Volume 1, Issue 3. December, 2021. www.jbbt.org

RECENTTRENDS IN BIODIESEL PRODUCTION: CHALLENGES AND ADVANCES

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ABSTRACT

Biodiesel is synthesized in transesterification reaction that uses vegetable oil or animal fat and methanol or ethanol in presence of catalyststhat may be either homogeneous or heterogeneous catalysts This brief review covers recent trends in the biodiesel production, its challenges and future perspectives of this eco-green process. The major existing obstacles of biodiesel synthesis process, current innovations, search of sustainable feedstock oil, catalysts role in biodiesel synthesis, separation of product from byproduct, to refine the produced fuel,to enhance chemical and mechanical strategies for quality enhancement of biodiesel,has been concisely reviewed.

Key words: Biodiesel, Feedstock oil/fat, Catalysts, Recent Trends, Quality

1. INTRODUCTION

The depleting fossil fuels, population explosion, urbanization have increased demand of renewable energy and in this regard biofuel has appeared as best alternate in combustion engines.The rising ecological distresses and deteriorating fossil fuel have declared renewable energy as an remarkable and favorable basis for future substitutes of energy (Atabani *et al*., 2013). Renewable fuels have tendency to resolve most of the modern social and environmental

glitches like distresses from effluence, climate change and sustainability issues (Gashaw and Lakachew, 2014). Biodiesel is the simply potential stand by to petro-diesel and favorablesubstitute resources for diesel engines (Farobie *et al.*, 2015). Biodiesel, mono-alkyl esters of long chain fatty acids, is derived from vegetable oils or animal fats and alcohol with or without a catalyst (Sadia *et al.*, 2013).Comparing with regular diesel fuel biodiesel has several benefits such as renewable, green, less poisonous, portable, low CO exhaust emissions, high flash point, low sulphur content, inherent lubricity that extends the life of diesel engine. Major demerits of biodiesel includes low energy contents, engine compatibility, higher NOx emission, high price, high pour and cloud point, low power and engine speed, high engine wear, high viscosity and injector coking. Among biofuel sources, first, second and third generation feedstocks of biofuel include edible sources, vegetable and animal fat; second includes non-edible sources and cellulosic biomass; third generation biofuels include solid wastes, sludges and algae. Recently, edible plants are chief source for biodiesel production but they have competition with food supply and to avoid this economic imbalance and price hike due to edible feedstock, non-edible sources are preferred. Moreover, high FFA content is major challenge that reduces the biodiesel yield.Biodiesel is prepared mainly from four ways i.e. micro-emulsion, thermal cracking, direct use and blending and tans-esterification. Triglyceride in oil or fat reacts with three moles of methanol to form methyl ester of respective fatty acids and glycerol (Leung *et al.* 2010).

$$TRIGLYCERIDE + METHANOL \rightleftharpoons MIXTURE\ OF\ FATTY\ ESTERS + GLYCEROL$$

The basic biodiesel synthesis process is mentioned in flow sheet diagram(**Figure *1***)

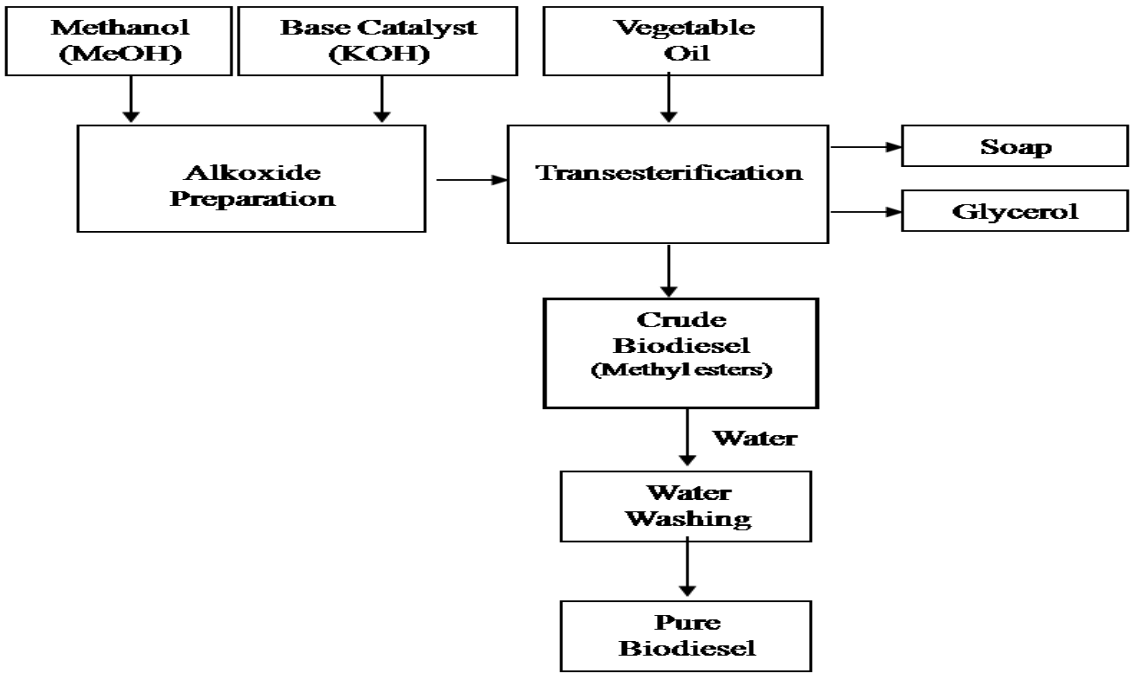

Figure 1 schematic diagram for biodiesel synthesis process

1. Trends in feedstock selection

More than 95% biodiesel is produced from edible sources because the characteristics are suitable for the alternate of petroleum based diesel fuel. Figure 2 shows world productivity of vegetable oil. The problemassociated with edible feedstock is its competition with food market hence increasing the fuel cost and leading to deforestation because these crops need specific fertile land to grow properly. In contrast, non-edible sources overcome such disadvantages because these are harmful for human consumptiondue to toxic compounds. Non-edible crops are grown at waste land and production cost is low however due to their high free fatty acid content they give high yield without specific care while growing at barren lands even. Animal fats with saturated fatty acids are solid at room temperature and their pretreatment enhances its production cost as compared to vegetable oils.

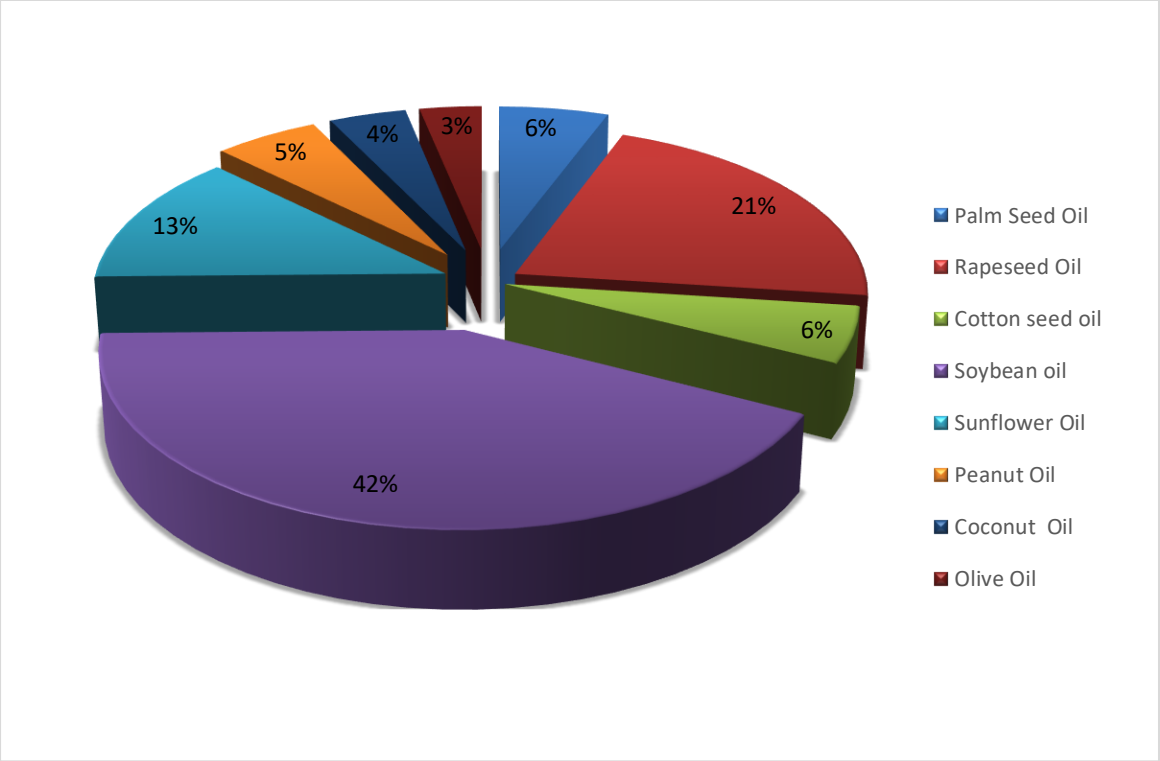

Figure 3 World productivity of vegetable oil

Microalgae is also best feedstock that helps to overcome food security issues as compared to edible non-edible feedstock. As microalgae are easily cultivated in short time round the year and give high yield of feedstock oil and productivity of algal biomass. Dewatering and harvesting algae is major bottleneck for algal biomass commercialization that is attributed to algal cell size and low conc. in culture medium therefore it is major obstacle in commercialization of algal biodiesel.

Table 1 Physicochemical properties of biodiesel from different edible sources

Edible fuel Sources	Edible oil	Density (g/cm^3)	Kinematic viscosity cSt. @40℃	Flash point ℃	Acid value mg KOH /g	Reference	Non-edible sources	Non-Edible oil	Density (g/cm^3)	Kinematic viscosity cSt. @40℃	Flash point ℃	Acid value mg KOH /g	Reference
	Soybean	0.91	32.9	254	0.2	(Niehaus, Goering, Savage, & Sorenson, 1986; Singh & Singh, 2010)		Jatropha	0.92	29.4	225	28	(Tiwari, Kumar, & Raheman, 2007)
	Rapeseed	0.91	35.1	246	2.92	(Winayanuwattikun et al., 2008)		Pongamia	0.91	27.8	205	5.06	(Sahoo & Das, 2009)
	Sunflower	0.92	32.6	274	-	(Winayanuwattiku		Sea mango	0.92	29.6	-	0.24	(Kansedo, Lee,

						n et al., 2008)						& Bhatia, 2009)
	Palm	0.92	39.6 @38℃mm^2 /sec	267	0.1	(Singh & Singh, 2010)		Polanga	0.90	72.0	221	44	(Sahoo & Das, 2009)
	Peanut	0.90	22.72	271	3	(Rao et al., 2009)		Tallow	0.92	-	-	-	(Goodrum, Geller, & Adams, 2003)
	Corn	0.91	34.9 @37℃mm^2 /sec	277	-	(Patil & Deng, 2009)		Nile tilapia	0.91	32.1@37℃ mm^2/sec	-	2.81	(Goodrum et al., 2003)
	Canola	0.91	38.2	-	0.4	(Issariyakul, Kulkarni, Meher, Dalai, & Bakhshi, 2008)		Poultry	0.90	-	-	-	(Goodrum et al., 2003)

2. Trends in catalyst selection

Transesterification is reaction that uses vegetable oil or animal fat and methanol or ethanol in presence of catalyststhat may be either homogeneous or heterogeneous catalysts (Leung *et al*., 2010).Among above mentioned, efficient catalysts are nano-catalysts have been appeared as promising catalyst to give high yields. *Figure 4* shows fundamental distinction between efficiency between three basic types of catalysts. Homogeneous catalysts are more selective, active, less stable, modern reaction time, difficult to recover and moderate reaction time but heterogeneous catalysts are less active, so take more reaction time, recoverable and more stable as compared to homogeneous catalysts. Table 2 shows depicts the contrast of competency of homogeneous catalysts with heterogeneous catalysts in transesterification reaction.

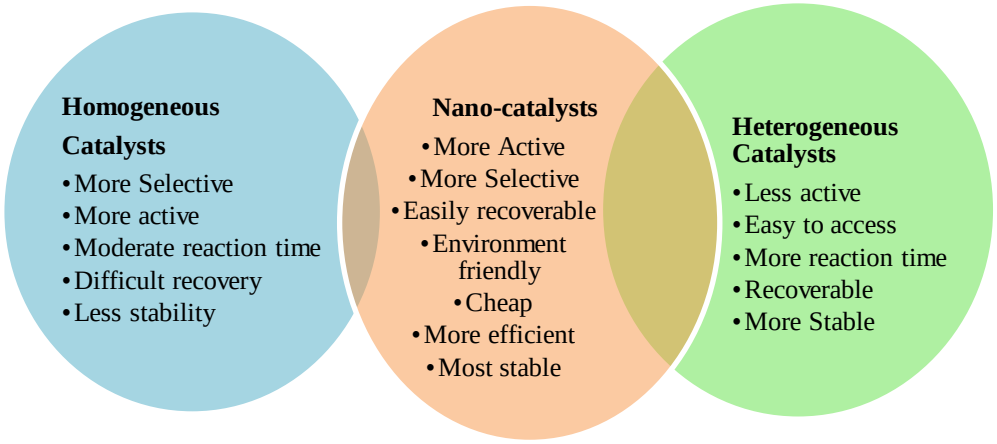

Figure 4. Difference in efficiency between three basic types of catalysts.

Table 2. Comparison of efficiency of homogeneous catalysts with heterogeneous catalysts in biodiesel synthesis

Factors	**Homogeneous Catalyst**	**Heterogeneous Catalyst**
Processing Methodology	Continuous process faces limitations	Continuous fixed-bed Operation
Concentration	Low	high

Reaction Rate	Fast Conversion	Medium Conversion
Thermal stability	Low	high
Life time	Variable	Long

The main strategies to overcome the demerits of biodiesel include use of nanosized catalysts i.e. more active, more selective, easily recoverable, environment friendly, cheap, more efficientand most stable. Features and advantages of nanocatalysts are shown in Figure 5

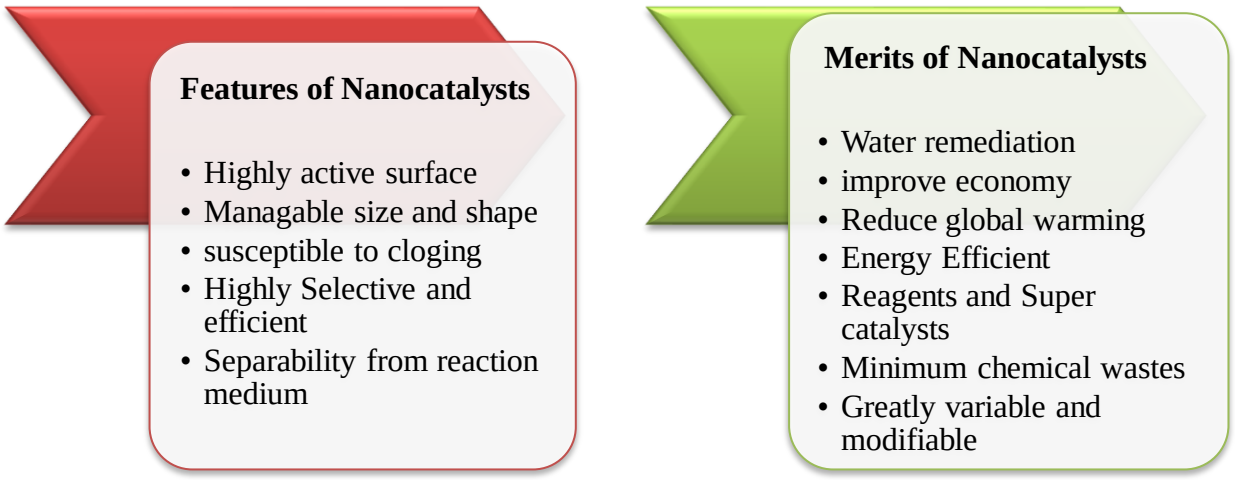

Figure 5 Features and Broad term merits of Nano-catalysts

3. Trends in biodiesel qualityenhancement

Purification and quality enhancement is major point of concern to be addressed as feedstock oil, byproduct, side reactions, high FFA content, reaction conditions have considerable effect. Chemical strategies involve cosolvent e.g. tetrahydrofuran, 1,4 dioxane, di-iospropyl ether and methyl tertiary butyl ether. Co-solvent usability is aimed for alcohol-oil single phase achievement. Separation of glycerol from biodiesel is slow and it is done by NaCl assisted gravitational settling 1g salt in 100ml glycerol reduced the glycerol settling time up to 100% without affecting the methyl ester yield (Shirazi *et al.*, 2013). Electrocoagulation is done for acceleration of decantation rate of glycerol. The high FFA is dealt with esterification with an acid along with transesterification via twostep process. (Pisarello *et al.*, 2018)To meet international standards ASTM D6751 biodiesel purification is done either by wet washing or dry washing and MST i.e. membrance separation technology. Electrospun polystyrene membranes are

more promising for industrial scale application due to their easy availability and low cost. To improve biodiesel properties oxygenated additives, cold flow improvers, combustion improvers, cetane no. improvers has been used for biodiesel quality enhancement (Mirzajanzadeh *et al.*, 2015)

Conclusion

In spite of detailedstudyconductedon different aspects of biodiesel synthesis process for improvement of economic viability of exclusive sustainable energy carrier yet its future feasibility is uncertain. It is because of inadequate feedstock oil accessible to meet the rising demand of biodiesel, and at the same time controversial matter of fuel and food supply competition. Furthermore, maintenance of biodiesel's market price with fluctuation in oil cost competitive to petro-diesel would appear as a challenge. Along with this, integrated strategies are vital to enhance the economic aspects of process.

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Paper 5

Volume 1, Issue 3. December, 2021. www.jbbt.org

THERAPEUTIC POTENTIAL OF HYDROETHONOLIC EXTRACT OF *NERIUMOLEANDER* FLOWERS FOR THEIR ANTIDIABETIC ACTIVITY

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ABSTRACT:

Diabetes Mellitus is a complicated metabolic illness that has greatly troubled human health and quality of life worldwide. Conventional methods are being used for treatment of diabetes; however they are not completely effective. These drugs maintain glucose level partially or temporarily. Medicinal plants with anti-hyperglycemic activities are being used at global level. In the present study the therapeutic potential of ethanolic extract of *Nerium oleander* flowers waschecked on diabetes *in vivo*. Alloxan was used to induce the diabetes in rabbits. Treatment rabbits were divided into two groups and given 200mg/kg body weight and 400mg/kg body weight per day respectively for 14 days. The decrease in blood glucose level was more significant in group II as compared to group I. Glucose level and histopathology was done to check the effect of extract of *N. oleander*.

Result showed decrease in blood glucose level in treatment group II (118.7 ± 7.4) and group I (150.64 ± 8.67) as compared to diabetic control (308 ±7.75). There was more pronounced decrease in blood glucose level in treatment group II as compared to group I. Serum insulin level was also improved in both treated groups i.e. in group I (7.67 ± 0.18) and group II (7.39 ± 0.9) versus diabetic controlled group (3.73 ± 0.26). Improvement in morphology of pancreas of treatment groups was observed. It was conclude that hydroethonolic extract of *N. oleander* flowers has tremendous effect against diabetes as it reduces the blood glucose level, improves glucose tolerance and also improves serum insulin level. the current study showed the hypoglycemic effect of ethanolic extract of *N. oleander*. OGTT was also performed and the results were quite satisfactory for treatment groups.

Key words*:* Diabetes mellitus, Glucagon likes peptide-1, Non-insulin dependent diabetes mellitus, Oral glucose tolerance test

INTRODUCTION:

Diabetes mellitus (DM) is a chronic disorder which is due to combination of hereditary and environmental factors. It is characterized by chronic hyperglycemia caused by deficiency in insulin action or secretion. It is found to damage most of the organ and tissue of body, such as circulatory system and nervous tissues (Cade, 2008). It is reported that more than 285 million people were affected with diabetes (Tariq *et al.*, 2013) and diabetes will be the seventh leading cause of death in 2030 (WHO, 2011).

There are two major types of diabetes Type 1 diabetes (T1D) and Type II diabetes (T2D). T1D is associated with insulin deficiency by destruction ofpancreatic β- cells. T1D is normally due to autoimmune damage of β cells of pancreas (George *et al.*, 2002). It can be managed with insulin injection. In T2D body cells fails to utilize insulin properly due to abnormal functioning of β-cells that leads to insulin deficiency and this condition called insulin resistance accompanied by decreased transport of glucose into fat cells and muscles due to which hepatic glucose output increased, all of which contribute to hyperglycemia(Setter *et al.*, 2003). Over body weight and lack of physical exercise are major causes of diabetes. Serious health problems due to diabetes are cardiovascular disease, blindness, kidney failure and lower limb amputation.

Diabetes mellitus is main threat to the world. There are different methods and practices to cure diabetes, but all these methods are effective temporarily or partially. Oral administration of immunosuppressive drugs is frequently used to treat diabetes. These drugs cannot maintain the blood glucose level accurately. Furthermore these drugs are very expensive and also have side effects. In severe conditions insulin injections are given to the patient. Although glucose level can be controlled by using insulin, but there is a risk of hypoglycemia (Sexena and Kishore, 2004). It is very panic process. Moreover patients have to

monitor blood glucose level several times in a day.

Another treatment strategy is β cell or stem cell transplantations. Islet/β- cells transplantation is the only successful therapy currently available (Tariq *et al.*, 2013). Stem cell therapy is potential replacement of β cell transplantation, but this 2 strategy is very expensive and is still in its initial stages may be available in near future. Another possible cure that may come in future is the use of nanoparticles.

Currently there is rapid interest in herbal remedies due to side effects related with therapeutic agents such as oral hypoglycemic agents and insulin for the treatment of diabetes mellitus (Khurshid *et al.*, 2012). Herbal and natural therapies are considered more safe and useful, and less expensive as compared to synthetic drugs.

Herbal medicine treatment is oldest form of health-care known to humans. Phytomedicines have great potential and had been used by all civilization throughout history. It was a basic part of the development of modern cultures/ civilization. Ethno botany studies the complex relationship between plants and cultures. It is multidisciplinary science defined as interaction between plants and people. The primitive man systematically collected information on the herbs and developed well-defined herbal drugs. Many drugs that are commonly used by humans today are of herbal origin. Most of drugs contain at least one active ingredient derived from plant extracts.

N. oleander is evergreen shrub and is widely used as a medicinal plant for many years in the treatment of different diseases. Some studies have investigated its toxicity and its cure action in diluted preparations (Haeba*et al.*, 2002).

N. oleander produces secondary metabolites; some of them have pharmacological interest. The important pharmacological activities are antifungal(Derwic *et al.*, 2010), antimicrobial (Hussain and Gorsi, 2004), antitumor (Ali *et al.*, 2010) and anti-inflammatory and anti-nociceptive (Erdemoglu*et al.*, 2003).Although *N. oleander* has great effects against different diseases but still there is alack of knowledge about antidiabetic activity of *N. oleander.* Few studies havebeen conducted on *N. oleander* to explore its anti-diabetic potential. Therefore, thepresent work will provide some novel aspects about *N. oleander* and in the field oftraditional medicine.

The present study was designed to explore anti-diabetic potential of hydro ethanolic extract of *N. oleander* flower in drug induced animals. The *N. oleander* plant has been used

traditionally as folk remedies for wide ranges of maladies and conditions including dermatitis, eczema, sores, abscesses, warts, skin cancer, asthma, heart tonics, and epilepsy. Anti-diabetic activity evaluation of *N. oleander* extract on insulin, glucose level and some liver enzyme activities was used by

Yassin and Mwafy, (2007). To check the therapeutic potential of *N. oleander* diabetic models were prepared by intravenous administration of alloxan. Hydroethanolic extract of *N.oleander flower* was orally administered to diabetic animals. The dose of *N.oleander* extract was based on toxicological studies (Haeba *et al.*, 2002; Adam *et al.*, 2011). Their blood glucose level was checked every day and serum insulin was also checked. Moreover histopathology of pancreas was also done to check the effect of *N. oleander* flowers extract.To evaluate the efficacy of hydroethanolic extract of *N. oleander* againstalloxan induced diabetic model.

1. To identify and extract of plant material from *N. oleander*.2. To induce diabetes in rabbits.3. To asses therapeutic potential of extract of flowers of *N.* oleander after treatment.4. To study histopathology of pancreas.

MATERIAL AND METHODS:

Sampling and Collection of Plants Material

The samples of plant were collected from local areas of Mirpur AJK andKasguma. The collected Samples were identified by an expert botanist Dr. RehanaAsghar, Chairperson of Biotechnology Department, MUST AJK.

Extract Formation from Flowers*:*

After collection of plants flowers were separated, washed and were dried inshady places for ten days. Later flowers were grounded and then dip its powderform in 500ml solution containing 70:30 of ethanol and water respectively. Thesolution was suspended in Soxhlet apparatus. By heating solvent was evaporated,transformed into condenser where it was converted in liquid and was collected inextraction chamber. The solid material was slowly filled with worm solvent. Whenthe Soxhlet chamber was almost full it was automatically emptied, with solventrunning back to the distillation flask. The above cycle was repeated several timesin a day. The filtrate was concentrated and solvent was recovered using rotaryevaporated. The paste was obtained and weighted by means of electrical balance.The whole material was placed in refrigerator to avoid any type of contamination.

Figure 1: Soxhlet Apparatus

Experimental Animals Used

Inbred male domestic rabbits (*Oryctolaguscuniculus*) with weight of 1-2kg approximately from Mirpur and surrounding areas were used. They werebrought three months prior to start of research in order to minimize the stresseffect. These rabbits were provided with natural habitat fresh water, vegetablegrasses, maize and some amount of glucose was also provided to these animals.The rabbits handled and treated carefully. The cages of these animals were cleanedon daily bases, fresh air and light was provided to these animals with reasonabletemperature.

Acute Toxicity Testing of Extract

For acute toxicity testing experimental animals were fasted overnight andwere provided only water and then the extract was given to the respective groups orally at the dose level of200mg/kg body-weight through digestive tract. Thesegroups were continuously observed for 24 hours for their behavior, neurologicaland autonomic profiles and then were studied for 72 hours in a week to check anytype of lethality. According to the guidelines if mortality is observed in 2 to 3animals, then the dose provided is toxic dose. If mortality is observed in oneanimal then the same dose is repeated to confirm the toxicity of that particulardose. If mortality is not observed at all, the plant extract is considered as non-toxic.No mortality was observed in experimental animals.

Induction of Diabetes in Rabbits

A group of rabbits with weight 1-1.5 kg were selected for alloxan injectionintravenously. 85mg/kg of alloxan was dissolved in 0.9% normal saline for makingit dilute solution. This solution was injected in jugular vein of rabbits with the helpof 3cc BD syringe. Winter green oil and a vasodilator methyl salicylate wereapplied to jugular vein before injection to make vein swell and prominent. The20% glucose in water was provided orally by means of small bottles for one day.The aim of glucose supply to animals was done to prevent them fromhypoglycemic shocks. The blood glucose level was routinely observed for five days with the help of glucometer. The test animals with blood glucose level more25than 250 mg/dl were considered as diabetic. At least two times the

blood glucoselevel greater than 250mg/dl confirms animals as diabetic model.

Experimental Design

The research animals were divided into five groups

Normal Group: Given no alloxan and no treatment

Diabetic Group: Given alloxan but no treatment

Controlled Group: Given alloxan and treated with insulin @ 5 IU/kg/day

Treatment Group1: Diabetic animals treated with 200mg/kg *N. oleander*flower *e*xtract.

Treatment Group 2: Diabetic animals treated with 400mg/kg of *N. oleander*flower *e*xtractThe animals of different groups were tagged for their easy recognitionduring study. The above mentioned groups were provided with vegetables, fruitsand glucose during experimentation of three weeks.

3.7: *Treatment of diabetic animals:*

In each group 3 animals were used for research work. Group 1 normal andgroup 2 diabetic were not provided treatment. Group 3 animals diabetic wereprovided with daily insulin of 5IU/kg group 4 animals were treated with 200mg/kgweight extract of *N. oleander* flower per day through oral route, per day. Thetreatment was started after a week on those animals which had hypoglycemicconditions. Flower extracts was present in the form of semisolid gel likeappearance. Before administration, extract was mixed with distilled water to makeit dilute. A dropper and food pipe was used to transfer the extract into mouth of therabbit. The procedure was repeated daily for two weeks and blood glucose levelwas checked on daily basis.

Measurement of Blood Glucose Level during Treatment

Blood glucose level of all experimental groups was monitored by glucoseoxidase method using Glucometer and standard glucose strips of ABBOT Ltd. Theblood glucose level was checked at every day after the start of treatment. Thenormal group was also detected at different times to observe any type of change inblood glucose level.

Measurement of Serum Insulin Levels*:*

Animals were handled with great care and blood samples were collectedimmediately from jugular vein. The jugular vein was then pressed with cottonsoaked with spirit to save the rabbits from infection.Samples of all model groups were collected after at the 10th day oftreatment in order to study any type change in pancreatic beta cells in to secreteinsulin. Serum was separated from the samples and insulin level was measured byELISA.

Oral Glucose Tolerance Test

Oral glucose tolerance test was applied to both diabetic controlled andtreated models of rabbits with *N. oleander*. The aim of this test was to observefailure of diabetic animal to metabolize the orally given glucose, and also observethe effect of glucose homeostasis in diabetic treated animals with *N. oleander*flower extract 200mg/kg and 400mg/kg body-weight. The rabbits were kept infasting condition for 12 hours and before giving them glucose solution, theirbaseline glucose level was checked with glucometer. The glucose solution wasthen provided to these rabbits orally 3g/kg their

body weight with the help ofdropper. the blood was withdrawn from ear veins after 30 minutes, 60 minutes, 90minutes and 120 minutes of glucose administration via glucometer the glucoselevel for all groups was measured and results were compared of controlled andtreated animals.

3.8: Chemicals and Apparatus Used*:*

1. Rotary evaporator(Heidolph)
2. Refrigerator
3. Grinder machine
4. Graduated beakers
5. Filter paper
6. Ethanol
7. Alloxan monohydrate (Sigma Aldrich chemical, Saint Louis, MO,USA)
8. Glucose
9. Insulin
10. Weighing balance
11. Glucometer and standard glucose strips of ABBOT Ltd.
12. Glucose measuring strips
13. Soxheletapparatus(set 55/50pcs by Laboy)
14. Urine Strips from Bayers Diagnosis Ltd.

Statistical Analysis:

The data obtained from various experiments was recorded and wasevaluated statistically using one way analysis of variance (ANOVA); mean valueswere determined along with standard deviation. Level of significance was kept asP>0.05.

RESULTS AND DISCUSSION

Comparison of Blood Glucose Levels of Normal Verses Diabetic Rabbits:

To induce diabetes in rabbits, they were induced with alloxan @ 85mg/kgbody weight. These rabbits showed symptoms of diabetes like polyurea with 3-4days. Diabetic status was confirmed by measurement of blood glucose level atfourth day alloxan induction by using ABBOT Glucometer. Rabbits with bloodglucose level > 250 mg/dl were confirmed to be diabetic. To establish a base linevalue of blood glucose in normal and diabetic rabbits, the blood glucose level oftwo groups was monitored for 14 days. The measurements were taken on dailybases. The results showed that rabbits induced alloxan have elevated level of bloodglucose as shown in Figure 4.1.

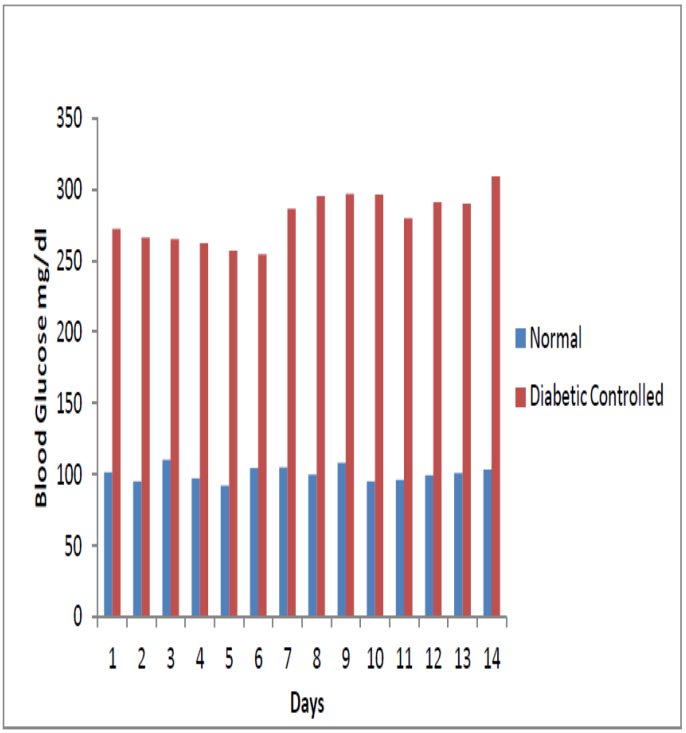

Figure 1. Evaluation of Blood glucose of normal and diabetic group.

N. oleander Shows Hypoglycemic Activity*:*

To evaluate the hypoglycemic activity of extract of *N. oleander* flowers, theblood glucose level were measured. The result showed the blood

glucose level oftreatment group 1(150.64 ± 8.69) and treatment group 2 (118.66 ± 7.38) weremarkedly lower than diabetic group (308± 7.75) at day 14 as shown in Figure 4.2

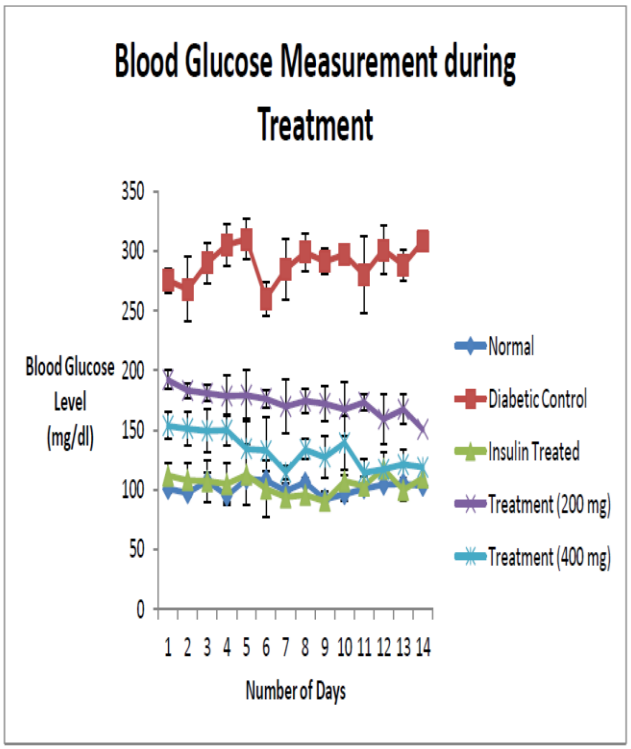

Figure 2. A Comparison of blood glucose levels of Normal, Controlled and Treatmentgroups

4.3: *Oral Glucose Tolerance Test:*

To check the impairment in glucose homeostasis, overnight fasted rabbitswere given glucose solution @ 3g/kg body weight. The blood glucose levels weremeasured at regular intervals of 30min for four times wise 30, 60, 90 and 120mins.The result showed a significant decrease in blood glucose levels of treatment group1 (271.53 ± 10.5), (248.83 ± 9.87), (188.22 ± 8.29) and (170.22 ± 7.38) andtreatment group 2 (210.21 ± 18.45), (180 ± 17.9), (153.15 ± 14.25) and (151 ±13) as compared to diabetic group (328 ± 25.35), (288.52 ± 13.42), (272.42 ±9.88) and (255 ± 12) at 30, 60, 90 and 120mins respectively as shown in Figure 4.3

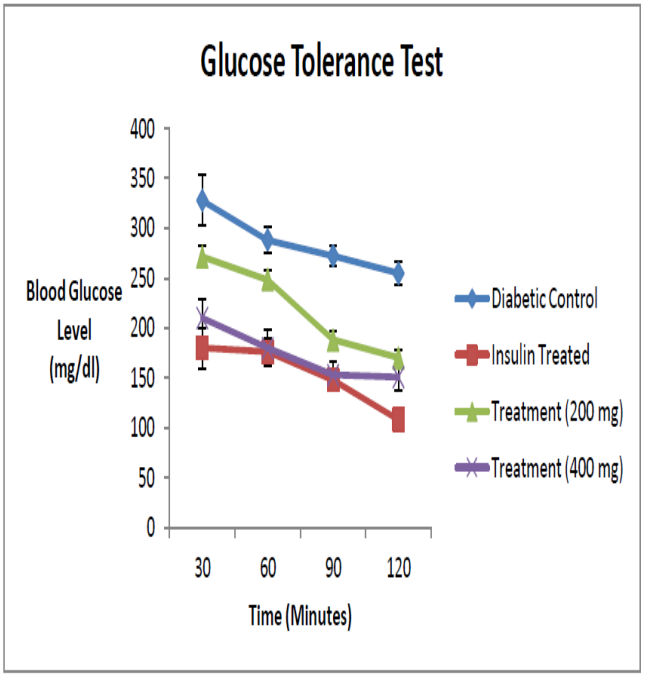

Figure 3. A Comparison of blood glucose of normal, control diabetic and treated groups. Allvalues are expressed in mean ± SEM.

4.4: Measurement of Serum Insulin Levels*:*

To assess the functionality of treated rabbits, the serum insulin levels weremeasured at the end of treatment. The results showed that there was a slight ornon-significant increase in the serum insulin level of treatment group 1 (5.67 ±0.18) and treatment group 2 (5.39 ± 0.9) as compared to diabetic group (3.73 ±0.256) as shown in Figure 4.4

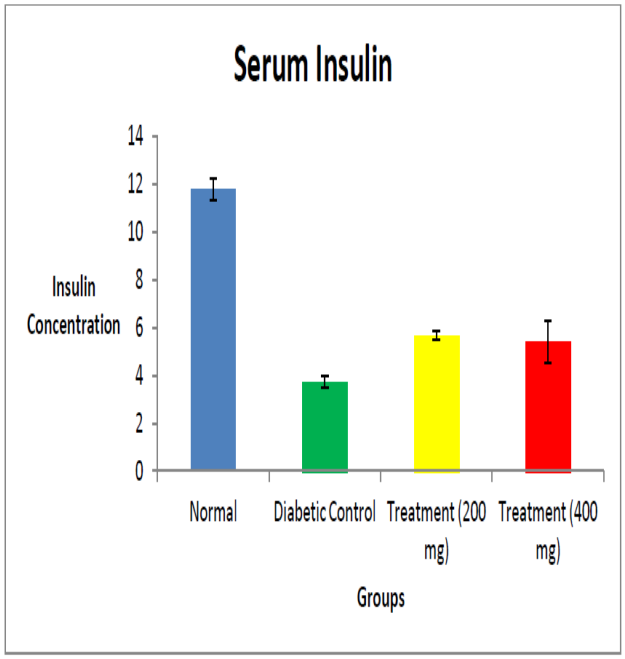

Figure 4. Serum Insulin Measurements (IU/ml) of Normal, Diabetic Control and Treatedanimals by ELISA. All values are expressed in mean ± SEM.

Histopathological Studies*:*

To observe the changes in morphology of pancreas after *N. oleander* flowersextract treatment, pancreata of rabbits from different groups were excised,processed, sectioned and stand with hematoxylin and eosin. The resultant standsections were observed under digital microscope. Images were taken and observedthe result showed slight improvement in the morphology of treatment groups ascompared to diabetic groups as shown in Figure 4.5

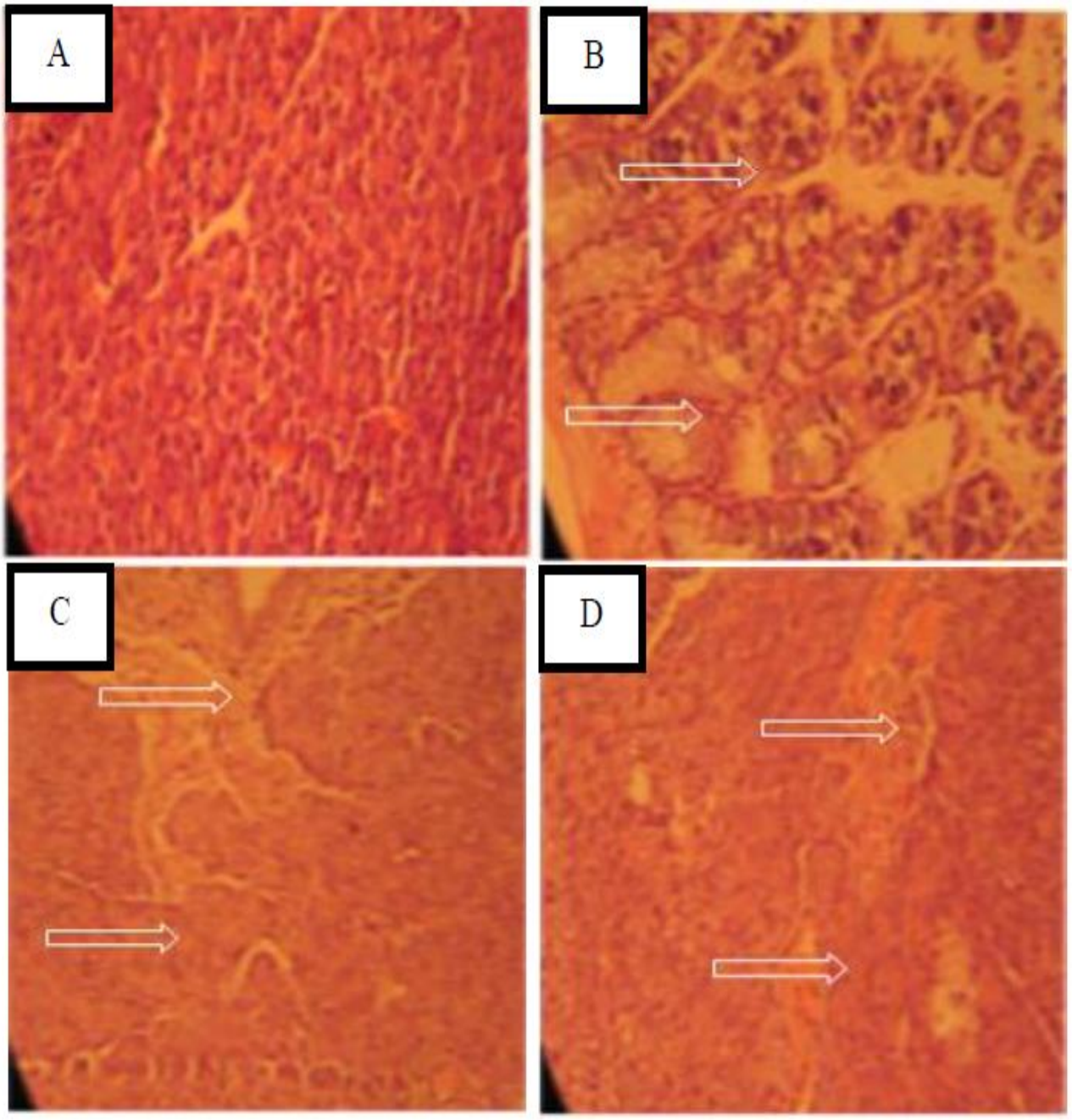

Figure 5. Histopathological micrographs of pancreatic section. Image A and B of Diabetic Control, C Treated with 200 mg/kg and D treated with 400 mg/kg

Conclusion:

After completion of present research it can be concluded that *NeriumOleander* is very effective against diabetes. The extract of flowers reduces theblood glucose level and also improved serum insulin levels. Histopathology ofpancreas also showed that there were significant changes after using the extract of*Nerium oleander*. Diabetes is a worldwide disease and the number of patients isincreasing rapidly. Although allopathic medicines are being used for treatment butit is common observation that these medicines have many side effects. So it ishighly recommended that phytomedicines should be preferred instead of allopathicmedicines and insulin injections. However the factor which limits the use of herbalmedicine is their standardization at commercial level.

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Paper 6

Volume 1, Issue 3. December, 2021. www.jbbt.org

BIOINFORMATICS BASED INDICATION OF *BACILLUS LENTUS* NCTC4824EXPRESSINGSIRTUIN-LIKE PROTEIN

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ABSTRACT

Bacillus species as part of probiotics of the so-called "live drugs" and food additives are approved for human and animal consumption. *Bacillus* genus members are commonly employed in industry for large-scale enzyme synthesis.*Bacillus lentus* is aerobic, Gram positive bacteria. *B. lentus* has two industrially significant strains: NCTC 4824 and NBRC 16444. It can be used in a variety of biotechnology sectors, including detergent and food manufacturing. It is utilized in industry to make different enzymes, like proteases and peptidases. Household detergents are the furthermost well-known use of these alkaline proteases. In modern biology and medicine, bioinformatics is critical for data management.Hypothetical proteins of bacteria present in NCBI Protein and UniProt databases are identified and characterized by bioinformatics. In biochemical and physiological pathways, hypothetical proteinsmay play a significant role.In our study, NCBI Protein database was used to download the complete genome of *B. lentus* NCTC4824 in FASTA format. After retrieving hypothetical proteins from the NCTC4824 genome,NCBI

Protein BLAST was performed for sequence similarity search against locally-designed BIPs dataset. The obtained best hithaving highest percent identity, and query coverage, and e-value was found to NAD-dependent protein deacetylase of another specie of *Bacillus*. This enzyme has a significant importance in biotechnology.Sirtuin Sir2 (deacetylase enzyme for NAD+ dependent acetate consumption) influences cell ageing, chromosomal stability, energy metabolism in response to dietary cues, gene silencing, and stress response.The findings have elevated the importance of an industrially significant bacterial strain i.e. *B. lentus* NCTC4824.

Keywords: *Bacillus lentus*, Sirtuin, hypothetical proteins, gene silencing

INTRODUCTION

Species of *Bacillus* as part of probiotics of the so-called "live drugs" and food additives approved for human and animal consumption include only certain types of bacilli *B. subtilis, B. licheniformis, B. coagulans, B. toyoi (cereus), B. clausii (lentus),* and *B. polyfermentans*. The bacteria *B. lentus*are part of the non-pathogenic transit microflora of the intestines of humans and animals, but have their own characteristic difference. *B. lentus* are known to be alkaline tolerant and are known producers of highly alkaline protease. Different strains of *B. lentus* are ATCC 10840, NG121 and NCTC 4824 (Sharma *et al.*, 2006; Xu and Jean, 2003).

B. lentus is aerobic, gram positive bacteria that produce endospores. It is found in soil. Considered non-pathogenic to human. *B. lentus* grow on solid media (e.g. nutrient agar) at 37°C, within 24-48 hours. *B. lentus* also grow on liquid media (e.g. nutrient broth) at 37°C.The complete genome assembly of *B. lentus* NCTC4824 is sequenced. The accession number of bacterium is LS483476.1. Total sequence length is 4,384,366 base pairs (bp.) and total un-gapped length is 4,384,366 bp. Total number of chromosomes and plasmids are 1.

Bacillus genus members are commonly employed in industry for large-scale enzyme synthesis. *B. lentus*is utilised in industry to make a variety of enzymes, including proteases and peptidases (Jorgensen *et al.*, 2000).One of these industrial organisms is the alkalophilic*B. lentus*, which produces BLAP(*B. Lentus* Alkaline Peptidases), a commercial peptidase preparation. Nowadays, one of the furthermost well-known applications of BLAP is in household detergent (Jorgensen *et al.*, 2000).It was observed that BLAP is inhibited by its products in a competitive manner, as a result, it was used to hydrolyze casein in an enzyme membrane reactor (EMR) (Eisele *et al.*, 2013).Proteases are found in many industrial processes and products.The most major bacterial supplier of proteases is *Bacillus*, providing large quantities of proteolytic enzymes that are neutral and alkaline with unique features for example, excellent stability in the presence of severe temperatures, pH, organic solvents, detergents, and oxidising chemicals. As a result, numerous ways for producing *Bacillus* proteases at a low cost have been devised, including optimising fermenter conditions. Protease enzymes are employed in many different applications, such as detergent, food processing, and skin dehairing. From various *Bacillus* species, many commercial proteases have been described, purified, and produced (Contesini *et al.*, 2018).

The use of computation and analysis tools to record and understand biological data is known as bioinformatics.Bioinformatics is necessary for data management in modern biology and

medicine.Software programs for computers,the bioinformatics toolkit, like BLAST and Ensembl, which require internet connectivity. Bioinformatics is becoming more widely used for a variety of other crucial functions, such as analysinggene expression and variation, analysing and predicting structure and function of genes and proteins, as well as gene regulator networks prediction and detection, in addition to genome sequence data processing (Bayat, 2002).

Sequence analysis is a wide field with numerous sub-domains. Sequence alignment can disclose crucial information about the structural and functional areas within a sequence. It is used to investigate the evolutionary course of sequences by finding orthologs and homologs of the sequences. Statistical approaches are used to generate sequence profiles and more precisely identify distantly related sequences. The advancement of sequencing technologies has ushered in the next-generation era, which has allowed for personalised medicine and the finding of haplotypes and quasi-species (Speed, 2003).

The field of molecular biology known as functional genomics explores the roles and interactions of genes (and proteins).The field of functional genomics focuses on the dynamic aspects of genomic data, such astranscription of genes, and translation, gene control, and significant interactions between proteins, rather than static aspects, such as the sequence or the structures of DNA. Characteristics of the genome that are connected to its function, such as polymorphism and mutation (for example, single nucleotide polymorphism (SNP) analysis), in addition to measuring molecular activity, all of this is a part of functional genomics. Transcriptomics (gene expression), proteomics (protein production), and metabolomics(metabolite production) are some of the "-omics" that are included in this category (Mlecnik *et al.*, 2018).

Hypothetic proteins (HPs) are proteins that are anticipated to be expressed using an open reading frame but without any experimental evidence of translation. Only about 2% of the genetic material in the genome proteins are coded, the others are either non-coding or have not yet been detected.(Ijaq *et al.*, 2019). Because these proteins demonstrate weak link to known annotated proteins, they are referred to Putative Conserved Proteins (PCPs) or Hypothetical Proteins (HPs) (Shahbaaz *et al.*, 2013). Despite the fact that they lack functional characterization, they serve a critical role in comprehending biochemical and physiological mechanisms, such as discovering novel structures and functions, markers and pharmaceutical

targets, as well as early identification and advantages for proteomic and genomic studies (Ijaq *et al*., 2019).

Proteins play a role in every biological process that takes place in living organisms. Protein interacts with a wide range of substances. Enzyme, a protein that catalyses chemical reactions, is the most well-known example of protein function. (Ismaya and Wangsa 2011). Microbial enzymes have attracted interest for their broad use in industries and medicine due to their stability, catalytic activity, and ease of manufacturing and optimization.

Enzymes use in a variety of industries (such as food, agriculture, chemicals, and medicine) is rapidly increasing due to their shortened processing time, minimal energy input, cost effectiveness, non-toxic, and environmentally benign features. Chemicals contained in industrial and household trash can be degraded or converted by microbial enzymes (phenolic compounds, nitriles, and amines, etc) (Singh *et al*, 2016).

MATERIALS AND METHODS

Working System

All computational experiments were carried out using Google Chrome version 87.0.4278.0 (Official Build) dev (64-bit) on a 64-bit processor Intel(R) Core(TM) m3-7Y30 CPU @ 1.00GHz 1.61GHz and 8.00 GB RAM on a Windows 10, version 2004 (OS Build 19041.508) operating system.

Literature Mining

In order to carry out my research work, firstly I choose bacterial specie after comprehensive study through research articles, a bacterium named *B. lentus* that is rod shaped and gram-positive. It has two strains NCTC 4824 and NBRC 16444. I selected it because of its interesting features like it is amongst the biggest known bacteria. Currently, it is used as industrial organism to produce a variety of proteins like proteases and peptidases. It has a wide range of uses in biotech sectors, including detergent and food processing. I carried out my research work with *B. lentus* NCTC 4824 strain.

Retrieval of Genomic Datasets

The complete proteome of *B. lentus* NCTC 4824 under the accession number LS483476.1was retrieved from the NCBI Protein Database (https://www.ncbi.nlm.nih.gov/protein/) (Benson

et al., 2016). Hypothetical proteinswere extracted from complete proteome in FASTA format using FaBox tool (https://users-birc.au.dk/~palle/php/fabox/index.php) (Villesen, 2007).

Dataset of Biotechnological Important Proteins

Dataset of biotechnological important proteins (BIPs) was made through an extensive study of literature of industrially significant proteins. The protein sequences were retrieved in FASTA format from NCBI Protein database(Benson *et al*., 2016). Proteins datasetwas compiled along with references, industrial uses, and proteomic sequences.

Sequence Similarity Search

For sequence similarity search perform NCBI Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of two datasets in which Query Sequence is Hypothetical proteins of *B. lentus* and Subject Sequence is BIPs dataset of 100 proteins. Search for the best hits using three values: percent identity (>70%), e-value (near to 0) and query coverage (near to 100%). Also the query hypothetical protein should be of large size as to avoid partial submissions in NCBI Protein database.

Validation Experiments

The obtained best hit from BIPs was searched in the NR database using NCBI Protein BLAST in which Query Sequence is accession number of best hitagainst NCBI NR database. This is done to check the consensus of naming and similarity with other proteins.

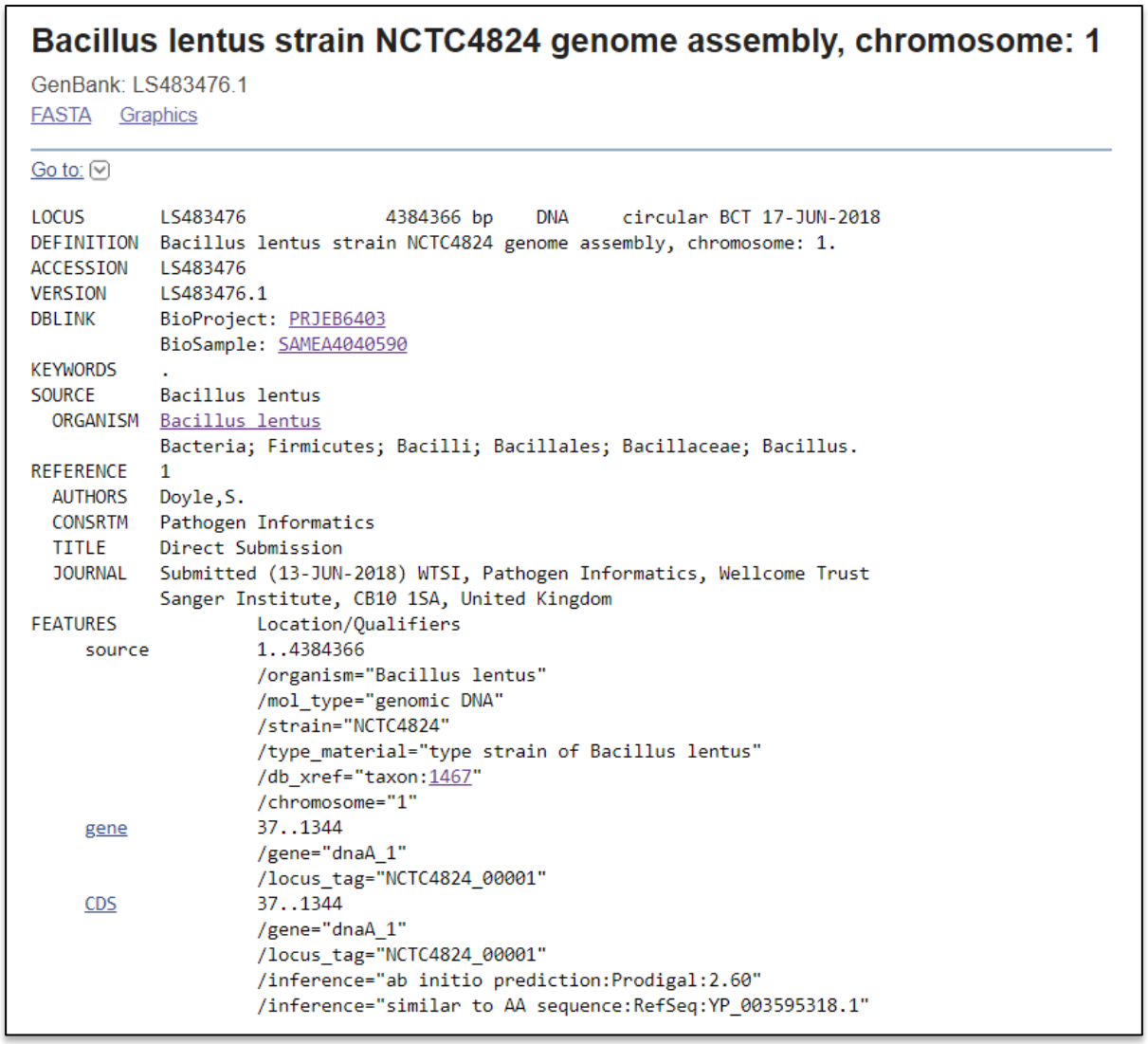
RESULTS AND DISCUSSION

Literature Mining

The genome size of*B. lentus*NCTC 4824 strain is 4,383,366 bp. It was retrieved from NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome) under accession number LS483476.1 (Fig.1) (Kitts *et al*. 2016).

Retrieval of Genomic Dataset

The complete proteome of *B. lentus* NCTC4824 contains 4,072 proteins out of which 483 were hypothetical proteins, which were extracted from complete proteome in FASTA format using FaBox tool (Villesen, 2007).



Bacillus lentus strain NCTC4824 genome assembly, chromosome: 1

GenBank: LS483476.1

FASTA Graphics

Go to:

```
LOCUS       LS483476             4384366 bp    DNA     circular BCT 17-JUN-2018
DEFINITION  Bacillus lentus strain NCTC4824 genome assembly, chromosome: 1.
ACCESSION   LS483476
VERSION     LS483476.1
DBLINK      BioProject: PRJEB6403
            BioSample: SAMEA4040590
KEYWORDS    .
SOURCE      Bacillus lentus
  ORGANISM  Bacillus lentus
            Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1
  AUTHORS   Doyle,S.
  CONSRTM   Pathogen Informatics
  TITLE     Direct Submission
  JOURNAL   Submitted (13-JUN-2018) WTSI, Pathogen Informatics, Wellcome Trust
            Sanger Institute, CB10 1SA, United Kingdom
FEATURES             Location/Qualifiers
     source          1..4384366
                     /organism="Bacillus lentus"
                     /mol_type="genomic DNA"
                     /strain="NCTC4824"
                     /type_material="type strain of Bacillus lentus"
                     /db_xref="taxon:1467"
                     /chromosome="1"
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                     /locus_tag="NCTC4824_00001"
     CDS             37..1344
                     /gene="dnaA_1"
                     /locus_tag="NCTC4824_00001"
                     /inference="ab initio prediction:Prodigal:2.60"
                     /inference="similar to AA sequence:RefSeq:YP_003595318.1"
```

Figure 1: Complete Genome of *B. lentus* NCTC4824

Sequence Similarity Search

For sequence similarity search performed NCBI Protein BLAST of two datasets in which Query Sequence is Hypothetical proteins of *B. lentus*NCTC4824and Subject Sequence is BIPs dataset of 100 proteins. The obtained best hit is NAD-dependent protein deacetylase of *B.onubensis*. The NAD-dependent protein deacetylase of *B. onubensis* have percent identity is 85.37%, e-value is 0.0 and query coverage is 100% (Fig. 2). Results with good score were checked for pairwise alignments using dot representation for identities (Fig. 3).

Hypothetical Protein

- Query Accession No: LS483476.1
- Query Name: Hypothetical Protein

- Query Organism: *B. lentus* strain NCTC4824

>gi|1054047867|ref|WP_066139086.1| hypothetical protein [Bacillus lentus]
MLTQQYQNIIGTILEKIEEADAIVVGGAAGMSAAGGYNWYLDDENFREHFNVFAEKYGIDSIFGGFYYKF
RTEEERWAYLATLINFVTEVPIGQPYKDLYEIIKDKNYYILTTNQDTQFLQVFPEEKVSAIQGNWTYLQC
SGPCHDGIYPYAEQAKELCAHIDGTKIPSDMVPKCPECGGPMELWVRSFVFLEGEKYRDEHHKYRTFLLE
NQNKKILFLELGVGQMTPMFIKEPFWNMTYTWPDAYYITINPKDALLPQELKNKGLAVHEDIATVLSNVL
TEQQKERKQGAV

Best Known Hit in BLASTp Results

- Subject Accession No: WP_099353704.1
- Subject GI: 1272174496
- Subject Name: NAD-dependent protein deacetylase
- Subject Organism: *B.onubensis*
- Research Article(s) for Biotech Significance (PubMed IDs): PMID: 23226010

>WP_099353704.1 NAD-dependent protein deacetylase [Bacillus onubensis]
MLPHQYQNNIDTILQKIKEADAIVVGGASGMSAAAGYNWYRDDENFRKYFNAFAVEYGIDSIFGGFYYKF
RTEEERWAYLATLINFVADVPIGQPYKDLYEILKDKNYYILTTNQDTQFLQVFPEEKVSAIQGNWTYLQC
SGPCHDGIYPYAEQAKELCNHIEGTKIPSSMVPTCPECGGPMELWVRSFVFLEGSKYHDEHRKYREFLLE
NHNKKVLFLELGVGRMTPMFIQEPFWNMTYSWPDAYYITINPKDALLPQQLKDKNKGIAIHEDIAPVLRD
VLTKQQTEGEKGAV

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
hypothetical protein [Bacillus lentus]	610	610	100%	0.0	100.00%	WP_066139086.1
NAD-dependent protein deacetylase [Bacillus onubensis]	531	531	100%	0.0	85.37%	WP_099353704.1
NAD-dependent protein deacetylase [Lysinibacillus sp. OL1]	507	507	99%	5e-180	81.23%	WP_131520440.1
hypothetical protein [Bacillus sp. MRMR6]	496	496	100%	1e-175	84.69%	WP_075688611.1

Figure 2: BLAST results of BIPs

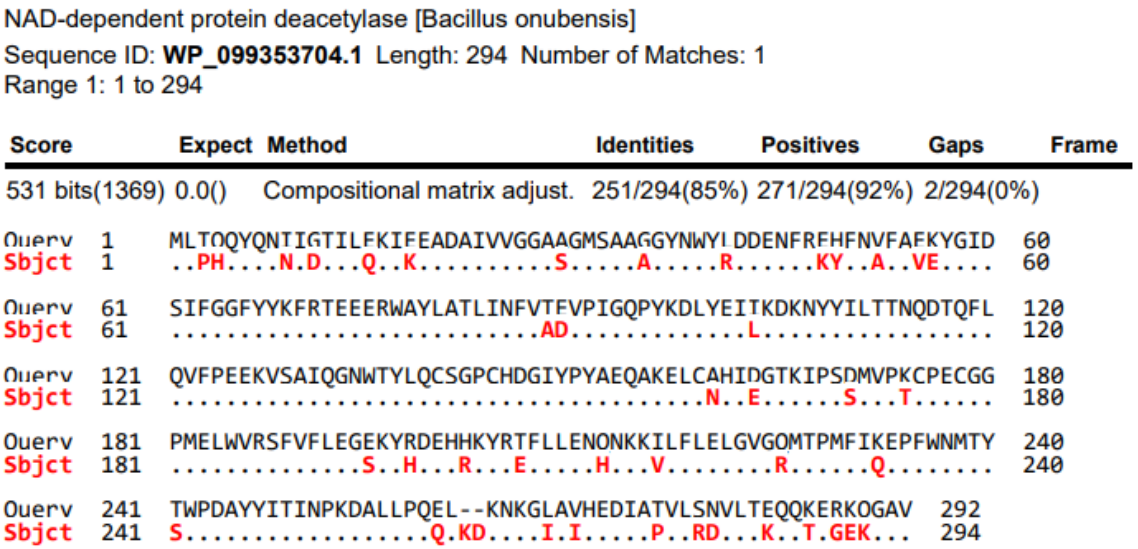
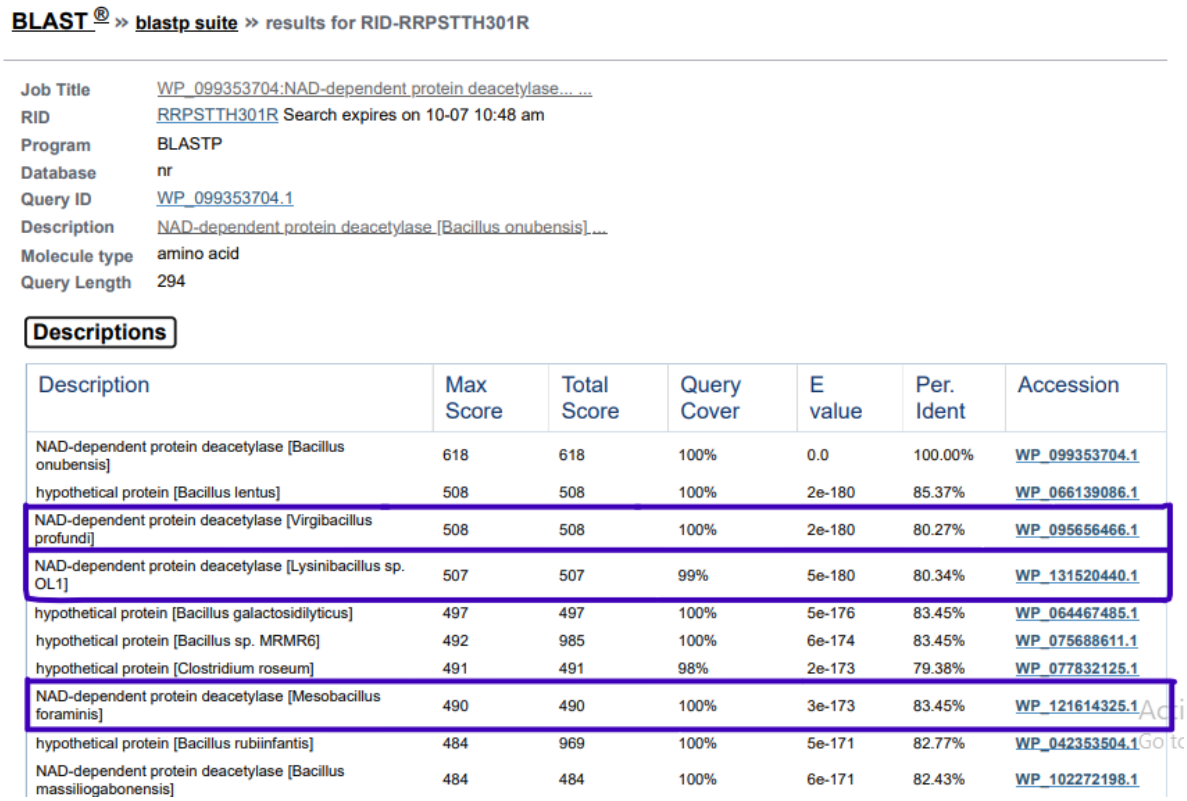


Figure 3: BLAST result in pairwise alignment format

Validation Experiments

The NAD-dependent protein deacetylase was searched in NR database using NCBI Protein BLAST.It was observed that NAD-dependent protein deacetylase [*Virgibacilusprofundi*], NAD-dependent protein deacetylase [*Lysinibacillus sp. OL1*] and NAD-dependent protein deacetylase [*Mesobacillusforaminis*] has the close homologs with same name (Fig. 4). So, the hypothetical protein WP_066139086.1 of *B. Lentus* is predicted to be having the function of NAD-dependent protein deacetylase. This prediction came from having the sequence similarity of 85%from *B. Onubensis.*



BLAST® » **blastp suite** » results for RID-RRPSTTH301R

Job Title	WP_099353704:NAD-dependent protein deacetylase... ...
RID	RRPSTTH301R Search expires on 10-07 10:48 am
Program	BLASTP
Database	nr
Query ID	WP_099353704.1
Description	NAD-dependent protein deacetylase [Bacillus onubensis] ...
Molecule type	amino acid
Query Length	294

Descriptions

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
NAD-dependent protein deacetylase [Bacillus onubensis]	618	618	100%	0.0	100.00%	WP_099353704.1
hypothetical protein [Bacillus lentus]	508	508	100%	2e-180	85.37%	WP_066139086.1
NAD-dependent protein deacetylase [Virgibacillus profundi]	508	508	100%	2e-180	80.27%	WP_095656466.1
NAD-dependent protein deacetylase [Lysinibacillus sp. OL1]	507	507	99%	5e-180	80.34%	WP_131520440.1
hypothetical protein [Bacillus galactosidilyticus]	497	497	100%	5e-176	83.45%	WP_064467485.1
hypothetical protein [Bacillus sp. MRMR6]	492	985	100%	6e-174	83.45%	WP_075688611.1
hypothetical protein [Clostridium roseum]	491	491	98%	2e-173	79.38%	WP_077832125.1
NAD-dependent protein deacetylase [Mesobacillus foraminis]	490	490	100%	3e-173	83.45%	WP_121614325.1
hypothetical protein [Bacillus rubiinfantis]	484	969	100%	5e-171	82.77%	WP_042353504.1
NAD-dependent protein deacetylase [Bacillus massiliogabonensis]	484	484	100%	6e-171	82.43%	WP_102272198.1

Figure 4: BLAST results of NAD-dependent protein deacetylase

Significance of NAD-dependent protein deacetylase

A transcriptional unit with two genes including Sirtuin Sir2 (deacetylase enzyme for NAD+-dependent acetate consumption) and a universal stress protein genes. The proposed transcriptional units for stress-sensitive inorganic sulphate absorption and acetate utilization could explain biological mechanisms that restrict *Bacillus* species survival by sulphate and acetate. Given the relevance of sirtuins in mammalian physiology, more research on the USP-Sir2 transcriptional unit of *B. megaterium* should help explain mammalian acetate metabolism in glucose-limiting circumstances like caloric restriction.

B. megaterium soil inhibiting bacteria has six USP genes, one of which is on a plasmid. Another *Bacillus* species that lives in the soil, *B. subtilis*, has two USP genes. A transcriptional unit consisting of sirtuin (Sir2) gene and a USP gene discovered solely in *B. megaterium* was of particular interest. Deacetylase enzyme Sir2removes acetyl groups from lysine amino acids in proteins, found in bacteria and higher eukaryotes,using the nicotinamide adenine dinucleotide ion (NAD+). Sirtuin (SrtN), a NAD+-dependent deacetylase, and AcuC, a non-NAD+-dependent deacetylase, are necessary in *B. subtilis* to

keep the enzyme acetyl coenzyme A (Ac-CoA) synthetase (AcsA) active (i.e. deacetylated), so that the cell can grow at low acetate concentrations.

Sir2 proteins influence cell ageing, chromosomal stability, and energy consumption in response to nutritional cues, gene silencing, and stress response. The sirtuin gene family has been studied intensively as a potential therapeutic target for age related illnesses, obesity, cardiovascular disease, and cancer(Williams *et al.*, 2012).

CONCLUSIONS

The hypothetical protein WP_066139086.1 of *B. lentus* is predicted to be having the function of NAD-dependent protein deacetylase which is also referred as Sirtuins. This prediction came from having the amino acid sequence identity of 85% and similarity of 92% from *B. onubensis.*Only 23 out of 294 amino acids are found to be different. Further investigation is needed to inquire this uniqueness of WP_066139086.1.

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