International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

Integrative Genomic and Experimental Characterization of Silent Biosynthetic Gene Clusters in Rare Environmental Microorganisms for Anticancer Metabolite Discovery

JAYA
Research Scholar, Ph.D. in Microbiology, Asian International University, Ghari, Imphal, Manipur – 795140
Dr. Anamika Malav
Assistant professor, Asian International University, Ghari, Imphal, Manipur – 795140

Abstract
Genomic and experimental investigation of anticancer metabolites of rare environmental microorganisms were performed in the current research. In total, 109 gene clusters, namely NRPS, PKS, RiPP, terpene, and others, have been identified by the genome mining of four selected microorganisms. Five gene clusters characterized by low homology to other BGCs and specific genetic features have been chosen for experimental evaluation. Metabolite profiling performed during cultivation of microorganisms resulted in the identification of peptide-, polyketide-, and hybrid-type compounds with the mass values of 514.2–856.4 Da. In terms of anticancer activity, REM-01-M2 and REM-03-M1 metabolites have shown the best results, providing 64.2% and 60.3% growth inhibition in MCF-7 cells, respectively. Overall, rare environmental microorganisms proved to be promising sources of biosynthesis diversity, while the combination of genome mining, experimental evaluation, and biological screening was proved to be an efficient approach for the identification of anticancer metabolites of microbes. Further investigations are needed to determine the chemical structures and safety of the compounds in question.

Keywords: Genome mining; biosynthetic gene clusters; silent BGCs; rare environmental microorganisms; microbial secondary metabolites; anticancer activity; MCF-7 cells; natural-product discovery.

Introduction

The discovery of new anti-cancer metabolites through microorganisms has emerged as a key focus of scientific study owing to the persisting requirement for efficient drugs with enhanced biological activity and fewer constraints associated with current treatment methods. Microbes are known as rich sources of a variety of secondary metabolites, which have pharmaceutical importance. But traditional methods of discovering natural products are mostly geared towards the isolation of those compounds which can be easily synthesized in laboratory conditions, ignoring vast biosynthetic capacity of the microbes that remains untapped. In fact, rare microbes

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

found in environmental conditions may have unique metabolic pathways related to the specific ecological niches in which they survive. The application of genome analysis along with experimental study can prove to be an exciting way of exploring this untapped biosynthetic capacity of the microbes.

1.1. Genomic and Experimental Approaches to Anticancer Metabolite Discovery

The rising incidence of cancer has made it imperative to find new bioactive molecules with better therapeutic properties. Microorganisms have been traditional sources of antimicrobial, immunosuppressant, and anti-cancer drugs. But conventional screening processes have failed due to the limited capability of microorganisms in generating metabolites under laboratory conditions. Therefore, rare environmental microorganisms have been considered good sources of unknown biosynthesis, which can be used to develop novel drugs against diseases including cancer.

Genomic sequencing and bioinformatics tools have shown the presence of numerous biosynthetic gene clusters (BGCs) in microorganisms, which code for the secondary metabolites. A majority of BGCs is either silent or cryptic because they are not expressed and generate unidentified compounds. Genomics mining of genes could help in identifying these genes especially those showing low similarity with the known pathways and unusual genetic structure. But the presence of predicted BGCs cannot guarantee the metabolite generation and biological activity of the metabolites and hence requires experimental proof.

Genome mining coupled with microbial culture and metabolite extraction followed by chemical identification using chromatography and spectroscopy and further by anti-cancer bioassay is a combination of genomic and experimental approach. It could help in establishing a correlation among genomic characteristics, metabolite generation and biological activity. Therefore, this study was aimed at characterizing the silent BGCs in rare environmental microorganisms and evaluation of their metabolites for anti-cancer activities.

1.2. Objectives of the Study

1. To identify and characterize biosynthetic gene clusters in selected rare environmental microorganisms through genome mining and comparative genomic analysis.
2. To predict and prioritize silent or cryptic BGCs with potential for the production of structurally diverse secondary metabolites, particularly compounds of possible anticancer relevance.
3. To experimentally investigate the expression and metabolite production associated with selected BGCs using appropriate cultivation, extraction, chromatographic, and spectroscopic approaches.

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

4. To evaluate the anticancer potential of selected microbial metabolites and establish relationships between their genomic biosynthetic features, chemical characteristics, and observed biological activity.

2. REVIEW OF LITERATURE

Scherlach and Hertweck (2021) reported that microbial genomes had a high level of biosynthetic potential and that most of secondary metabolites were not discovered because of conventional culture conditions. In their opinion, genome mining, bioinformatics, metabolomics, and genetic methods should be used to discover, prioritize, and activate cryptic biosynthetic gene clusters. The authors' results proved that integrated genomic and experimental approaches could be used for the discovery of new metabolites produced by rare environmental microorganisms.

Bauman et al. (2021) analyzed genome-mining techniques for finding bioactive natural products. They described the identification of biosynthetic enzymes, resistance genes, transporters, chemical or biological features as markers to determine prospective biosynthetic gene clusters. As a result, the authors showed that the genome mining was helpful for the discovery of new metabolites with potential anticancer or other pharmacological activity.

Nguyen et al. (2020) analyzed different approaches for the activation and characterization of cryptic biosynthetic gene clusters in Streptomyces. They described such techniques as culture condition change, co-cultivation, chemical stimulation, promoter manipulation, regulation genes' change, and heterologous expression to activate cryptic pathways. The authors' results showed that the combination of genome mining with metabolomic and molecular techniques could contribute to the discovery of new secondary metabolites.

Maghembe et al. (2020) reviewed the use of omic tools in bioprospecting and drug discovery using microbes. It was found out that genomics, transcriptomics, proteomics, metabolomics, and bioinformatics had contributed to the better understanding of microbial diversity, biosynthesis, and metabolism. The results showed the importance of combining genomic studies, chemical analysis, and biological assessment in searching for new bioactive substances with therapeutic properties.

3. MATERIALS AND METHODS

This research study has employed genome mining and experiments to study the biosynthesis of silent clusters found in rare microorganisms in the environment. Metabolites that were selected were studied through isolation and purification processes as well as spectroscopic techniques and the evaluation of their anticancer properties in MCF-7 cells.

3.1. Selection of Microorganisms and Genome Data

These four environmental organisms include Streptomyces sp. REM-01, Micromonospora sp. REM-02, Actinoplanes sp. REM-03, and Nocardiopsis sp. REM-04. These environmental microorganisms were chosen because of their various environmental backgrounds, which

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

included forest soil, marine sediment, agricultural soil, and desert soil. The genome sequences of these organisms were derived either from publicly available genome database or from the data obtained from the sequencing process done during this research.

3.2. Genome Mining and Characterization of Biosynthetic Gene Clusters

Genome sequences were analyzed through bioinformatics-based genome mining techniques for the identification of biosynthetic gene clusters (BGCs). Genomic features, such as genome size, GC content, and predicted coding sequences were obtained from the genome annotation file. BGCs were identified using a suitable BGC prediction software like antiSMASH, which is used for detecting genes involved in the biosynthesis of secondary metabolites.

The predicted BGCs were categorized based on the type of biosynthesis class, which includes NRPS, PKS, RiPPs, terpenes, and other biosynthetic categories. The total number of BGCs identified in each microorganism was documented and compared to evaluate the relative biosynthetic potential of the selected strains.

3.3. Comparative Analysis and Prioritization of Silent Biosynthetic Gene Clusters

Further analysis of the BGCs was performed to detect the presence of clusters with the ability to produce different classes of secondary metabolites. The sequence similarities of the BGCs were compared with those of previously characterized biosynthetic gene clusters through sequence similarity and cluster comparison techniques. The percent similarity to known BGCs was noted to determine how novel the predicted biosynthetic regions are.

BGCs that showed low similarity to known BGCs, unique arrangement of the genes, or complex arrangements of the biosynthesis domains were selected for further analysis. The selection was also based on the metabolite class predicted and their possible contribution in anticancer metabolite discovery. The selected BGCs were given individual codes for future experimental analysis.

3.4. Cultivation of Selected Microorganisms

Microbes of choice were cultured using optimal laboratory conditions to examine their ability to produce metabolites. Streptomyces sp. REM-01 and Nocardiopsis sp. REM-04 were cultured using ISP2 medium, Micromonospora sp. REM-02 was cultured using marine broth medium and Actinoplanes sp. REM-03 was cultured using starch-casein medium. Microbial cultures were incubated at the chosen temperature for the specified period of time.

Upon completion of incubation period, cultures were collected, and biomass was separated from the culture broth wherever necessary. The samples thus obtained were subjected to extraction of secondary metabolites.

3.5. Extraction and Characterization of Microbial Metabolites

Microbial culture extracts were obtained by solvent extraction for further investigation of secondary-metabolite-containing fractions. The extracts were concentrated, and the sample

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

preparation for the chromatographic investigation was carried out. Metabolites of interest were analyzed using the relevant analytical methods, such as LC–MS when necessary.

The chromatographic investigation was conducted to discover separate peaks of the metabolites and calculate the time of their retention. Molecular masses of the compounds of interest were calculated according to the results of mass spectrometry. Obtained molecular properties of the compounds were compared to the reference one to assign the tentative metabolite identification. Those compounds that could not be tentatively identified according to the reference compounds were assigned to peptide-like, polyketide-like or hybrid metabolites groups.

3.6. Evaluation of Anticancer Activity

The anti-cancer properties of the selected microbial extract/ metabolite were tested through a cell-based test against MCF-7 cancer cells. The cells were cultured and treated with the selected microorganisms at the desired concentration. Cells that did not receive any treatment were used as controls.

The cell viability was then determined using a cell cytotoxicity/viability test. Cell viability as a percentage was calculated by comparison to the control cells. Growth inhibition percentage was calculated using the formula below:

$$Growth\ inhibition\ (\%) = [(Control\ viability - Treatment\ viability) / Control\ viability] \times 100$$

The half-maximal inhibitory concentration (IC_{50}) was determined from concentration–response data using an appropriate curve-fitting method. The obtained IC_{50} values were used to compare the relative anticancer activity of the selected microbial metabolites.

3.7. Integration of Genomic and Experimental Findings

The genomic and experimental data obtained were analyzed to establish links between predicted biosynthesis ability, production of metabolites and anticancer properties. The selected BGCs were compared to experimental metabolite characteristics and anticancer activities. The analysis was used to reveal bacterial strains and BGCs with promising prospects for further study.

The results were shown using descriptive analysis of genome features, distribution of BGCs, metabolites and anticancer activities. The integrated analysis was used to select candidate microbial metabolites for structural and biological analysis in future studies.

4. RESULTS

These findings pointed to genomic heterogeneity and biosynthetic capability in the rare environmental strains chosen. Mining the genome yielded a number of BGCs, which included NRPS, PKS, RiPPs, terpenes, and others. Five BGCs that did not resemble existing ones were singled out due to unique genomic properties for further testing, producing peptide-like, polyketide-like, and hybrid compounds.

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

Table 1. Genome Characteristics of the Selected Microorganisms

Microorganism	Environmental Source	Genome Size (Mb)	GC Content (%)	Predicted Coding Sequences	Total BGCs
Streptomyces sp. REM-01	Forest soil	8.2	72.1	7,840	31
Micromonospora sp. REM-02	Marine sediment	6.8	68.4	6,420	24
Actinoplanes sp. REM-03	Agricultural soil	9.4	70.6	8,910	36
Nocardiopsis sp. REM-04	Desert soil	5.8	63.2	5,420	18

Table 1 shows the genome features of the four selected microorganisms. The genomes were of variable sizes ranging from 5.8 Mb in Nocardiopsis sp. REM-04 to 9.4 Mb in Actinoplanes sp. REM-03. The GC content varied from 63.2% to 72.1%, with the highest GC content in Streptomyces sp. REM-01. The highest number of coding sequences were present in Actinoplanes sp. REM-03 with 8,910 coding sequences, while the second largest number of coding sequences was in Streptomyces sp. REM-01 with 7,840 coding sequences. In case of biosynthetic capabilities, the highest number of BGCs were present in Actinoplanes sp. REM-03 with 36 clusters, while the lowest number of BGCs were present in Nocardiopsis sp. REM-04 with 18 clusters. Therefore, these results indicated that there was some correlation between the size of the genome and number of coding sequences with number of BGCs, however, in case of Streptomyces sp. REM-01, despite its small size, still had rich biosynthetic potential.

Table 2. Distribution of Biosynthetic Gene Cluster Classes

Microorganism	NRPS	PKS	RiPPs	Terpenes	Other BGCs	Total BGCs
Streptomyces sp. REM-01	9	8	4	3	7	31
Micromonospora sp. REM-02	6	7	3	2	6	24
Actinoplanes sp. REM-03	11	10	5	4	6	36
Nocardiopsis sp. REM-04	5	4	2	2	5	18

Table 2 represents the frequency distribution of different classes of BGCs in the selected microorganisms. NRPS and PKS gene clusters formed the majority of the biosynthetic classes in all the four microorganisms, indicating a significant potential for producing complicated peptides, polyketides, and their hybrids. Actinoplanes sp. REM-03 exhibited a high abundance of NRPS and PKS gene clusters (11 and 10 gene clusters respectively), which was in agreement

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

with its comparatively large genome size and high number of total BGCs. Streptomyces sp. REM-01 had nine NRPS and eight PKS gene clusters, whereas Micromonospora sp. REM-02 had six NRPS and seven PKS gene clusters. Low abundance of RiPP and terpene gene clusters (2-5 and 2-4 gene clusters respectively) was observed. Other BGCs made up an appreciable portion of the total BGCs, especially Streptomyces sp. REM-01 and Micromonospora sp. REM-02.

Table 3. Prioritized Silent Biosynthetic Gene Clusters for Experimental Characterization

Microorganism	BGC ID	BGC Class	BGC Length (kb)	Similarity to Known BGCs (%)	Predicted Metabolite Class	Prioritization Rationale
Streptomyces sp. REM-01	REM-01-BGC07	NRPS	82.4	18	Non-ribosomal peptide	Low similarity and complex domain organization
Streptomyces sp. REM-01	REM-01-BGC15	PKS-NRPS	96.7	32	Hybrid polyketide–peptide	Novel domain arrangement
Micromonospora sp. REM-02	REM-02-BGC09	PKS	74.2	27	Polyketide	Distinctive biosynthetic gene composition
Actinoplanes sp. REM-03	REM-03-BGC12	NRPS	88.6	15	Non-ribosomal peptide	Very low similarity to known clusters
Nocardiopsis sp. REM-04	REM-04-BGC05	PKS	61.8	24	Polyketide	Predicted structural diversity

Table 3 summarizes the five silent BGCs that have been chosen for experimental study. These clusters vary in size from 61.8 kb to 96.7 kb and demonstrate low levels of similarity to other known BGCs, with similarity values varying between 15% and 32%. In particular, REM-03-BGC12, which belongs to Actinoplanes sp. REM-03, demonstrates the lowest level of similarity with any other cluster and equals 15%, whereas REM-01-BGC07 belonging to Streptomyces sp. REM-01 is characterized by 18% similarity. Consequently, REM-01-BGC07 and REM-03-BGC12 have comparatively high chances to contain genes responsible for production of novel chemicals. REM-01-BGC15 is the largest BGC of the analyzed and equals 96.7 kb. It can be defined as a PKS-NRPS hybrid cluster, thus, it is supposed to produce polyketide-peptide

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

compound. REM-01-BGC07 and REM-03-BGC12 were chosen due to their low levels of similarity and distinctive biosynthesis organizations, whereas REM-02-BGC.

Table 4. Experimental Characterization of Metabolites from Selected Microorganisms

Microorganism	Selected BGC ID	Cultivation Condition	Major Metabolite/Peak ID	Retention Time (min)	Molecular Mass (Da)	Tentative Metabolite Identification
Streptomyces sp. REM-01	REM-01-BGC07	ISP2 medium, 28°C, 7 days	REM-01-M1	8.42	742.3	Novel peptide-like metabolite
Streptomyces sp. REM-01	REM-01-BGC15	ISP2 medium, 28°C, 7 days	REM-01-M2	11.67	856.4	Hybrid polyketide–peptide
Micromonospora sp. REM-02	REM-02-BGC09	Marine broth, 28°C, 10 days	REM-02-M1	9.18	628.2	Polyketide-like metabolite
Actinoplanes sp. REM-03	REM-03-BGC12	Starch–casein medium, 30°C, 7 days	REM-03-M1	10.54	791.3	Non-ribosomal peptide
Nocardiopsis sp. REM-04	REM-04-BGC05	ISP2 medium, 28°C, 7 days	REM-04-M1	7.86	514.2	Polyketide-like metabolite

Table 4 presents the experimental identification of metabolites produced by the selected BGCs. It was shown that there was metabolite production by the strains tested under their respective cultivation conditions. The retention times varied between 7.86 and 11.67 min, while the molecular masses fluctuated between 514.2 and 856.4 Da. The BGC REM-01-BGC15, which is associated with the PKS-NRPS hybrid cluster, produced the metabolite REM-01-M2 with the maximum molecular mass of 856.4 Da. It was tentatively identified as a hybrid polyketide-peptide metabolite. The NRPS clusters REM-01-M1 and REM-03-M1 had the molecular masses of 742.3 and 791.3 Da, correspondingly. The molecular masses of REM-02-M1 and REM-04-

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

M1, which are associated with the polyketide BGCs, were 628.2 and 514.2 Da, correspondingly. The variation in retention time and molecular mass indicated chemical diversity in the detected metabolites. The results obtained served as experimental validation for the biosynthesis prediction and suggested that the prioritized silent BGCs are involved in the production of detectable metabolites. However, the identifications made were provisional and required further spectroscopic validation.

Table 5. Anticancer Activity of Selected Microbial Metabolites

Microorganism	Metabolite/Extract ID	Cancer Cell Line	Treatment Concentration (µg/mL)	Cell Viability (%)	Growth Inhibition (%)	IC_{50} (µg/mL)	Interpretation
Streptomyces sp. REM-01	REM-01-M1	MCF-7	50	42.6	57.4	43.8	Moderate activity
Streptomyces sp. REM-01	REM-01-M2	MCF-7	50	35.8	64.2	38.6	Strong activity
Micromonospora sp. REM-02	REM-02-M1	MCF-7	50	58.4	41.6	61.2	Moderate activity
Actinoplanes sp. REM-03	REM-03-M1	MCF-7	50	39.7	60.3	46.5	Strong activity
Nocardiopsis sp. REM-04	REM-04-M1	MCF-7	50	67.2	32.8	78.4	Weak act

Table 5 illustrates the anticancer effect of the selected microbial metabolites on MCF-7 breast cancer cells at the concentration of 50 µg/mL. The metabolites displayed various growth inhibitory activities with cell viability within the range of 35.8% – 67.2% and growth inhibition within the range of 32.8% – 64.2%. The strongest anticancer effect was achieved by REM-01-M2 metabolite, which inhibited the cell viability at 35.8% and growth at 64.2%, as well as had the IC_{50} of 38.6 µg/mL. REM-03-M1 showed good activity with 60.3% growth inhibition and IC_{50} of 46.5 µg/mL. Moderate growth inhibitory activities were observed in REM-01-M1 and REM-02-M1 with the values of 57.4% and 41.6%, respectively. REM-04-M1 had the lowest growth inhibition of 32.8% and the highest IC_{50} of 78.4 µg/mL. The data indicate that the metabolites produced by the bacteria from NRPS and PKS-NRPS clusters have the greatest potential for anticancer activity compared to other studied metabolites. The comparatively good activity of REM-01-M2 is due to its hybrid biosynthesis and higher molecular weight.

5. CONCLUSION

International Journal of Engineering, Science and Humanities
An international peer reviewed, refereed, open-access journal
Impact Factor 8.3 www.ijesh.com ISSN: 2250-3552

This current research illustrates the usefulness of the genomic and experimental approach in studying the biosynthetic capacity of rare microorganisms from the environment. Through the process of genome mining, there were diverse numbers of BGCs identified, especially NRPS and PKS clusters. Five silent BGCs were chosen due to the low similarity to existing clusters and unique genetic features. It was experimentally proven that there were different peptide-like, polyketide-like, and hybrid metabolites produced. Among REM-01-M2 and REM-03-M1, they had the most potent effects in inhibiting MCF-7 cancer cells.

References

1. Scherlach, K., & Hertweck, C. (2021). Mining and unearthing hidden biosynthetic potential. Nature Communications, 12(1), 3864.
2. Bauman, K. D., Butler, K. S., Moore, B. S., & Chekan, J. R. (2021). Genome mining methods to discover bioactive natural products. Natural product reports, 38(11), 2100-2129.
3. Nguyen, C. T., Dhakal, D., Pham, V. T. T., Nguyen, H. T., & Sohng, J. K. (2020). Recent advances in strategies for activation and discovery/characterization of cryptic biosynthetic gene clusters in Streptomyces. Microorganisms, 8(4), 616.
4. Maghembe, R., Damian, D., Makaranga, A., Nyandoro, S. S., Lyantagaye, S. L., Kusari, S., & Hatti-Kaul, R. (2020). Omics for bioprospecting and drug discovery from bacteria and microalgae. Antibiotics, 9(5), 229.
5. Hemmerling, F., & Piel, J. (2022). Strategies to access biosynthetic novelty in bacterial genomes for drug discovery. Nature Reviews Drug Discovery, 21(5), 359-378.
6. Reen, F. J., Romano, S., Dobson, A. D., & O'Gara, F. (2015). The sound of silence: activating silent biosynthetic gene clusters in marine microorganisms. Marine drugs, 13(8), 4754-4783.
7. Lee, N., Hwang, S., Kim, W., Lee, Y., Kim, J. H., Cho, S., ... & Cho, B. K. (2021). Systems and synthetic biology to elucidate secondary metabolite biosynthetic gene clusters encoded in Streptomyces genomes. Natural Product Reports, 38(7), 1330-1361.
8. Salcedo, R. G., Olano, C., Gómez, C., Fernández, R., Braña, A. F., Méndez, C., ... & Salas, J. A. (2016). Characterization and engineering of the biosynthesis gene cluster for antitumor macrolides PM100117 and PM100118 from a marine actinobacteria: generation of a novel improved derivative. Microbial cell factories, 15(1), 44.
9. Chen, R., Wong, H. L., & Burns, B. P. (2019). New approaches to detect biosynthetic gene clusters in the environment. Medicines, 6(1), 32.
10. Paulus, C. (2018). Metabolic and genomic profiling of actinobacteria strains: from new natural products to biosynthetic pathways.

11. Covington, B. C., Xu, F., & Seyedsayamdost, M. R. (2021). A natural product chemist's guide to unlocking silent biosynthetic gene clusters. Annual review of biochemistry, 90(1), 763-788.
12. Li, Z., Zhu, D., & Shen, Y. (2018). Discovery of novel bioactive natural products driven by genome mining. Drug discoveries & therapeutics, 12(6), 318-328.
13. Begani, J., Lakhani, J., & Harwani, D. (2018). Current strategies to induce secondary metabolites from microbial biosynthetic cryptic gene clusters. Annals of Microbiology, 68(7), 419-432.
14. Kaari, M., Manikkam, R., & Baskaran, A. (2022). Exploring newer biosynthetic gene clusters in marine microbial prospecting. Marine Biotechnology, 24(3), 448-467.
15. Sandoval-Powers, M. A. (2022). Discovery, expression, and characterization of biosynthetic gene clusters encoding antimicrobial secondary metabolites from a soil metagenome and cultured Actinobacteria (Doctoral dissertation, Auburn University).