results indicate that there may have been an issue with primer design, in which no change in PCR conditions would have yielded a successful experiment. This leaves plenty of room for further research into this organism, and in particular, the Pol α gene.

IV. Conclusion

The purpose of this study was to investigate the catalytic subunit of the DNA polymerase α gene in *T. lanuginosus*. To achieve this, PCR was performed in an attempt to amplify a segment of the target gene sequence. These results were then visualized using agarose gel electrophoresis. Three sets of primers were tested under a variety of PCR conditions, yet none were successful at amplifying the expected regions of the genome. The most likely reason for these results is the use of ineffective primers. Each set of primers underwent strict analysis before ordering, but as scientific research often proves, real life conditions tend to be much more complicated than theory. Nonetheless, this study provides insight into the *T. lanuginosus* genome and opens a pathway for additional research. Future studies should be advised to take caution in designing PCR primers, ensuring that they are of good quality. There is plenty of potential to be found in this area of research, as it can help us gain a more complete understanding of this particular gene. In addition, this research can offer insight into *T. lanuginosus* as a whole, potentially finding new industrial applications and advancements in the field of biochemistry.

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