BIOE Seminar
Final - NIH R01 Proposal
Christopher Garland - 4385504
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Utilizing Genetically Modified Multicellular Eukaryotes with Morphological Dimorphism for Programmable Production of Insulin Encapsulated Nanoparticles

A University of Pittsburgh Student’s Approach to Making Medicine More Accessible for All

SPECIFIC AIMS and BACKGROUND

Ever since the 1920's and the days of Frederick Banting, insulin has been a lifesaving treatment for those who are diagnosed with diabetes [1]. Recent years have shown an increasing prevalence and morbidity of both diabetes and obesity, especially in the United States. More than ever before, there is a desperate need for affordable treatments and treatment plans. Despite it being more than a century old and even its own creator coining the phrase, "Insulin does not belong to me, it belongs to the world.", there has been a continuous increase in its price since its inception. Luckily, a recent move in 2024 by the major manufacturer Eli Lilly has finally put a price cap on insulin, ending a century of extortion by pharmaceutical companies [2]. Despite the sudden hopeful outlook, the current administration in the United States threatens to undo many of the advancements the scientific community has made over the past century. Insulin's methods of large-scale production mean that it is vulnerable to economic shocks, regulatory changes, and supply chain bottlenecks. With this in mind, maybe it's time for the development of treatments and research to move underground, literally. I would like to propose the development of a new modular, ex-vivo, biosynthetic system, built from genetically programmed fungal reactors and microfluidic systems.

In the mid-to-late 1970's there was an exciting and rapid development of technology to use recombinant deoxyribonucleic acid methods to clone and express human genes in E. *coli*. The pioneer molecules for the development of this technology were somatostatin, the insulin A and B chains, and proinsulin. The goals of most studies were focused around inserting novel synthetic genes into bacteria in order to force them to produce these functionally active molecules for humans. Ironically, the company that is responsible for the recent price drop, Eli Lilly, is also responsible for being the first to produce insulin (brand name Humulin) from modified bacteria on a large scale [3]. Keeping the achievements of the past and the challenges of the future in mind, I believe we as bioengineers can begin developing new protocols and methods with the new technology we have access to, in order to provide better access to insulin.

Although insulin is an extremely effective treatment, it suffers from extremely poor bioavailability. Additionally, patients must check their blood-sugar levels constantly to ensure that their doses are effective. While rising costs are an issue, patients could also benefit from drugs that employ active targeting or controlled and extended release, in order to better help patients manage their blood-sugar levels. The need for better patient tailored medications is clear.

The focus of this proposed study is to employ genetically engineered eukaryotes (specifically fungi) within a compartmentalized system that will allow for the creation of a new ex-vivo model for high throughput production of active pharmaceutical intermediates (API's). The novelty of this proposal lies in the biosynthetic pathways being utilized for production, as well as the lack of a need for further purification or tuning, due to it inherently being integrated within the system's design. The central hypothesis of this study is that genetically engineered fungal strains can be physically separated within a new bioreactor, in order to independently produce proinsulin and chitosan-based scaffolds for nanoparticle encapsulation. This proposal takes inspiration from recent advancements in metabolic engineering, along with our ever-expanding knowledge of the complex and overlapping processes which take place daily in the natural world.

AIM 1: Engineer and characterize dimorphic *Mucor lusitanicus* strains for conditional expression of proinsulin and chitin under morphology-linked conditions.
Hypothesis: Morphological states of fungi and their resulting change in gene expression can be leveraged as a biological switch to upregulate heterologous biosynthesis of proinsulin and chitin production independently.
Approach: Using known regulators of dimorphism associated with *Mucor lusitanicus*, develop a dual-strain fungal bioreactor with transcriptional switches tied to environmental or chemical stimuli. We hope to use this platform to validate the expression and secretion of proinsulin. Additionally, we hope to validate and quantify chitin and chitosan production. Both will be done using specific immunoassays.

AIM 2: Design and implement a modular microfluidic system downstream of our dual-strain bioreactor, in order to facilitate fusion of bioactive proinsulin and chitosan for nanoparticle assembly.
Hypothesis: By streamlining the production of a biomaterial scaffold and a bioactive molecule within the same microfluidic system, and using flow-control and machine-learning algorithms, we can facilitate nanoparticle formation for high-throughput pharmaceutical synthesis.
Approach: Incorporate the previously mentioned dual-strain bioreactor into a multi-channel microfluidic system with flow-control, mixing chambers, and an optional enteric coating step. We hope to characterize the size, morphology, encapsulation, and biological activity of the resulting nanoparticles. Additionally, to test the active targeting capability and oral availability of our drug, we will simulate gastrointestinal (GI) conditions.

Impact: If our study proves to be both feasible and successful, it will demonstrate the first known implementation of a modular and adaptive, living biosynthetic pipeline, which employs multicellular eukaryotes, to both manufacture and then assemble a clinically relevant API, which would theoretically, be immediately ready for use in living patients. Additionally, it represents breaking ground in the newly emerging field of synthetic ecology, while also helping us gain a better understanding of programmable bioproduction using microfluidic controlled systems.

SIGNIFICANCE

The escalating comorbidity of diabetes and obesity represents an urgent need for affordable and widely accessible insulin therapies. Most of the existing systems and platforms for insulin production rely on the foundational research mentioned earlier. They employ prokaryotic systems which use well-studied strains such as *E. coli* or *S. cerevisiae*. It is also worth noting that these systems have been optimized for scale, not tunability. Large-scale production is still an undertaking only possible for mega-corporations and large entities that can procure continuous funding. Facilities must be centralized to include not only the equipment required for initial production, but also the strenuous multi-step purification procedures associated with making a single biologically active molecule at volume. Please note these immense requirements are for just a single, known, well-studied biologically relevant molecule. Any small company or private institution has no way of undertaking such a task. Our proposed platform diverges from previous schools of thought. By leveraging the unique morphologies and

gene expression of the fungal world, I believe we can present a new and improved production model that is programmable, built around eukaryotes, and more importantly, sustainable.

Before we continue with the innovation of this proposal, I would like to make note of some significant challenges posed by this solution. Utilizing filamentous fungi for heterologous protein production, especially those active in humans, is not the most attractive offer for patients or researchers. Fungal infections are still prevalent and are estimated to cost roughly $4 billion annually. Additionally, the complex secretory pathways of fungi which can be highly upregulated based on environmental cues, can serve as both an aid and detriment to our goal. The classical secretion route that fungi employ involves translocation into the endoplasmic reticulum (ER), folding and modification, and then transport through the Golgi apparatus to the extracellular environment. There are multiple chances, at every level, for proinsulin or insulin, to be destroyed. Proinsulin and other bioactive molecules require further processing and cleavages to ensure biological activity. Fungi typically perform hyperglycosylation on their proteins, which could drastically change the activity of proinsulin. Additionally, once transported to the extracellular matrix, enzymes associated with proteolytic degradation could completely destroy any non-native molecules. These challenges are not trivial, and could stop this proposal in its tracks if not properly handled. We propose a few different approaches that might ensure that insulin can be effectively produced at scale in different strains of fungi. The first and simplest would be to utilize protease-deficient strains. This could minimize the effects the host system has on our desired products. Alternatively, we could explore an alternative secretion pathway, such as the multivesicular body (MVB) secretion, which acts independently of the pathway associated with the Golgi apparatus. The last option, and perhaps the most realistic, would be to utilize chaperone coexpression and human prohormone convertase. It will require extensive research of the specific fungal strain; however, by pairing coexpression of proinsulin and human prohormone convertase with the correctly upregulated molecular chaperones, one could theoretically help facilitate proper protein folding and processing of proinsulin into its active form, all in a non-native host. Other methods could be employed, as the main chemical reaction that catalyzes the activation of proinsulin is a simple cleavage followed by disulfide bonding.

Another significant road-block of note if every aim proves to be successful, is that due to the fact we are using chitin and chitosan as a scaffold for our nanoparticle, any part of the general population that suffers from a shellfish allergy is completely excluded. I chose chitin because with the bulky modification associated with producing insulin, it seemed the most reasonable, as fungi already produce chitin in very large volumes.

INNOVATION

As mentioned earlier, most of the existing models for insulin production rely on extremely old and foundational research. We hope to improve upon these systems by adding a level of modularity, programmability, and robustness. The three key innovations of note are as follow: A dual-reactor system which leverages morphological control for biosynthesis, A chimeric microfluidic system which includes both bioactive proteins and biopolymer scaffolds, A framework for artificial intelligence (AI) or machine-learning guided synthetic ecology. Very briefly, I would like to expand upon the third innovation. Should every hypothesis be proven correct and every aim met, as the system grows more complex and begins to include genetic regulatory circuits, complex flow rates, and unexpected biosynthetic outputs, an AI is required to

serve as its central nervous system. These new technologies excel at identifying optimal production windows and could be used to adjust growth parameters or even suggest new genetic edits. Additionally, with research being done with these systems in order to create small-scale 3D molecular synthesizers, one could see a machine-learning algorithm suggesting the synthesis of new signaling peptides which could aid in overcoming some of the challenges associated with the secretory pathway and post-translational processes. Many proposals hope to optimize production or delivery of drugs independently. I aim to approach the two concepts as an inseparable biological and chemical challenge that must be overcome.

APPROACH

Specific Aim 1: Engineer and characterize dimorphic *M.lusitanicus* strains for conditional expression of proinsulin/insulin, and chitin/chitosan under morphology-linked conditions.

A. Rationale: *M.lusitanicus* exhibits an interesting dimorphic switch between yeast and hyphal growth depending on the oxygen availability of its habitat [4]. The morphology is coupled with gene regulation pathways, specifically cAMP/PKA. Another pathway it is coupled with is the calcineurin-dependent networks, which some researchers like, Homa et. al., have shown may be linked to virulence as well [5]. Other studies have characterized native genes in the *Mucor* family, notably *hsbA* family for hydrophobins, and *chs3* and *chs5* for chitin biosynthesis. Both are of note, as hydrophobins are responsible for controlling secretory pathways, while chitin biosynthesis is the basis for our nanoparticle scaffold. Both of these pathways are upregulated in specific morphologies, making them ideal for manipulation. The studies I am basing my hypothesis off of were done by de Graaff and Scholtmeijer et. al.; however, their work was performed using *T.reesei* and *A.niger* [6]. These species of fungus are much better researched and hydrophobin activity is not conserved across this domain of the animal kingdom. To further clarify, this means some tailoring may need to be done on our part to ensure the genes we are targeting for coupling are actually upregulated in whatever morphology we choose to induce.

B. Study Design:

B.1-1a. Bio-Reactor Specifications and Design Considerations: (The “Fungal-Flow” System)

Both bioreactors require some similar specifications. The first is high sugar content, to ensure continuous and accelerated growth. This could be done using a classical liquid culture used for commercial mushroom growth, or a common agar, fitted with a higher concentration of glucose. Another consideration is the growth states we are inducing. To prevent mixing, each bioreactor will be isolated to its own closed loop, and only its supernatant collected in order to ensure the integrity of the biomass. This is an important consideration. We are creating a synthetic environment, and its value is in its robustness. In order to introduce another layer of protection, all microfluidic designs directly interfacing with any bio-reactors must maintain laminar flow and low shear stresses. This ensures no interruption to natural secretions and allows for real-time harvesting of the target molecules. Another very important design consideration.

B.1-1b. Bio-Reactor Chamber A: Proinsulin/Insulin Production:

This chamber of the bioreactor features *M.lusitanicus* in its anaerobic, or yeast-like state. The temperature range should not vary outside of 28°-30°. The pH must be maintained within a range of 5.5-6.0. Agitation should not exceed approximately 50 rpm. The carbon to nitrogen ratio should be 15:1 in order to favor formation of a biomass that can produce large amounts of insulin. Ammonium salts could be employed to help encourage such an environment. A notable genetic modification to this strain would be the inhibition or deletion of calcineurin. Homa et. al. found that by removing the genes responsible for the expression of calcineurin, *M.lusitanicus* is unable to transition to its aerobic morphology. This not only adds another layer of protection to our system, but also gives a site for insertion of genes relating to insulin production. If the bioreactor is fitted with a smart system to monitor its production, oxygen can be safely introduced into the environment in small quantities, in order to tailor insulin production. Another site for insertion would be near genes relating to hydrophobin or MVB secretion pathways, which need further research. Assuming insulin is properly secreted into the supernatant of our bioreactor, precipitation via pH changes can be used to isolate insulin. Once the effluent has a desirable amount of product, it can be transferred to the next part of its loop, where the pH can be lowered to help precipitate insulin, which is only soluble between 5.5-6.0. After the insulin has been filtered and redissolved, it can be sent to the main Y-junction of our microfluidic system, where we can begin assembly of our nanoparticle. It is important the pH of the effluent is restored and then returned to the original bioreactor to ensure the integrity of the main biomass. Further codon optimization for genetic modification could be performed using AI's like FungiOPT or IDT Codon Optimizer.

B.1-1c. Bio-Reactor Chamber B: Chitosan Scaffold Production:

This chamber of the bioreactor features *M.lusitanicus* in its aerobic, or hyphal-mode. The temperature should be the same as bioreactor chamber A. The pH range should be 5-5.5. Dissolved oxygen should remain greater than 30% to ensure the correct morphology. The stir-rate of this chamber can be slightly higher to aid in high-throughput production. A range of approximately 150 rpm would likely be suitable. The carbon to nitrogen ratio should be 10:1 in order to favor chitin production. Inclusion of ammonium salts and oxidative stressors would provide the necessary building blocks to aid in chitin output. The genetic modifications done to this strain should be coupled in regard to oxygen availability and chitin synthase. If hydrophobins are correctly coupled with extra chitin synthases, one could ensure a large amount of chitin is secreted to the extracellular matrix so that the fungus does not use all of its chitin for growth. With that consideration, some level of growth arrest would want to be implemented so that the fungus can no longer grow, and only secrete chitin or chitosan to its environment. Since finished products will be floating around everywhere, we should also tailor this strain to be protease-deficient so that scaffold polymers aren't degraded. Additionally, overexpression of *chs3* and *chs5* can help boost chitin production. Similar to the way the supernatant for bioreactor chamber A is filtered, bioreactor chamber B must also have the same closed loop modifications. The only other important consideration is that chitin and chitosan precipitate at higher pH's, meaning we need to raise the pH, filter, and then lower the pH of the effluent from bioreactor chamber B.

B.2. Success Criteria:

The morphological states of both fungi can be verified using bright-field and fluorescence microscopy. The mRNA expression can be analyzed using qPCR. Protein secretion can be analyzed for production. ELISA assays for insulin, and Cibacron Brilliant Red assays for chitosan [7]. Unfortunately, the only way to analyze and verify protein folding processes would be western blots or very complicated mass spectrometry analysis. Data on glycosylation may aid in our understanding of folding processes, and this can be analyzed using lectin blotting. Success for this portion of our experiment would be each strain to show morphology-coupled expression of their respective target, with bioreactor chamber A producing bioactive insulin or proinsulin, and bioreactor chamber B producing chitosan-rich supernatant, or biomass itself.

B.3. Anticipated Results:

Bioreactor chamber A produces bioactive insulin or proinsulin. Bioreactor chamber B produces chitin or chitosan-rich supernatant or biomass, which can be easily processed for use in nanoparticle production.

B.4. Possible Complications:

Incomplete proinsulin cleavage or failure to secrete chitosan scaffold.

B.5. Alternative Strategies:

The closed-loop portion of bioreactor chamber A could have another branch following the filtration and isolation of proinsulin, where it is then incubated with a synthetic cleaver. Alternatively, if the machine-learning algorithm outfitted to the controller of the system is able to design synthetic genes, one could possibly create a synthetic self-cleaving linker that might act as an artificial human prohormone convertase. Also, if all of the chitosan scaffolds we wish to use are not properly secreted, we may have to end up sacrificing some of our biomass in order to get the chitin we need for production. This may result in two separate bioreactors being made, rather than the novel design proposed.

Specific Aim 2: Design and implement a modular microfluidic system downstream of our dual-strain bioreactor, in order to facilitate fusion of bioactive proinsulin and chitosan for nanoparticle assembly.

A. Rationale: Once each closed-loop bioreactor system is properly in effect, we can begin nanoparticle self-assembly via microfluidic design. Some pioneering research done by Zervantonakis et. al. here at the University of Pittsburgh have shown that this can be done with great success. It would require lots of trial and error, but with the help of machine-learning algorithms, flow-rates could be automatically modified, and their products analyzed. Since we are aiming to develop an API with oral bioavailability, we must include an enteric coating step to protect the nanoparticle from the harmful effects of the gastrointestinal tract. Some agents that could be included within another branch of the microfluidic system include hydroxypropyl methylcellulose phthalate, or cellulose acetate phthalate (CAP) [8].

B. Study Design:

B.1. Experimental Design:

A microfluidic system composed of a Y-junction and another optional branch for enteric coating could be created using classical photolithography techniques demonstrated by Zervantonakis et. al.. By including an insertion to a zetasizer outfitted with a controller using a machine-learning algorithm, which also tailors flow dynamics, one could allow this system to automatically optimize flow-rates to form the optimal nanoparticle.

B.2. Success Criteria:

Nanoparticles for insulin delivery have been developed, tested, and failed repeatedly. Luckily, this does provide us with a good understanding of what our particles need to be successful. We hope that analysis using a zetasizer and western blot (for encapsulation efficiency) would yield insulin-loaded chitosan nanoparticles. Perhaps most importantly, particles need to be resistant to extremely low pH solutions to ensure their enteric coating can resist the metabolism of the GI tract.

B.3. Anticipated Results:

A nanoparticle that could effectively facilitate oral delivery of insulin would need a PDI of less than 0.2, a size distribution between 200 and 300 nm, and an encapsulation efficiency greater than 75%. It should have a net charge between -7 to +7 mV, so as to prevent aggregation once in the blood-stream. Finally, the particle should withstand solvation in solutions of pH ranging from 1 to 3.

B.4. Possible Complications:

One major pitfall to this entire project is poor assembly of our chitosan scaffold. Another major complication is that once our particle reaches the bloodstream it can not be delivered to cells.

B.5. Alternative Strategies:

To aid in purification of chitosan, one could introduce a tangential flow filtration (TFF) system in order to produce a more uniform product. Additionally, one might also want to add ionic cross-linkers to promote chitosan gelation, like TPP, which is employed in many other chitosan nanoparticles, within the microfluidic system [9]. Also, one should consider adding a targeting ligand to our particle in order to facilitate active targeting of receptors overexpressed in patients with diabetes, in order to ensure delivery.

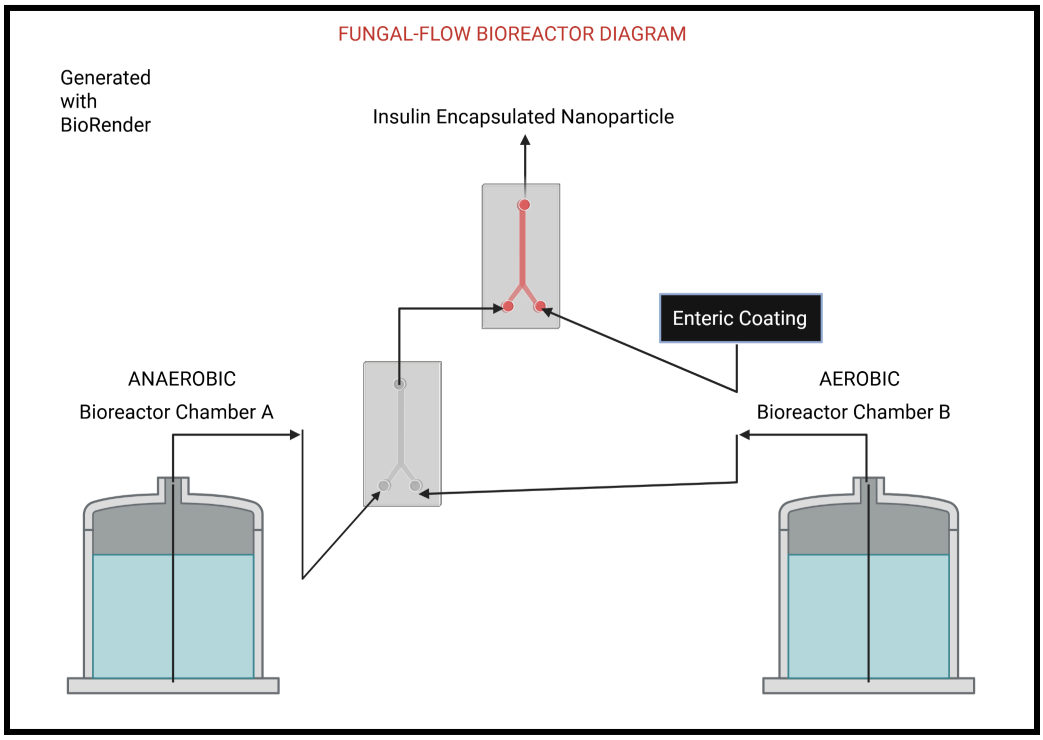
CONCLUSION

This proposal introduces a dual-track experimental design; however, I believe its broader implications and utility lie in its adaptability to future therapeutic needs. Aim 1 focuses on genetically engineering multicellular eukaryotic organisms to produce biologically active molecules for use in humans. Additionally, it leverages conditional expression under morphology-linked control. I recognize the success in this aim hinges on overcoming the significant challenge of post-translational processing in fungi. Should engineered strains fail to

produce properly folded and cleaved insulin, aim 2 remains independently valuable as a platform for biomanufacturing. Pre-purified insulin or therapeutic agents could be inserted into the system to aid in nanoparticle formation. The proposed microfluidic system could still act as a powerful tool for tuning drug delivery profiles, particle sizes, release mechanics, and enteric coating behavior. The true value of this project lies in the hopeful and continuous advancement of AI and machine-learning tools. Once a system such as this one is built and operational, its real-time sensor data would be invaluable if consistently monitored and analyzed by a predictive learning model. Such a system can use software and programs that have already been developed. Examples include, using COBRApy to simulate fungal metabolic fluxes to identify bottlenecks in insulin biosynthesis, using AlphaFold-Multimer to predict folding success, using Bayesian optimization algorithms for the flow control system to fine-tune reactor cycling, or even using AutoML platforms like Google's Vertex AI to try and uncover novel correlations between morphology and yield. To conclude, we are proposing a system that might not be successful initially, but a system that is both alive and programmable. Through orchestrated level by level tuning, we can create a synthetic environment that has AI acting as its nervous system. Being able to feed a live system commands and ask for information or connections not of obvious note, is a tool of immense value for us as researchers. As technologies in AI-based metabolic prediction and 3D molecular imaging and printing become more advanced, I am confident this is exactly where the future is heading. In time, biology itself can become programmable. This work is the first step. I hope to use it to make medicine not accessible for only those that can afford it, but those that need it.

FIGURES

Figure 1. Fungal-Flow Bioreactor Diagram:



REFERENCES

[1] “The Miracle Discovery That Reversed the Diabetes Death Sentence.” The Nobel Prize, www.nobelprize.org/the-miracle-discovery-that-reversed-the-diabetes-death-sentence/.

[2] Desjardins, Jeff. “The Rising Cost of Insulin in the U.S.” Visual Capitalist, 6 Apr. 2023, www.visualcapitalist.com/cp/rising-cost-of-insulin-us/.

[3] Slater, Simon C., et al. “Large-scale production of human insulin using recombinant DNA technology.” Trends in Biotechnology, vol. 5, no. 11, 1987, pp. 275–281. https://doi.org/10.1016/0167-7799(87)90136-0.

[4] Voigt, Kerstin, et al. “Transcriptome dynamics of the dimorphic zygomycete Mucor circinelloides during aerobic and anaerobic growth.” Fungal Genetics and Biology, vol. 44, no. 10, 2007, pp. 982–994. https://doi.org/10.1016/j.fgb.2007.04.007.

[5] Homa, Faezeh, et al. “Role of calcineurin in regulating morphogenesis and pathogenicity in Mucor lusitanicus.” Frontiers in Microbiology, vol. 10, 2019, article 1585. https://doi.org/10.3389/fmicb.2019.01585.

[6] Scholtmeijer, Karla, et al. “Characterization of novel hydrophobins from the industrially used fungus Trichoderma reesei.” Applied Microbiology and Biotechnology, vol. 78, no. 5, 2008, pp. 871–880. https://doi.org/10.1007/s00253-008-1362-y.

[7] Rinaudo, Marguerite. “Chitin and chitosan: properties and applications.” Progress in Polymer Science, vol. 31, no. 7, 2006, pp. 603–632. https://doi.org/10.1016/j.progpolymsci.2006.06.001.

[8] Sriamornsak, Pornsak. “Application of pectin in oral drug delivery.” Expert Opinion on Drug Delivery, vol. 8, no. 8, 2011, pp. 1009–1023. https://doi.org/10.1517/17425247.2011.580783.

[9] Akinc, A., et al. “Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms.” Nature Biotechnology, vol. 28, no. 12, 2010, pp. 1279–1286. https://doi.org/10.1038/nbt.1703.

[10] Ogasawara, Yasushi, et al. “Exploration of lavendamycin biosynthesis reveals unprecedented enzymes and biochemical logic.” The Journal of Antibiotics, vol. 73, 2020, pp. 1–10. https://doi.org/10.1038/s41429-020-0303-3.

[11] Linder, Markus B., et al. “Hydrophobins: the protein-amphiphiles of filamentous fungi.” FEMS Microbiology Reviews, vol. 29, no. 5, 2005, pp. 877–896. https://doi.org/10.1016/j.femsre.2005.01.004.

[12] Ebrahim, Ali, et al. “COBRApy: COnstraints-Based Reconstruction and Analysis for Python.” BMC Systems Biology, vol. 7, no. 1, 2013, article 74. https://doi.org/10.1186/1752-0509-7-74.

[13] Evans, Richard, et al. “Protein complex prediction with AlphaFold-Multimer.” bioRxiv, 2021. https://doi.org/10.1101/2021.10.04.463034.

[14] Carpenter, Anne E., et al. “CellProfiler: image analysis software for identifying and quantifying cell phenotypes.” Genome Biology, vol. 7, no. 10, 2006, article R100. https://doi.org/10.1186/gb-2006-7-10-r100.

[15] Alfatama, Mulham, et al. “A Comprehensive Review of Oral Chitosan Drug Delivery Systems: Applications for Oral Insulin Delivery.” Nanotechnology Reviews, vol. 13, no. 1, 2024, pp. 1–18. https://doi.org/10.1515/ntrev-2023-0205.

[16] Riggs, Arthur D. “Making, Cloning, and the Expression of Human Insulin Genes in Bacteria: The Path to Humulin.” Endocrine Reviews, vol. 42, no. 3, 2021, pp. 374–380. https://doi.org/10.1210/endrev/bnaa029.

[17] Zacchetti, Boris, Paul Smits, and Dennis Claessen. “Dynamics of Pellet Fragmentation and Aggregation in Liquid-Grown Cultures of Streptomyces lividans.” Frontiers in Microbiology, vol. 9, 2018, article 943. https://doi.org/10.3389/fmicb.2018.00943.

[18] Kozma, Michael, Bishnu Acharya, and Rabin Bissessur. “Chitin, Chitosan, and Nanochitin: Extraction, Synthesis, and Applications.” Polymers, vol. 14, no. 19, 2022, article 3989. https://doi.org/10.3390/polym14193989.

[19] Hou, Jiaxin, Berk Emre Aydemir, and Ahu Gümrah Dumanli. “Understanding the Structural Diversity of Chitins as a Versatile Biomaterial.” Philosophical Transactions of the Royal Society A, vol. 378, no. 2183, 2020, article 20200331. https://doi.org/10.1098/rsta.2020.0331.

[20] Rong, Yixin, et al. “CRISPRi-Mediated Metabolic Switch Enables Concurrent Aerobic and Synthetic Anaerobic Fermentations in Engineered Consortium.” Nature Communications, vol. 15, 2024, article 53381. https://doi.org/10.1038/s41467-024-53381-4.

[21] Chen, Wei, et al. “Structural Basis for Directional Chitin Biosynthesis.” Nature, vol. 610, 2022, pp. 402–408. https://doi.org/10.1038/s41586-022-05244-5.

[22] Fetouh, Howida A., et al. “Formulation of New Drug Delivery Systems for Insulin from Natural Bioactive Biocompatible Polymers.” Scientific Reports, vol. 15, 2025, article 86938. https://doi.org/10.1038/s41598-025-86938-4.