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Characterization of Thermophilic Bacteria *Anoxybacillus rupiensis* and Cultivation in Agroindustrial Wastes Isolated from Hot Spring in Chakwal, Pakistan

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Authors' Contribution
FJ designed and performed the experiments and wrote the manuscript. BM performed some experiments, collected and analyzed the data. JIQ collected samples and guided for the performance.

Key words
Thermophiles, *Anoxybacillus rupiensis*, Hot spring, Agro-dairy wastes.

ABSTRACT

Two thermophilic bacteria were isolated from hot spring located near the city Chakwal in Pakistan. The cells of both strains were rod-shaped, stained Gram positive and formed endospores. The isolates were able to utilize sugars like fructose, Maltose, mannose, xylose, sacchorose, sorbitol, mannitol, Arabinose and polysaccharides like gelatin and starch. Phylogenetic analysis based on 16S rRNA gene sequences revealed that both isolates belong to the same specie *Anoxybacillus rupiensis* with 99% similarity under Accession number KF254911 (JF82) and KC849452 (JF83). The DNA G + C contents were 56.854% and 56.754% for *A. rupiensis* JF82 and *A. rupiensis* JF83, respectively. The isolates grew over a wide range of temperatures (40-70°C) and pH (4-9). The isolates exhibited growth with variety of nitrogen sources. Both thermophilic strains were grown on agro-dairy wastes to attain their cheap production by utilizing the wastes nutrients. Protein production was determined using 1% (w/v) of various agro-dairy wastes in production medium both with and without nutrients at pH 7.0 for 48 h at their respective suitable temperatures. They yielded enough thermostable protein suggesting their potential in production of various enzymes and proteins in unconventional and economical substrates suitable for various industrial uses.

INTRODUCTION

Extreme environment inspired microbiologist to study on hot spring microbial diversity throughout the world. Many researchers have reported thermophilic microbes from diverse hot springs and geothermal sites of the earth. In recent years usage of thermophilic bacteria has become prominent. They are mostly generated in hot spring water and can produce unique catalyst in extreme conditions (Aanniz *et al*., 2015; Rehman *et al*., 2018). Thermostable enzymes of these bacteria have been used in variety of fields of food, detergent, textile and molecular biology (van den Burg, 2003) as they are usually resistant to chemical.

Pakistan lies over the junctions of the tectonic plates of the sub-continent and is rich in geothermal resources. Major tectonic elements during the Cenozoic and Mesozoic era have shaped the Geological structures that observed in Pakistan today (Javed *et al*., 2012). Tectonic movements also give rise to hot springs like in Chakwal, Pakistan. Thermophiles have been produced thermostable enzymes of industrial use. Their thermostable enzymes withstand the harsh condition of industrial processing. Cultivation of thermophiles at high temperature is suitable as it minimizes the contamination risk and maximizes the substrate solubility (Zeldes *et al*., 2015; Fotouh *et al*., 2016). Thermostable enzymes and microorganisms have been topics for much research during the last two decades, but the interest in thermophiles and how their proteins are able to function at elevated temperatures actually started as early as in the 1960's by the pioneering work of Brock and his colleagues (Brock and Freeze, 1969). In this regard, *Anoxybacillus* relatively new genus is alkali tolerant thermophiles suitable for many industrial applications. These thermophiles are revealing new capabilities and are being manipulated by biotechnologist in utilizing them in different unique ways (Gursahani and Gupta, 2015).

The use of inexpensive and cheap raw materials is important to the overall economics of whole the process production as they account for 50% of the final cost. The best way to reduce substrate cost for biotechnology at present is to use wastes with the right balance of carbohydrates and lipids to support optimum bacterial growth and protein production. It is known, millions tons of hazardous and non-hazardous wastes are expelled out

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of industries throughout the world. There is a great need for better management of these wastes through reuse and recycle (Gursahani and Gupta, 2011; Nadeem *et al*., 2013; Jadhav and David, 2016; Verma *et al*., 2018). So far, several renewable substrates include various agricultural and industrial by-products and waste materials have been intensively studied for microorganism cultivation at a laboratory scale for example sugarcane bagasse, wheat bran, rice bran, molasses, corn cob, whey, wheat straw, vegetables and fruits waste etc. which also reduces the waste management problem in very useful and productive way.

Keeping in view the biotechnological potential of the thermophiles the present study was conducted to exploit them as potential candidates. The study was aimed at the development of economical methods for protein production by the use of unconventional substrates in submerged fermentation produced every year in million tones as "waste products". The research investigated the potential of utilizing agro-industrial wastes to replace synthetic media for cultivation of thermophilic *Anoxybacillus rupiensis* and thermostable protein production.

MATERIALS AND METHODS

Sampling and isolation of thermophilic bacteria

In this study, two strains of *Anoxybacillus* sp. were isolated from the two hot springs located in the city Chakwal, Pakistan. Water samples were collected in screw capped tight sterile bottles transported to laboratory and was kept at 4°C until use. Samples were processed for isolation of thermophilic bacteria on nutrient agar medium by incubating the initial inoculums at 60°C for 3 days. Two thermophilic bacterial isolates were isolated, pure cultured and stored in slants and glycerol stocks.

Morphological and biochemical analyses

The isolates were characterized through morphological and biochemical tests for catalase, oxidase, nitrate reduction, starch and gelatin hydrolysis, methyl red, Voges Proskauer test and citrate utilization as described by Benson (1994). The isolates were ultimately identified by 16S rDNA sequencing.

For genomic DNA extraction, a single colony was grown in nutrient broth at 60°C in shaker incubator with 100 rpm for overnight. Genomic DNA extraction was done by the method described by Sambrook and Russel (2001). For the amplification of 16S rDNA gene in bacterial isolates universal primer (Tamura *et al*., 2007) were used. The 50 µl of reaction mixture consisted of 50 ng of genomic DNA, 2.5 units of *Taq* polymerase, 5 µl of 10 X buffer (100 mM Tris-HCl, 500 mM KCl pH-8.3), 200 µM dNTP, 1.5 mM MgCl2 and 10 pmoles of each primer. The forward primer 27F (50-AGAGTTTGATCCTGGCTCAG30) and reverse primer 1492R (50-TACGGTTAC CTTGTT ACGACTT-30) (Miller *et al*., 2013) were used. Amplification was performed under the following thermal (PCR System 2720, Applied Biosystems, Singapore) conditions: initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1.5 min, extension at 72C for 2 min and a final extension at 72°C for 7 min. Amplified PCR products (5 µl) were resolved on a 1.2% (w/v) agarose gel. It was purified using PCR purification kit (QIAquick PCR purification kit). In order to establish the genetic relationship, we used 16S rDNA gene sequence of isolated species along with reference strains retrieved from ribosomal database project available at Gene Bank, using BLAST tool in NCBI for recognition of the highest % similarity with the described species. Multiple sequence alignment was done by Clustal W and MEGA 4.0 software for construction of phylogenetic tree with 500 bootstrap replication. The evolutionary distances were computed using the Maximum Composite Likelihood method (Nei *et al*., 1985).

Optimization of growth

Effects of temperature and pH on thermophilic bacterial growth were studied by growing the isolate in cultural broth at different temperature ranging from 40 to 70°C and different pH ranging from 4 to 9 for 5 days. Different nitrogen sources such as trypton, gelatin, peptone, yeast extract, urea, ammonium chloride, ammonium oxalate and ammonium dihydrogen phosphate were supplemented to the medium to study their influence on growth (Bayoum *et al*., 2008; Jabeen and Qazi, 2014).

Cultivation in agro-dairy wastes

Sugarcane bagasse was collected from the hostel juice shops of the University of the Punjab, Quaid-e-Azam campus, Lahore, Pakistan while whey was collected from different dairy shops from local markets. Wheat straw was collected from waste of wheat mills from different places of Lahore.

Sugarcane bagasse (SCB) was washed with water to remove dust and soil particles, and then subjected to sun drying. SCB and wheat straw were kept in an oven at 80°C for 1 to 2 weeks till consistent weight was achieved. After drying, the substrates were crushed into fine powder with the help of a grinding mill and stored in air tightened containers.

For preparation of inoculum, the bacterial cultures were grown in 20 mL of ntrient broth taken in a 100-mL Erlenmeyer flask and inoculated with a loop full of cells from a 24 h old slant and kept at 60°C in a rotary shaker (120 rpm). After 10 h of incubation, 1 mL of this culture

was used as inoculum. By serial dilution and plating, the number of viable colonies in the inoculum was found to be 3×10^8 CFU/mL.

Growth in prepared sugarcane bagasse

The selected bacterial strains were cultivated in 2% sugarcane bagasse. For this purpose 2% pulverized sugarcane bagasse was autoclaved at 15 lb/inch^2 for 15 minutes and filtered through Whattman Filter Paper No. 1. Then all the ingredients were added to the SCB extract and employed for cultivation of the bacteria. Desired pH of a medium optimum for respective bacterium was adjusted with 1M HCl/NaOH solution. The pH adjusted media were again autoclaved and the media 10 ml/test tube were inoculated with 0.1 ml of 5 days old bacterial culture broth. After incubating at their respective optimum conditions the bacterial cultures were harvested after 5 days and processed for the determination of growth, extracellular protein and final pH.

Growth in whey

First whey was autoclaved and then filtered through Whattman Filter Paper No. 1. The filtrate was processed for media preparation, cultivation of the bacteria and processing of the culture samples as described above.

Growth in sugarcane bagasse and whey

Autoclaved and filtered aqueous extract of whey and 2% SCB were taken in 1:1 ratio. The mixture was then processed for media preparation, cultivation of the bacteria and processing of the culture samples as described above.

Growth in wheat straw

Two percent wheat straw was autoclaved and filtered through Whattman Filter Paper No. 1. The filtrate was processed for media preparation, cultivation of the bacteria and processing of the culture samples as described above

Estimation of extracellular protein

The protein content was estimated by the method described by Bradford (1976). Bovine serum albumin (BSA) prepared from 2 µg/ml to 10 µg/ml range with 2 class intervals was used as standard. Calibration curve was then plotted by performing regression analysis of A_{595} absorbance versus corresponding concentrations of the standards.

RESULTS

Biochemical characteristics of the isolated thermophilic bacterial strain

Two bacterial isolates were obtained from water samples collected from hot spring of Chakwal, Pakistan. The bacterial isolates were screened for their thermo-tolerance property by incubating at 60°C. The bacteria were characterized by morphological observation and biochemical tests. Gram staining, microscopic and biochemical characterization revealed it to be a Gram positive rod. The bacteria tested positive for catalase activity, oxidase, endospore production, starch hydrolysis and negative test from nitrate reduction, citrate utilization, Voges-proskauer, methyl red, gelatin hydrolysis anaerobic and sulphate reduction. The biochemical characteristic of the bacteria is presented in Table I. Morphological and biochemical characterization of the isolate indicated it to be *Anoxybacillus* sp.

Table I.- Morphological, physiochemical and sugar utilization tests.

Characteristics	***Anoxybacillus rupiensis***	
	JF83	**JF82**
Configuration	Round with raised margin	Round with raised margin
Margin	Almost smooth	Irregular
Elevation	Raised	Raised
Consistency	Creamy	Butyrous
Optical feature	Translucent	Opaque
Surface	Little shiny	Dull
Colour	Off white	Off white
Size in diameter (mm)	2.0	2.5
Gram staining	+	+
Cell shape	Rods	Rods
Endospore staining	+	+
Oxidase test	+	+
Growth in anaerobic condition	+	+
Nitrate reduction	-	-
Citrate utilization	-	-
Voges-proskauer	-	-
Starch hydrolysis	+	+
Gelatin hydrolysis	-	-
Methyl red	-	-
Fructose	+	+
Lactose	-	-
Maltose	+	+
Sucrose	-	-
Galactose	-	-
Mannose	+	+
Xylose	+	+
Sacchorose	+	+
Sorbitol	+	+
Mannitol	+	+
Arabinose	+	+

Molecular identification of the isolated strain

Species level confirmation of the isolate was done by 16S rDNA sequencing. Based on BLAST search analysis of the sequence, both isolates showed maximum 99% identity with *Anoxybacillus rupiensis*. The 16S rDNA sequences of all the bacteria have been submitted to the NCBI GenBank databases under the accession number of KC849452 (*Anoxybacillus rupiensis* JF83) and KF254912 (*Anoxybacillus rupiensis* JF82).

Based upon 16S rDNA sequence alignment, phylogenetic tree was constructed for the isolated strain. The phylogenetic trees are shown in Figure 1. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in ClustalW. From the construction of the phylogenetic tree it was revealed that closest homolog of *Anoxybacillus rupiensis* JF83 was *Anoxybacillus rupiensis* R-32636 strain 412 (GenBank Accession no AM988775) and closest homolog of *Anoxybacillus rupiensis* JF82 was *Anoxybacillus beppuensis* JF84 (GenBank Accession No KF254912).

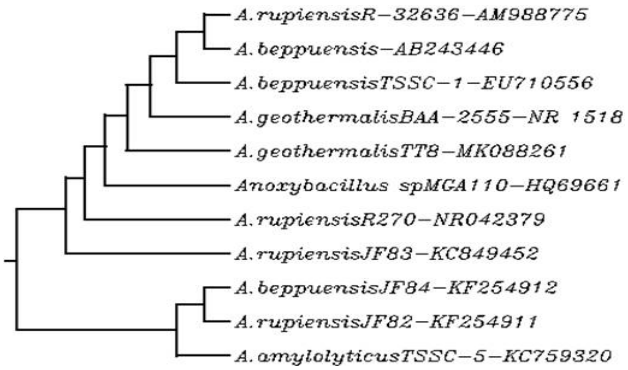

Fig. 1. Phylogenetic tree of the isolates which have the highest homology with *Anoxybacillus*. Consensus neighbour-joining phylogenetic unrooted tree from sequences of the 16S rRNA gene, encompassing *Anoxybacillus* species.

Optimization of growth

Maximum extracellular protein production by *A. rupiensis* JF83 was observed at initial pH 7 while *A. rupiensis* JF82 showed at initial pH 6. However, significant amount of protein was also produced by *A. rupiensis* JF83 at pH 5 and 6 and could attain 88.73 and 95.36% of the level obtained at pH 7, respectively. Similarly considerable amount of protein was also created by *A. rupiensis* JF85 at pH 7 and could attain 90.61% of the level obtained at pH 6. The bacterial growth for *A. rupiensis* JF83 and *A. rupiensis* JF82 could approach CFU of 259x10^2 /ml and of 289x 10^2 /ml, respectively, following inoculation in the nutrient broth with initial pH 4. While highest growth upto 132 x 10^6 CFU/ml was recorded with pH 7 in case of *A. rupiensis* JF83 and 37x10^6 CFU/ml with pH 6 in case of *A. rupiensis* JF82.

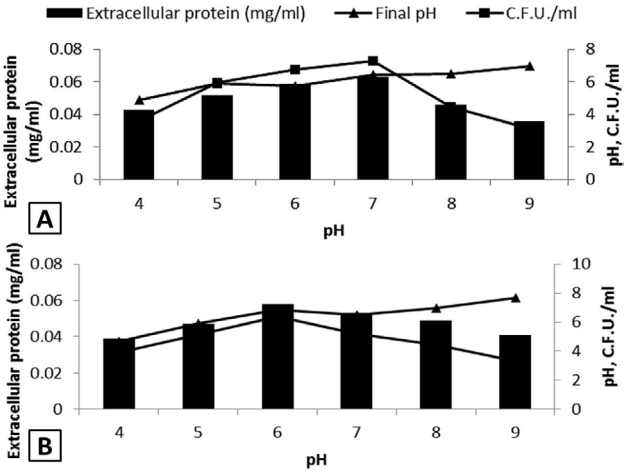

Fig. 2. Effect of different pH on growth and extracellular protein produced by *Anoxybacillus rupiensis* JF83 (A) and JF82 (B) following 24 h of incubation at 60°C and 140 rpm.

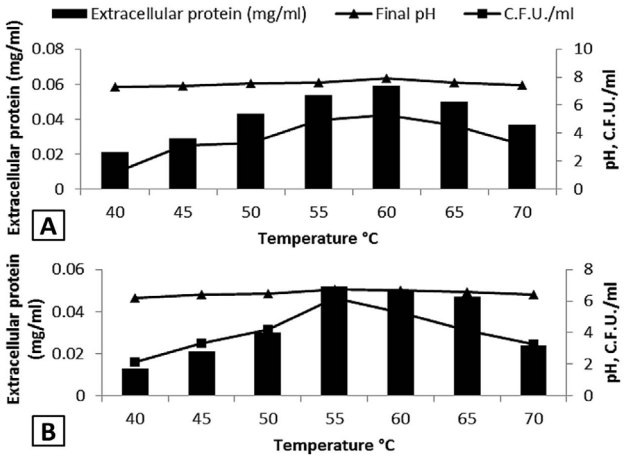

Fig. 3. Effect of different temperatures on growth and extracellular protein produced by *Anoxybacillus rupiensis* JF83 (A) and JF82 (B) following 24 h at optimized pH and 140 rpm.

Highest protein production up to 0.059 and 0.052 mg/ml was achieved at 60°C and 55°C for *A. rupiensis* JF83 and JF82, respectively. Considerable decrease in protein production for both strain *A. rupiensis* JF83 and *A. rupiensis* JF82 were observed at low temperatures so that at 40°C incubation temperature 77.17% and 62.77% reduction in the yield and CFU reach to 183 and 132/

ml was recorded, respectively. Among various organic and inorganic nitrogen sources tested, yeast extract was identified as the best nitrogen source for *A. rupiensis* JF83 supporting the extracellular protein leading to 0.058 mg/ml with 272 x 10^6 CFU/ml while supplementation of peptone in the media for *A. rupiensis* JF82 increased protein to 0.067 mg/ml with 34 x 10^7 CFU/ml (Figs. 2, 3, 4).

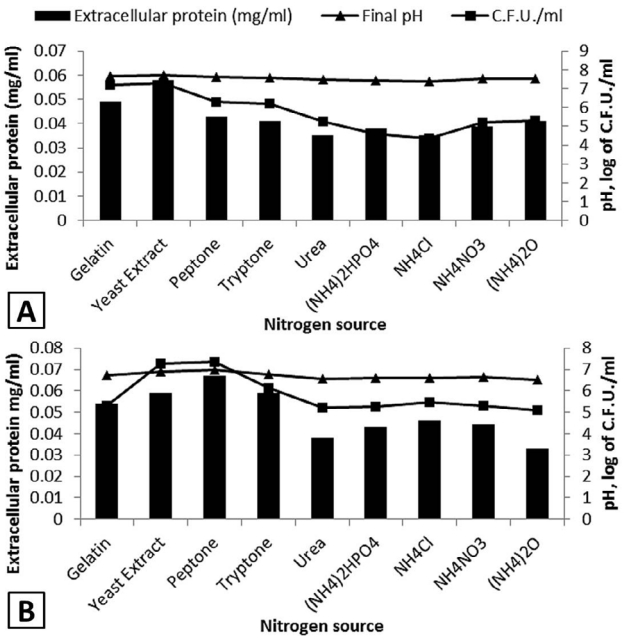

Fig. 4. Effect of different nitrogen sources on growth and extracellular protein production produced by *Anoxybacillus rupiensis* JF83 (A) and JF82 (B) at optimized conditions.

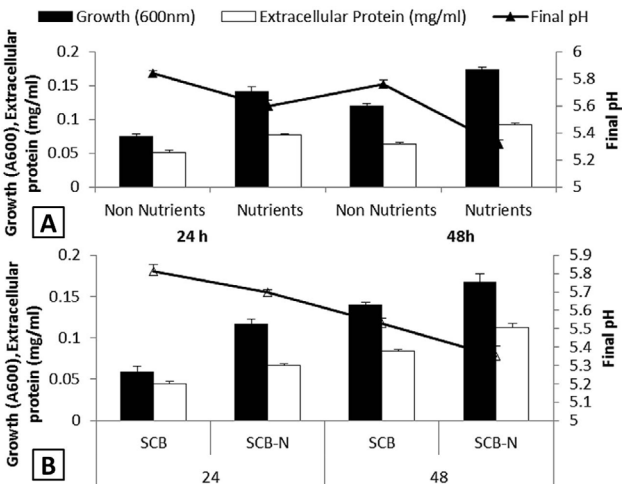

Fig. 5. Cultivation of *Anoxybacillus rupiensis* JF83 (A) and JF82 (B) in 2% sugarcane bagasse without nutrients (SCB) and sugarcane bagasse containing nutrients (SCB-N) for 24 and 48 h at initial pH 6.

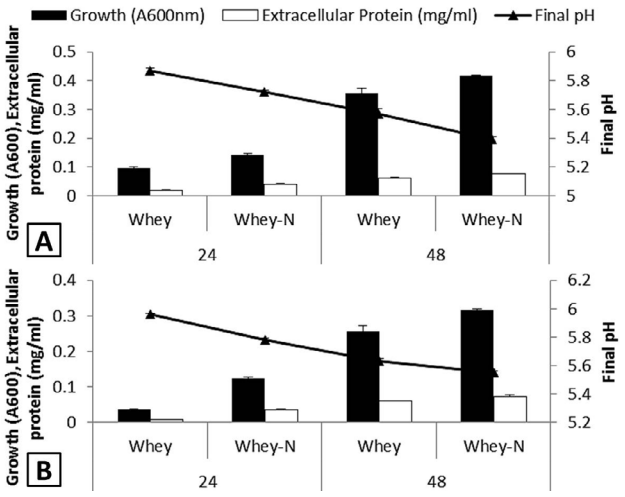

Fig. 6. Cultivation of *Anoxybacillus rupiensis* JF83 (A) and JF82 (B) in 2% whey without nutrients and whey with nutrients (whey-N) (A), whey containing all the ingredients of FJ medium except chitin (whey-N) (B) for 24 and 48 h at initial pH 6.

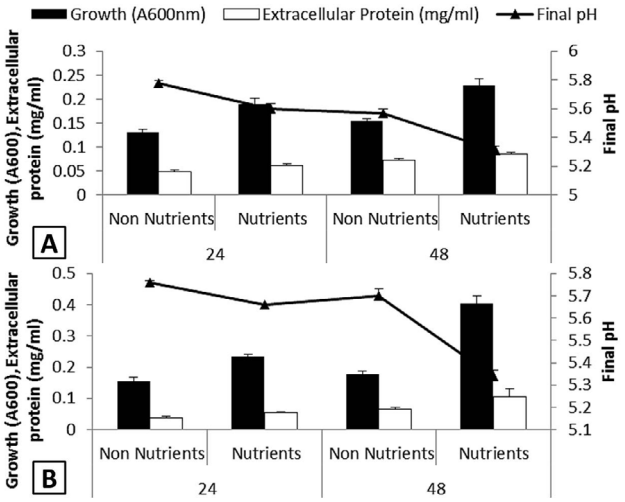

Fig. 7. Cultivation of *Anoxybacillus rupiensis* JF83 (A) and JF82 (B) in 2% SCB and whey nutrients (SCB+whey-N) (A) and SCB and whey containing all the ingredients of FJ medium except chitin (SCB+whey-N) (B) for 24 and 48 h at initial pH 6.

Production of protein by agro-dairy wastes

To check the suitability for protein production by *Anoxybacillus* was performed by adding 2% sugarcane bagasse (SCB), whey (W) and wheat straw (WS) separately or in combination in the production medium by replacing carbon source with and without nutrients. In all agro-dairy wastes growth and protein production was increased with time after 48 h both strains exhibited

better growth and protein production than 24 h similar trend was seen with nutrients as these minerals support the growth and protein production in both strains. Wheat straw was found most suitable for protein production for both strains. Extracellular protein approached to 0.118 mg/ml with wheat straw by *A. rupiensis* JF82 while *A. rupiensis* JF83 represented maximum 0.092 mg/ml protein with sugarcane bagasse. The order of substrate suitability for both strains *A. rupiensis* JF83 was SB>WS>SB+W>W but for *A. rupiensis* JF82 was found WS>SB+W>SB>W shown in Figures 5, 6, 7 and 8. It is also observed that without the addition of nutrients, these agro-dairy wastes support the growth of bacteria but adding of other nutrients enhance the protein production and bacterial growth as well (Figs. 5, 6, 7, 8).

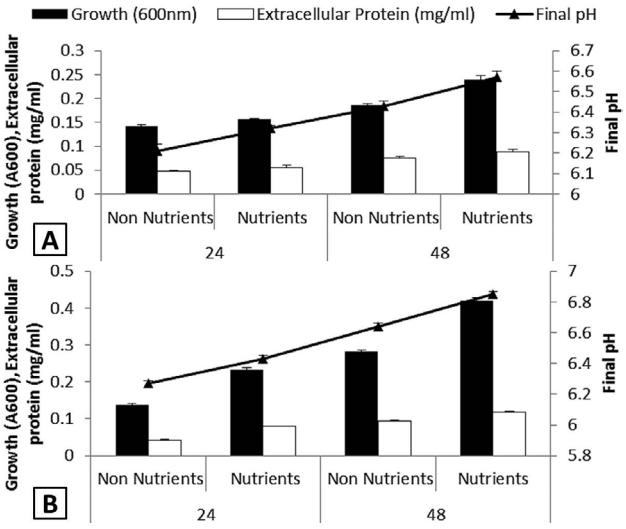

Fig. 8. Cultivation of *Anoxybacillus rupiensis* JF83 (A) and JF82 (B) in 2% wheat straw and wheat straw containing nutrients (wheat straw-N) for 24 and 48 h at initial pH 6.

DISCUSSION

Thermophiles have the ability to exist in hot environmental conditions due to their molecular change at cellular and subcellular level. In our present study we investigated water samples from hot spring in Chakwal to isolate thermophilic bacteria. The maximum growth rate at high temperature indicated to be thermophilic. The bacteria were identified as *Anoxybacillus rupiensis* by both morphological and molecular characterization. Awan *et al*. (2018) also collected water samples from hot spring in Chakwal and isolated thermophilic bacteria that were identified as *Bacillus licheniformis by* 16S rRNA gene sequencing. Gursahani and Gupta (2015) and Yohandini *et al*. (2015) isolated the thermophilic bacteria from hot spring and identified as *Anoxybacillus rupiensis*.

Generally *Anoxybacillus* and *Geobacillus* are thermophilic, gram-positive, spore forming, rod-shaped cells, and aerobic or facultative anaerobic bacteria. *Anoxybacillus* are thermophiles found in hot springs, mines and hot reservoirs. Suitable conditions are always required for maximum growth and enzyme production. Maximum growth of these *A. rupiensis* JF83 and *A. rupiensis* JF82 was observed at 60°C and 55°C, respectively, showed its thermophilic nature. Awan *et al*. (2018) observed 50°C the most suitable temperature for maximum growth of thermophilic bacteria. Similarly, Jabeen *et al*. (2018) also worked on *Anoxybacillus* and found maximum growth at 60°C. It was observed *Anoxybacillus rupiensis* performed well at neutral pH. Acer *et al*., (2015) also reported the similar results with maximum growth at pH 7 by the same specie *Anoxybacillus* but Jabeen *et al*. (2018) found pH 6 the most appropriate for the *Anoxybacillus beppuensis*. Nitrogen source is an important factor influenced bacterial growth and protein production. Effect of different nitrogen sources on protein production was observed in order to get suitable nitrogen source. Yeast extracts and Peptone were found to be growth enhancer for both strains JF83 and JF82, respectively. Bakir and Metin (2015) and Acer *et al*. (2015) also found peptone the most suitable for the production of enzyme.

Thermophilic microbes have proven themselves as potential candidate of bioactive compounds and many secondary metabolites. Thermophilic bacteria have ability to produce wide range of thermostable enzymes. They are the most economically as well as biotechnologically valuable microorganisms. The increasing expansion of agro-dairy wastes has resulted in accumulation of huge quantity of wastes across the globe. In this study, we have described the isolated thermophilic strains of *Anoxybacillus* having ability to generate protein while using agro-dairy waste as a sole carbon source. It was also observed addition of other nutrients supported the growth and protein production. Various wastes and agricultural residues can be utilized as low-cost feed stock for production of industrially important extracellular enzymes. Singh *et al*. (2012) reported production of extracellular enzymes by using sugarcane bagasse, rice bran, corn cob, wheat bran, wheat straw as a carbon source with thermophilic actinomycetes. Saleem *et al*. (2002) described that various agro-industrial wastes (rice straw, wheat straw, bagasse, wheat bran and kraft pulp) at 2% concentration could be used for the production of xylanase enzyme by *Bacillus circulans* AB16. Shah *et al*. (2013) also used 2% waste for the production of invertase enzyme. Mixed or in combination wastes were also being utilized by many workers for the production of enzymes. Mixed wastes provide multiple

nutrients at a time to growing microbes that may support the growth and provide variety of enzymes. These thermozymes have showed great potential in industries due to their wide applications. Potential applications for some of *Anoxybacillus* species were reported previously, particularly for the production of bioactive molecules and biocatalysts that may be significant for industrial processes. This bacterial group had known to produce a variety of thermostable enzyme which showed it potential candidate in different industries. Either using single or mixed wastes, it's important to utilize the agro-industrial wastes for production of industrially valuable proteins/ enzymes for generating low cost processes as well as solving environmental issues.

CONCLUSION

Both strains of thermophilic bacteria *Anoxybacillus rupiensis* were isolated and their preliminary protein production potential was characterized. This is the first report on isolation of a Anoxybacillus strains from Chakwal hot springs. Protein production and growth level showed its capabilities to grow over wide range of pH, temperature and nitrogen sources. Utilization of agroindustrial wastes highlights its capabilities to replace expensive synthetic substrate to low cost medium for protein/enzymes production. These promising results can be exploited further for production of biotechnological important and industrially thermostable enzymes. This study widens the opportunities for further research to be conducted to explore more the immense significance of these strains where there is lack of intensive studies regarding this organism.

Statement of conflict of interest

The authors declare no conflict of interest.

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