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Genetic Manipulation of *Bacillus safensis* Isolated from Soil for Enhanced Production of Alpha Amylase

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Abstract
Bacillus species capable of extracellular amylase production were isolated from soil samples using rice bran and wheat bran as substrates. Using starch hydrolysis plate assay test the bacterial isolates were screened and were identified using maximum zone of clearance and further characterized according to standard methods and molecular analysis.The isolates were identified as Bacillus safensis. They were used to produce α-amylase. Bacillus safensis had maximum enzyme activity of 40.86 IU/mL and 43.87 IU/mL on rice and wheat bran respectively over the same period. Enhancement of enzyme production was done using two techniques; induction of mutation by UV irradiation and recombinant DNA technology. Six mutants each were randomly selected from each UV treatment time range. The mutant BSM_4 were higher α-amylase producers than parental strain. PCR amplification of the specific amylase gene (Amy) for the selected bacterial isolates was performed using specific primers AP-Amy F and AP-Amy R. The bands observed under UV-transilluminator confirmed the presence of amylase gene in the two bacterial isolates. The purified PCR product were cloned into the pBlueScriptSK(-) vector and transformed into Escherichia coli DH5α.to produce a cloned strain for both bacterial isolates. These were then used for α-amylase production. Recombinant strain, ECR_5 had maximum enzyme activity of 320.0IU/mL on wheat bran and 295.0 IU/mL on rice bran at 72hours, pH 7.0 at 40^oC while ECR_4 had maximum enzyme activity of 198.67IU/mL and 185.82IU/mL on wheat and rice bran over the same period. Results identified proteins with molecular weight within the range for alpha amylases (61.66-66.2kDa). With further purification in the food, pharmaceutical and clinical industries, these alpha amylases produced could be of great potential use to them.

INTRODUCTION

Biological substances or biological macromolecules also known as Enzymes are produced by living organisms which acts as catalysts to bring about a specific biochemical reaction. These are like the chemical catalysts in a chemical re action which help to speed up the biological/biochemical reactions inside as well as outside the cell. They are generally known as "biocatalyst" (Neelam *et al*., 2013). These days, enzymes are employed in many different areas such as food, feed, textile, detergent, laundry, tanning, pharmaceutical, cosmetics and fine chemical industries. Industrial applications account for over 80 percent of the global market of

enzymes and at least 50 percent of the enzymes marketed today are obtained from genetically modified organisms, employing genetic and protein engineering. Food enzymes are the most widely used and still represent the major share in enzyme market (Tiwari*et al*., 2015). The most important enzymes are the amylases which are of greater significance in the field of biotechnology. They can be obtained from several sources such as plants, animals and microorganisms. Amylases are used instead of chemical hydrolysis of starch because amylases are bio-catalysts, which increase the rate of any reaction in starch processing industries (Shalinimol, 2016). With the emergence of biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. These increased uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes (Aiyer, 2004). The major advantages of using microorganisms for production of amylases are their ability to produce in bulk and the ease at which it can be processed for desired products (Lonsane, 2009). The use of cassava-based and other agro wastes for the production of amylase enzyme have been explored by a number of researchers. The utilization of wastes as substrates for amylase production can be a solution to reduce the production costs. Numerous researchers have been carried out on the production of alpha amylase but little attention has been given to the enhanced production of the enzyme (Prasad, 2014). This has necessitated the search for possible ways to enhance the production of the enzyme locally using agro wastes to make it readily available.

MATERIAL AND METHODS

Isolation of Bacteria species from Soil

The soil sample used for this research was collected from Microbiology Laboratory 2 of Federal University of Technology, Minna, Niger State, Nigeria using a soil auger. The soil sample was stored at 4oC until use within 72 hours. Isolation of Bacillus species Serial dilutions of the soil samples were carried out by taking 1gram of soil sample into 10mL distilled water in a test tube. This was shaken properly and 1mL was transferred to another test tube containing 10mL distilled water until the 6th dilution. One milliliter (1mL) of the 6 th dilution was introduced into sterile Petri dishes and 25 mL of molten Nutrient agar was added. The Petri dishes were gently swirled to enhance homogeneity and then incubated in a pre-set aerobic incubator at 37oC for 24 hours. At the end of the incubation, visible colonies formed in the petri dishes were observed. The bacterials cultures were subjected to macroscopic examination for colonial morphology as described by Cheesbrough (2006). Pure cultures were obtained by repeated subculturing on media used for primary isolation and preserved on nutrient agar slants for further use.

Screening of Bacterial Isolates for Amylase Production

The isolates obtained were screened for ability to produce amylase by growing on starch agar (for amylase activity) at 37oC for 24 hours. Gram's iodine was added to microbial growth to screen for amylase production. A positive result was indicated by a yellow colour or a clearing, while a blue-black colour was observed in all areas where starch had 44 not been degraded (Oyeleke et al., 2010). The diameter of the clear zone was taken and this was used to select isolates with high potential for starch utilization. The potential amylase producing bacteria were characterized based on their Gram reaction.

Characterization and Identification of Amylase Producing Bacteria
The potential amylase producing bacteria were characterized based on their biochemical testsand molecular characterization.

Genetic Improvement of the Enzyme-Producing Potential of the Microbial Isolates
The bacterial isolates with proven potential for amylase production were subjected to relevant techniques aimed at improving their enzyme- producing abilities. The methods that were employed included gene cloning, and physically induced mutation.

Gene cloning
The purified PCR product were cloned into thepBlueScript SK (-) vector and transformed into Escherichia coli DH5α. Plasmid extraction was carried out with a 51 plasmid extraction kit. Sequencing of the 16S rRNA gene was carried out using Macrogen. The sequencing result was compared with published 16S rRNA sequences using NCBI Blast search (Frank et al., 2008).

Physical induced mutation (ultra-violet irradiation)
The bacterial isolates were subjected to UV- irradiation treatment using the method described by Okafor (2007). Loop full of parent Bacillus species were taken into a 20 mL Nutrient broth medium in a 100 mL conical flask and incubated for 24 hours at 37oC. The culture was serially diluted up to 10 -6 dilution and 0.5 mL was plated on Nutrient agar medium to find out the colony forming unit (cfu/mL) as control. 5mL of Nutrient broth culture was subjected to UV rays from a UV lamp of 15 watt and wavelength, 254.5 nm for various time intervals of 5, 10,15,20,25 and 30 minutes respectively. A distance of 20 cm was maintained between lamp and the culture. The exposed culture was mixed well separately and immediately diluted to 10 -5. The diluted culture samples were plated on the Nutrient agar plates and incubated for 24 hours at 37oC. After incubation, the plates were observed and the colonies were counted to determine survival of the target strain. Sub culturing was done and mutants were selected based on resistance to penicillin antibiotic and higher clearance zone than parental strain on starch agar. Selected mutants were then used for the production of the amylase.

Enhanced Production of Amylase using Solid State Fermentation
Preparation of inoculum

A volume of 20 mL of Nutrient broth contained in a 100 mL flask was inoculated with a loop full of colonies of the transformants or genetically improved strains of the organisms 52 from a 24 hour old slant and kept at 37oC in a rotary shaker (120 rpm). After 12 hours of incubation, 1 mL of this culture was used as inoculum (Sadin*et al*., 2009).

Solid state fermentation

Dry substrate (5 g) which passed through the sieve of 1500 micrometer was placed into 100 mL flaks. To adjust moisture levels 0.1M TrisHCl buffer (pH 7.0) was added. After sterilizing by autoclaving at 121oC for 15minutes and cooling to room temperature, the flask was inoculated with 2 mL of the inoculum and incubated at 37oC and 120 rpm for 12, 24, 48 and 72 hours respectively using a rotary shaker and an incubator (Sadin*et al*.,
2009).

Extraction of the enzyme

In order to extract the enzyme, the fermented mixture was thoroughly mixed with 0.1 M TrisHCl buffer (pH 7.0). Then contents were mixed in a shaker at 120 rpm. The slurry 53 was squeezed through a muslin cloth. The extract was centrifuged at 10000 rpm for 10 minutes and then the clear supernatant was used as crude enzyme (Sadin*et al*., 2009).

Test for total protein in the crude enzyme

A total protein test kit (R and ox Laboratories Ltd, CrumLin, UK), was used for the
estimation of total protein as described by Weichselbaum (1946).The content of the test kit are R1a (10 mL of bottled R1 (biuret reagent) in 40 mL distilled water (dH2O), R21 (10 mL of bottled R2 in 40 mL distilled water), and protein standard. The reaction mixture contains 1.0 mL of R1a and 0.02 mL of crude enzyme for sample's test tubes, while blank and standard test tubes contain 1.0 mL of R1a and 0.02 mL of dH2O or 0.02
mL of standard respectively. Each test tube was mixed and incubated at 25oC for 30 minutes. The absorbance of the standard, A standard and sample A sample were measured against reagent blank at 546 nm and protein concentration was calculated using

Total protein concentration (mg/dl) = Asample x Standard concentration Astandard
(Where standard protein concentration = 5.85 mg/mL)

Assay of enzyme activity using dinitrosalicyclic acid method

A mixture of 1 mL of crude enzyme and 1 percent soluble starch dissolved in 0.1 M phosphate buffer (pH 7), was incubated at 55oC for 15 minues. The reaction was stopped by adding 1mL of 3, 5 Dinitrosalicyclic acid, and then followed by boiling for 10 minutes. The final volume was made up to 12mL with distilled water and the reducing sugar released was measured using a spectrophotometer at 540 nm. One unit of amylase activity was defined as the amount of enzyme that released 1 micromole glucose 54 equivalent per minute under the assay condition. Reducing sugar (glucose) concentration
was determined from a standard curve under same condition using glucose (Senthilkumar et al., 2012). Enzyme was calculated using Equation

Enzyme activity Concentration of glucose produced Volume of enzyme
solution x incubation time

Partial purification of crude enzyme

To purify the enzyme extract, a solution of 50% (w/v) Ammonium sulphate was addedto the clarified supernatant and then centrifuged for 10,000 rpm for 10 minutes. The pellet was suspended in 0.1M Na2HPO2 (pH 6.0) to enhance the stability of the enzyme.
Further purification of the enzyme was carried out by adding ammonium sulfate to the mixture to precipitate the enzyme (protein). The proteins were harvested by centrifugation (15,000 rpm for 30 min at 40C). The pellets were dissolved in 0.1 phosphate buffer solution (Senthilkumar*et al*., 2012).

Characterization of the enzyme
Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect the partially purified enzyme (Khedr*et al*., 2017). The samples were mixed with loading dye. The samples and markers were loaded on the respective wells and were run for 1 hour. The gel was observed for determination of molecular weight.

RESULTS

Identification of bacterial isolates

Ten bacterial isolates were obtained from the soil sample, and were identified as *Bacillus* sp. (80%),*Staphylococcu*s sp. (10%) and *Pseudomonas* sp. (10%). The biochemical characteristics of the isolates are shown in Table 1. The *Bacillus* species were Gram positive rods, capable of fermenting glucose, lactose and sucrose.

Table 1: Biochemical characteristics and identification of bacteria isolated from soil sample

Isolate Code	Grxn	Sh	Cat	Oxi	Cit	Vp	Mot	Ind	MR	SF	Glu	Lac	Suc	Fru	Suspected Organism
B_1	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
B_2	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
C_3	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
C_4	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
T_5	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
C_6	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
C_7	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
C_8	+	Rod	+	+	+	+	+	-	-	+	AG	AG	AG	AG	*Bacillus* sp
C_9	+	Cocci	+	-	+	+	+	-	+	-	ND	ND	ND	ND	*Staphylococcus*sp

C_{10}	-	Rod	+	+	+	+	+	-	-	-	AG	AG	AG	AG	*Pseudomonas* sp

Key Grmrxn: Gram reaction, shp: shape, mot: motility, VP: Vogesprokauer, cat: catalase, Glu: Glucose, oxi: oxidase, SF: Spore formation, Lac: Lactose, Suc: sucrose Cit: citrate ND: Not determined, AG: Acid and Gas

Amylase activity of *Bacillus* species isolated from soil sample

Amylase activities of the isolates were evident by starch clearance zone of diameter ranging from 3.67±1.23 mm to 10.0±0.00 mm. Out of the eight isolates obtained, isolates T5 had the highest clearance zone of 10.0±0.00 mm followed by T_4 with 7.67±1.83 mm and B_2 and C_3 with 6.68mm each (Table 2). C_8 had the least diameter zone of clearing (2.67 mm).

Table 2: Screen test for Amylase activity of *Bacillus* species isolated from soil sample

Isolatecode	Zone of clearance on starch agar (mm)
B1	$3.67±1.23^{ab}$
B2	$6.68±2.34^{c}$
C3	$6.68±2.04^{c}$
T4	$7.67±1.83^{d}$
T5	$10.0±0.00^{e}$
C6	$4.25±1.34^{b}$
C7	$5.75±1.34^{bc}$
C8	$2.67±1.34^{a}$

Values are means ± SEM of triplicate determinations. Mean values with the same superscript on the same column are not significantly different from each otherby Duncan's multiple range test ($P > 0.05$).

Molecular identification of two bacterial isolates with high potential to produce amylase

Plate I shows the result of sequenced amplicons of bacterial species isolated from soil sample as the resulting alignment of the concatenated nucleotide (from5' → 3' and 3' → 5') withknown sequences previously blasted on International Institute of Tropical Agriculture (IITA), Ibadan data base. The gel documented images of the isolated bacterial DNA after electrophoresis shows lane Mk which represents molecular marker (ladder), lane T_4 and lane T_5 represents DNA extracted from isolate T_4 and T_5.

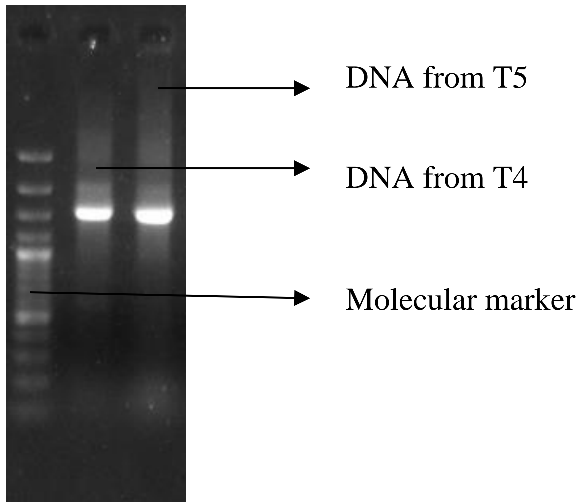

Plate I: Amplified image of the *Bacillus* isolates from soil sample

T_4: *Bacillus safensis* Strain BAB-6487

Table 3: Screening of fouragro wastes as substrates for production of Alpha amylase by *Bacillus safensis* and *Bacillus megaterium*

Agro wastes	Total protein(mg/mL) *Bacillus safensis* (T4)	Enzyme activity (IU/mL)
Rice bran	14.25±0.56 [b]	40.86±1.35 [b]
Wheat bran	15.96±0.45 [b]	43.87±0.95 [b]
Melon husk	8.17±0.54[a]	25.80±0.57 [a]
Corn cob	10.83±0.24[ab]	20.00±0.50 [a]

Values are means ± SEM of duplicate determinations. Mean values with the same superscript on the same column are not significantly different from each other ($P > 0.05$).

Table4: Alpha Amylase Production by *Bacillus safensis* and *Bacillusmegaterium using* wheat bran and rice bran

Bacterial isolates	Incubation period (hours)	Enzyme activity (IU/mL)	
		Rice bran	Wheat bran
	12	5.08±0.56[a]	3.0±0.10[a]
	24	14.89±0.00[b]	11.00±0.20[c]
Bacillus safensis	48	29.90±0.01[c]	23.00±0.20[d]
	72	40.86±0.10[e]	43.87±0.79[e]
	96	34.73±0.02[d]	10.20±0.10[b]
	12	22.0± 0.10[a]	7.00±0.00[a]
	24	41.0±0.02[b]	45.00±0.20[b]

Values are means ± SEM of duplicate determinations. Mean values with the same superscript on the same column are not significantly different from each other ($P > 0.05$).

Identification of mutants

The differences observed between the wild and mutant strains are represented on Table 5.

Table 5: Mutant and Wild Strains Reaction

Test	Wild strains	BSM
Gram reaction/ shape	+/ rods	+/ rods
Starch hydrolysis	+	+
Catalase	+	+
Citrate	+	-
Oxidase	+	+
Indole	-	-
Glucose	+	+
Lactose	+	+
Motility	+	+

MR/VP	-/-	-/-

+: Positive, -: Negative, MR: Methyl red, VP: VogesProskauer, BMM:*Bacillusmegaterium*Mutant, BSM: *Bacillussafensis* Mutant

Table 6:Selection and screening of mutant strains of *Bacillus megaterium* and *Bacillus safensis*

	UV exposure time (minutes)	Mutant designation	Clearance zone (mm)
	5	BSM_1	12.5 ± 1.40^e
	10	BSM_2	9.2 ± 0.10^c
Bacillus safensis	15	BSM_3	13.5 ± 1.00^f
	20	BSM_4	15.2 ± 0.20^g
	25	BSM_5	10.5 ± 0.10^d
	30	BSM_6	5.3 ± 0.10^a
Wild strain			7.6 ± 1.80^b

BSM- *Bacillus safensis* mutant, BMM- *Bacillus megaterium* mutant
Values are means ± SEM of duplicate determinations. Mean values with the same superscript on the same column are not significantly different from each other ($P > 0.05$).

Gene cloning of *Bacillus safensis* using the vector pBlueScipt SK (-) and *Escherichia coli* DH5α as host

The Alpha amylase gene identified from both bacterial isolates were inserted into *Escherichia coli* DH5α. The transformed and the untransformed *Escherichia coli* were identified by growing the on MaCconkey agar incorporated with ampicillin. Results showed that the untransformed *Escherichia coli* cells did not grow in the presence of the antibiotic (ampicillin) while the transformed *Escherichiacoli* cells grew.

Molecular identification of specific amylase gene in the bacterial isolates

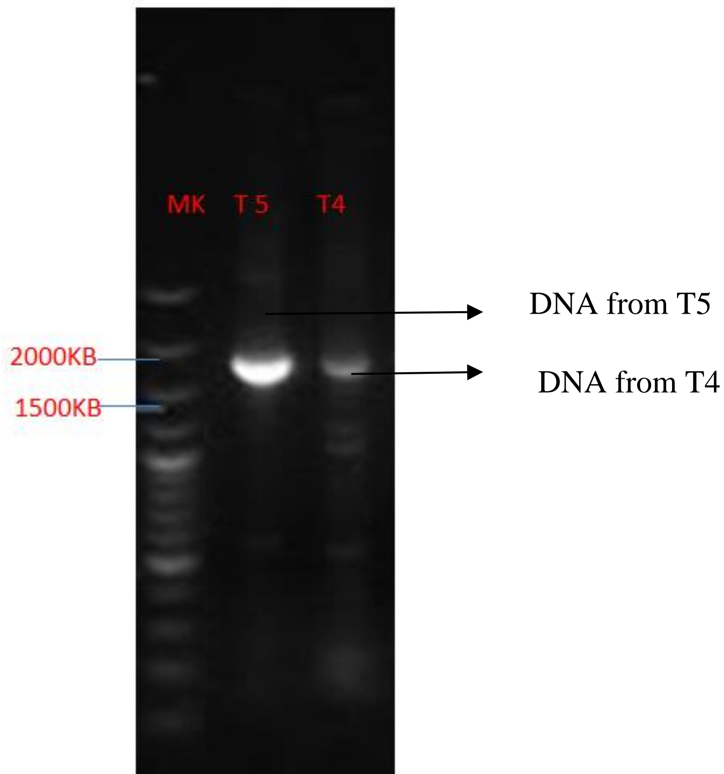

Plate II: Amplification of the amylase gene using primers AP-AmyF and AP-Amy R
Key: MK: DNA Ladder, T_4:*Bacillus safensis*
Alpha amylase production by recombinant and mutagenic *Bacillusafensis*

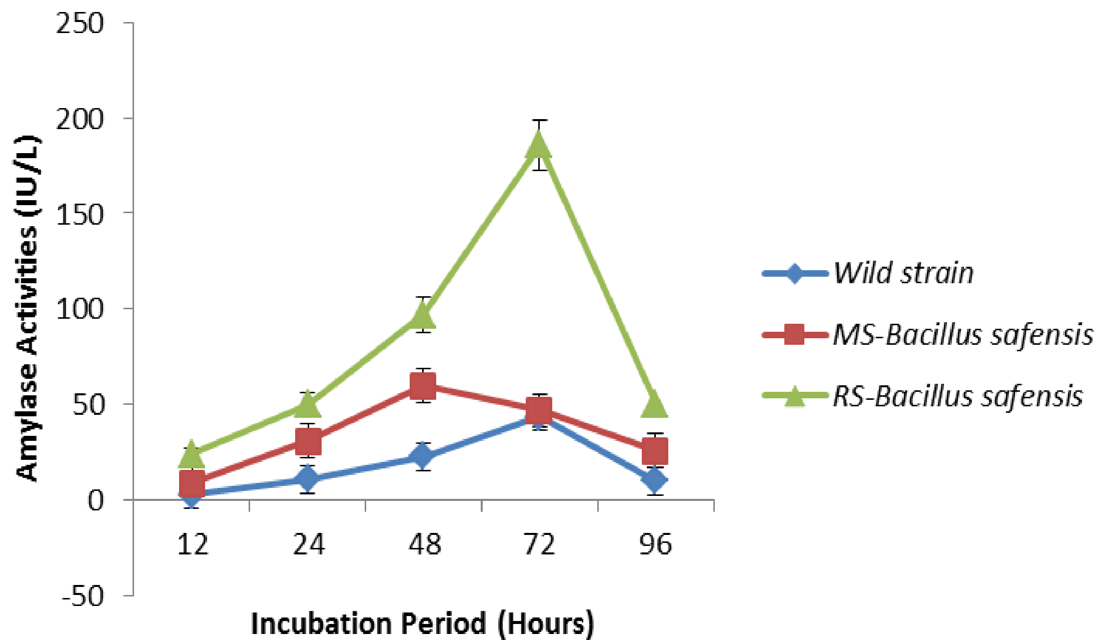

Figure 2: Comparing Alpha Amylase Production by Wild, Recombinant andMutagenic strains of *Bacillus safensis*using wheat bran

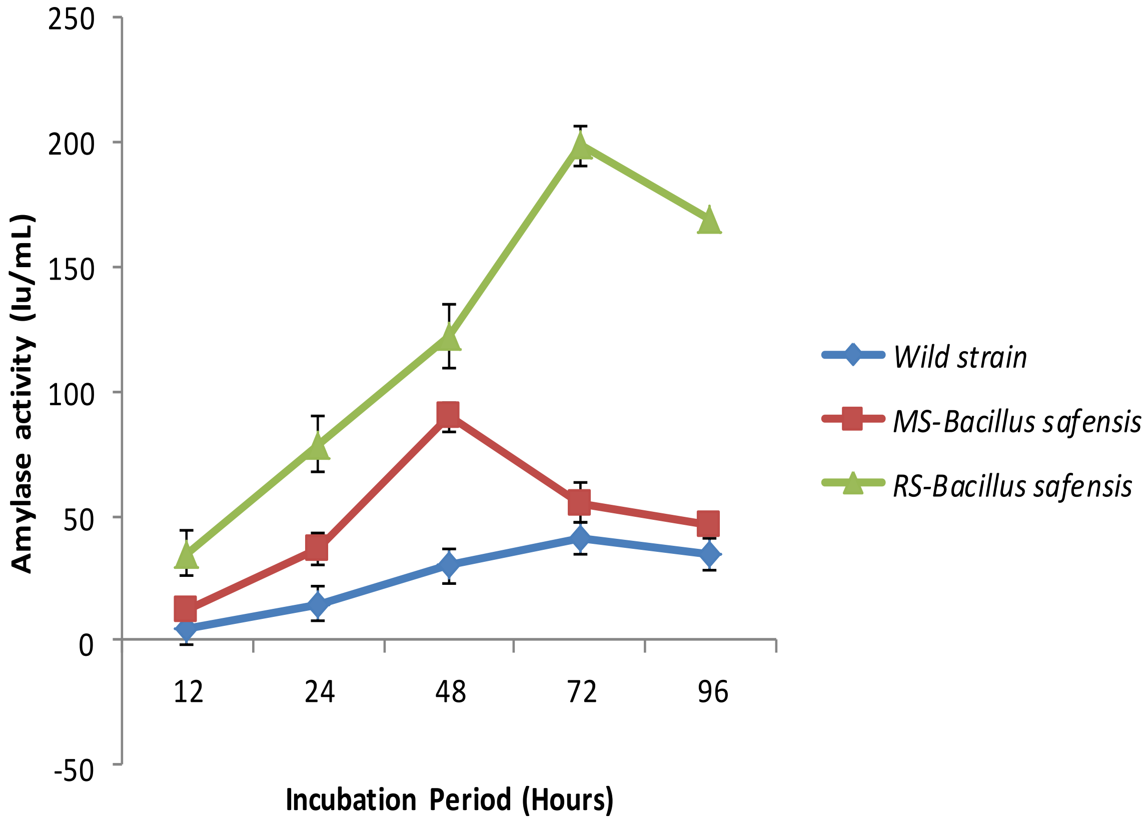

Figure 3:

Comparing Alpha Amylase Production by Wild, Recombinant and Mutagenic strains of *Bacillussafensis*using rice bran

Characterization of partially purified enzyme by SDS PAGE

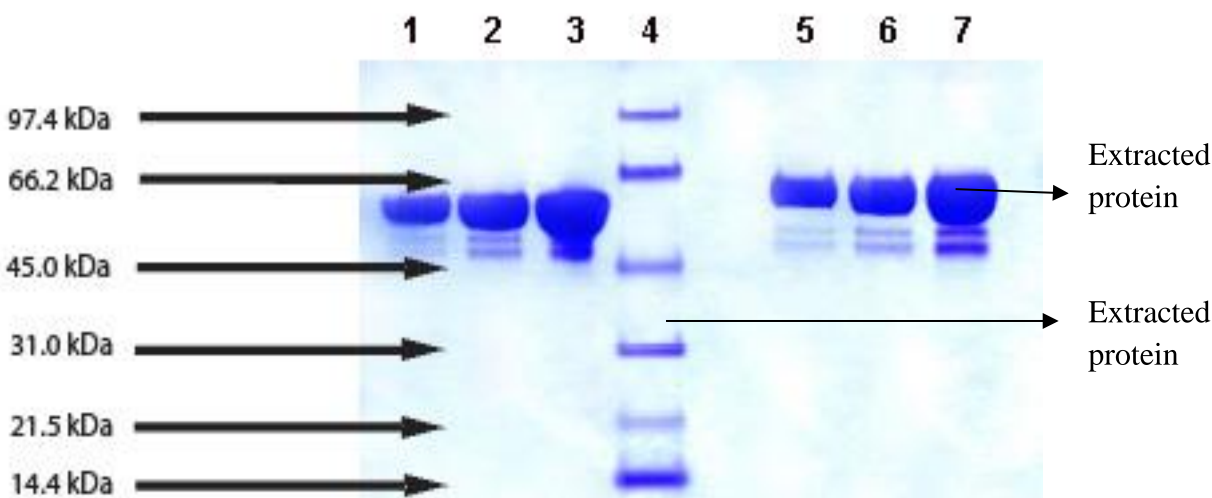

PLATE III: SDS PAGE for total proteins extracted from wild, mutants and recombinant strains Key: Lane 1: BSM_4, Lane 2:ECR_4, Lane 3: T_4, Lane 4: Marker, Lane 5: BMM_1, Lane 6: ECR_5, Lane 7: T_5

DISCUSSION

The ability of eight *Bacillus* species to hydrolyze starch with high zone of clearance on starch agar medium suggests their potential to produce amylase (Table 2). Out of the 8 isolates obtained, T4 (*Bacillus safensis)* had the highest clearance of 7.67±1.83 mm within 24hours meaning that they had strong ability to hydrolyze starch, since the larger the diameter the higher the amylase activity (Rajshree and Rajini, 2011). Similarly, Prasad (2014) investigated the potential of twelve bacteria species to hydrolyze starch out of which five (41.7%) were high starch hydrolyser and potential amylase producers. Four different substrates (rice bran, wheat bran, melon husk and corn cob) were screened for alpha amylase production, Table 3. Using *Bacillus safensis*, the highest total proteins contents (14.25±0.56 and 15.96±0.45) were observed from rice bran and wheat bran. Melon husk and corn cob gave total proteins contents of 8.17±0.54 and 10.83±0.24 respectively. Similarly the highest α- amylase enzyme yield (40.86±1.35 and 43.87±0.95 IU/mL) was observed from rice bran and wheat bran. However, melon husk and corn cob gave α- amylase enzyme yield of 25.80±0.57 and 20.00±0.50 respectively. Irfan*et al*. (2011) reported wheat bran as best substrate among other substrates such as rice bran and cottonseed meal. In the present study wheat bran and rice bran gave higher amylase production in comparison to melon husk and corn cob. UV mutagenesis was used in this study to enhance the production of alpha amylase. Table 4. Survival ratio was decreased as the time of UV exposure increased. It recorded 100% without UV exposure for both *Bacillus* species; six mutants each were selected randomly from the different exposure time. The BSM_4 were higher producers than wild strain with clear zone recorded as 22 mm (220%), and 15.2 mm (200%) respectively while wild strain had clear zone diameter 7.6 mm (100%) for *Bacillus safensis*. Many researchers have employed random mutagenesis for alpha amylase production by exposing the cultures to UV or chemicals (Markkanen and Enari, 1972; Haq*et al*., 2010a; Haq*et al*., 2010b) and results showed high yield of enzyme production In this study, the selected mutant (BSM_4) were used for the production of alpha amylase at various incubation periods of 12, 24, 48, 72 and 96 hours. It was observed that mutant BSM_4 had maximum

enzyme activity of 59.9 IU/mL at 48hours on wheat bran and 89.76 IU/mL at 48hours on rice bran. Production of amylase by mutant BSM_4 was found to be 2.6 times (61.6% increase) that of the wild strain on wheat bran and 3 times (66.7% increase) that of wild strain using rice bran. This also shows a high yield of enzyme production than the wild strain. Khedr*et al*. (2017) also reported high yield by UV mutant strains greater than the wild strain. Alpha amylase activity of mutants was more (Suntornsukand Hang,2008) as compared to the wild strain. The study showed that the enzymatic activity of mutant strains irradiated with UV radiation was different at different duration of radiation exposure. There was a maximum enhancement in amylase activity at 60 minutes exposure. Total enzymatic activity of the crude enzyme of wild strain was found to be 17.5 UmL/1 whereas in the mutant strain the enzymatic activity of the crude enzyme was 303 UmL/1 which was 17 times higher than parent strain. The differences in enzyme activities may be due to the different species of bacteria used and effect of varying UV exposure time.In this present study, the gene encoding amylase in the selected bacterial isolates (T_5 and T_4) were clone through PCR and expressed in *E. coli* using a pBlueScript SK(-) vector. The amylolytic activity of the recombinant (ECR_5 and ECR_4) and wild strains (T_4 and T_5) varied considerably among the two bacterial species. The results showed a high production of amylase by ECR_5 with enzyme activity of 320 IU/mL on wheat bran and 295 IU/mL on rice bran at 72 hours, pH 7.0 and 40°C . This was found to be three times higher than that produced by the wild strain. Similarly, ECR_4 recorded 185.82 IU/mL using wheat bran and 198.67 IU/mL rice bran as substrates respectively. This was found to be four times higher than that produced by the wild strain. Alpha amylase gene (amyBS-1) from *Bacillus subtilis* strain ASO1a was cloned and expressed in *Escherichia coli*. The recombinant enzyme produced was increased seven times that of the wild strain (Sameh, 2015). Characterization of α-amylase produced from wild strain, mutated and recombinant strains of *Bacillus safensis* by SDS-PAGE was analysed. The results showed that the α-amylase from *Bacillus safensis* was detected based on their molecular weights. Results identified proteins with molecular weight within the range for alpha amylases (61.66-66.2kDa). The single band protein identified for both bacterial species indicates a similar origin of a protein with a single polypeptide. Molecular weights of α-amylases are usually between 50 and 66.2kDa (Gupta *et al*., 2003).

CONCLUSION

The present study showed that The bacterial species identified as Bacillus safensis BAB-6487 were identified as producers of alpha amylase. Bacillus safensis having maximum enzyme activity at 72 hours. Enzyme activity of 43.87 IU/mL on wheat bran and 40.86IU/mL on rice bran was recorded. Their productivity was further enhanced by UV mutagenesis and recombinant DNA technology. The mutant (BSM4) recorded 68.8% increase than the wild strains. The recombinant strains had a much higher yield than the wild strains. Characterization of α-amylase produced from wild strain, mutated and recombinant strains of *Bacillus safensis* by SDS-PAGE identified proteins with molecular weight within the range for alpha amylases (61.66- 66.2 kDa). The alpha amylase produced had potential for use in the food and other industries. Further research should be carried out on the optimization of parameters affecting the optimum production of the enzyme by the mutant and recombinant strains.

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