



Dynamics of Phylogenetic Diversity and its influence on the production of Extracellular Protease by moderately Halotolerant Alkaliphilic Bacteria *Acinetobacter baumannii* GTCR407 Nov.

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Abstract: New characters emerge in the population of microorganisms living in the extreme environments due to its adaptation to ecological association. The microorganisms living in saline habitat utilize complex nutrients by adopting different strategies in Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA), which are related to their metabolic and ecological diversities. Isolation and characterization of the organisms producing extracellular protease from such environment were the prime focus of this investigation, which can indicate the importance of metabolic diversity in phylogeny. Norberg medium was used to isolate halotolerant microorganisms from salt-cured skin. The isolates were screened for high activity of protease and the strain showing maximum activity of protease was taken for further studies. The biochemical characterization and 16s ribosomal RNA sequencing studies confirm that the isolate is *Acinetobacter baumannii*. Moreover, hydrolysis positive for starch and casein, negative for gelatin shows that the organism is a variant form of *A. baumannii*. Cell growth parameters such as pH and temperature were optimized and their values are 8 and 37°C respectively. The extracellular production of protease was optimized in the suitable medium and its enzyme activity was 165µg/ml/min. The results imply that the isolate had acquired operational genes through lateral gene transfer (LGT) probably from unrelated species in the environment. This indicates that the isolate identified possesses metabolic and ecological diversities with values of phylogenetic delineation.

Keywords: Haloalkaliphilic, Extracellular protease, Phylogenetic diversity, Microbial population dynamics and *Acinetobacter baumannii*.

1. Introduction

The species *Acinetobacter baumannii* is a free-living saprophyte, characterized as a Gram-negative, non-fermentative bacterium found ubiquitously in hospital environments [1]. Here we report a species which is in contrast to the *A. baumannii* having intermediary characters and that was not reported so far from this organism. Such new characteristics could be important in elucidating certain phylogenetic relationships within the family of Neisseriaceae. Until now, species of *Acinetobacter* genus is named based on

distinguishable genotypic characteristics and consequently, seven genomic species have been named. In certain genotypes, the phenotypic characters merge and form phenotypic complex which is referred as *A. calcoaceticus*-*A. baumannii* complex [2, 3 & 4]. Due to the lack of classification methodology and confusion over phylogenetic character of this genus, *Acinetobacter* undergoes frequent taxonomic changes, and thus the delineation of species within this genus is still ongoing research [5, 6 & 7].

According to the *Bergey's Manual of Systematic Bacteriology*, the genus *Acinetobacter* was grouped in

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the Neisseriaceae [8]; however, it was recently proposed to include in the family Moraxellaceae of superfamily II of Proteobacteria [9, 10 and 11]. Moreover, the classification of this genus into nutritional groups [12, 13] would be close but not a complete delineation of phylogeny of the organism because the true extent of the domains of microbial life in the environment is still obscure. Likewise, the classification of genera in term of species characterization confined to cell morphology, physiology, and pathogenesis alone may lead to arbitrary assumption on their phylogenetic diversity and gene expression profiles [14]. Also, the taxonomical hindrance of this genus inhibits the understanding of their significant biological properties and commercially important enzymes for industrial processes [1]. Therefore, underpinning the metabolic diversity with reference to their habitat of ecology will extend our knowledge of delineation of this genus.

The purpose of the report is to define a member of the genus *Acinetobacter* having diversified new habitat and hence with some modified biochemical characteristics and differential gene expression. We propose that in addition to the conventional classification methods, the delineation of this genus based on species metabolic characteristics and ecological diversities would be more relevant to the organization of their phylogenies.

2. Materials and Methods

All the chemicals for the assay procedure and medium preparation were purchased from Sisco Research Laboratory (SRL), Himedia, India and Sigma Aldrich, USA.

2.1 Isolation of halophilic and halotolerant and haloalkaliphilic bacteria

The strains were isolated from salt-cured putrefied skin samples collected from the leather processing division of Central Leather Research Institute (CLRI) tannery, Chennai, India. The isolation was carried in Norberg medium (NM) [15] that contained the following in gram per liter; yeast extract, 1; MgCl₂, 4.88; KCl, 5; CaCl₂, 0.175; NaCl, 10; and Agar, 20. 1 ml of wash liquor obtained after shaking the soaked skin sample in an orbital shaker for 2 h was inoculated and incubated at 37°C for 48 h in the aforesaid medium. The colonies were isolated by pour plate technique and individual colony was taken in the loop and was streaked on the Norberg agar plate. Then, the pure line colony was cultured.

2.2 Screening for extracellular protease secretion and the growth of isolates in various salt concentrations

In order to screen the strains at various salt concentrations based on the extracellular protease

secretion and growth potential of all the isolates, NM was used as the screening medium supplemented with 1% (w/v) casein solution. Isolates were inoculated in the plates containing Norberg agar as well as NaCl from 0 to 4M concentrations and the plates were kept for incubation at 37°C for 24 h. The protease-producing efficacy was evaluated by measuring the zone area formed by radial diffusion on the NM agar plate according to modified Wikstrom *et al.*, method [16]. The density of colony formation in the particular NaCl concentration was defined physically.

2.3 Phenotypic identification

The isolate producing the maximum level of protease activity was chosen for phenotypic characters. The colony characteristics, cell morphology, Gram-staining reaction, nutritional susceptibility and other biochemical characteristics of the isolate were studied and the genus of the isolate was identified.

2.4 Molecular identification

The phenotypically identified genus producing extracellular protease with maximum activity was further analyzed by 16S rRNA amplification and nucleotide sequencing. The sequence after amplification was evaluated with Bioinformatics tools to identify the phylogenetic position and speciation of the strain GTCR407. The confirmed sequence was deposited in NCBI Gene Bank with accession no: GU319977.

2.5 Assay for proteolytic activity

The protease was assayed by Anson-Hagihara method [17] using casein as a substrate. One unit of enzyme activity (U) was taken as the amount of enzyme liberating 1µg of tyrosine per ml per minute under the assay condition. The estimation of enzyme activity was based on the tyrosine standard calibration curve. All the results of activities reported were the mean of triplicates.

2.6 Total protein content

The total protein content of each sample was determined according to Lowry *et al.*, [18]. The protein standard used was bovine serum albumin (Sigma Aldrich, USA).

2.7 Medium optimization for growth and protease production in various salt concentrations

The effect of different formulation of the referred mediums containing various combinations of organic and inorganic nutrients was evaluated for cell growth and production of protease. In all the compositions the NaCl in the concentration range of 0 to 3.5 M was included. In the autoclaved medium, 50 ml each was inoculated with 5 ml of the isolate. After incubation at 37°C and 150 rpm in orbital shaker, the growth and the protease production in each medium were monitored

for every 24 h interval up to 72 h. The cell growth was measured at 600 nm using a spectrophotometer. The protease production was evaluated by collecting 2-ml cell free supernatant after centrifuging the harvested medium at 4°C and 5000 rpm for 10 min, and the crude enzyme extract was assayed for proteolytic activity and total protein content. The optimization of the medium was based on the proteolytic activity to cell density.

2.8 Effects of pH and temperature on the growth of the organism

The effect of pH and temperature on the growth was studied. The culture was carried out at different initial pH values of the medium (pH 6.0–9.5) incubated at 37°C and 150 rpm for 72 h under shake flask condition and the pH of the medium was adjusted using autoclaved Na₂CO₃ (10%, w/v). The culture broths with different temperatures (25–45°C) of incubation were

kept in similar shake flask condition. The optimum physiological growth conditions were evaluated on cell growth at every 24 h interval for 72 h by measuring the optical density at 600 nm in spectrophotometer.

3. Results

3.1 Occurrence

A haloalkaliphilic bacterium was isolated and named tentatively as GTCR407 and its colony morphology was opaque, circular and convex surface, creamy colony with white pigmentation. Three other organisms were also isolated exhibiting different colony morphologies and they are identified as isolates GTCR107, GTCR207 and GTCR307. All the bacteria isolated were less than 3 mm in diameter and adhered tenaciously to the agar surface.

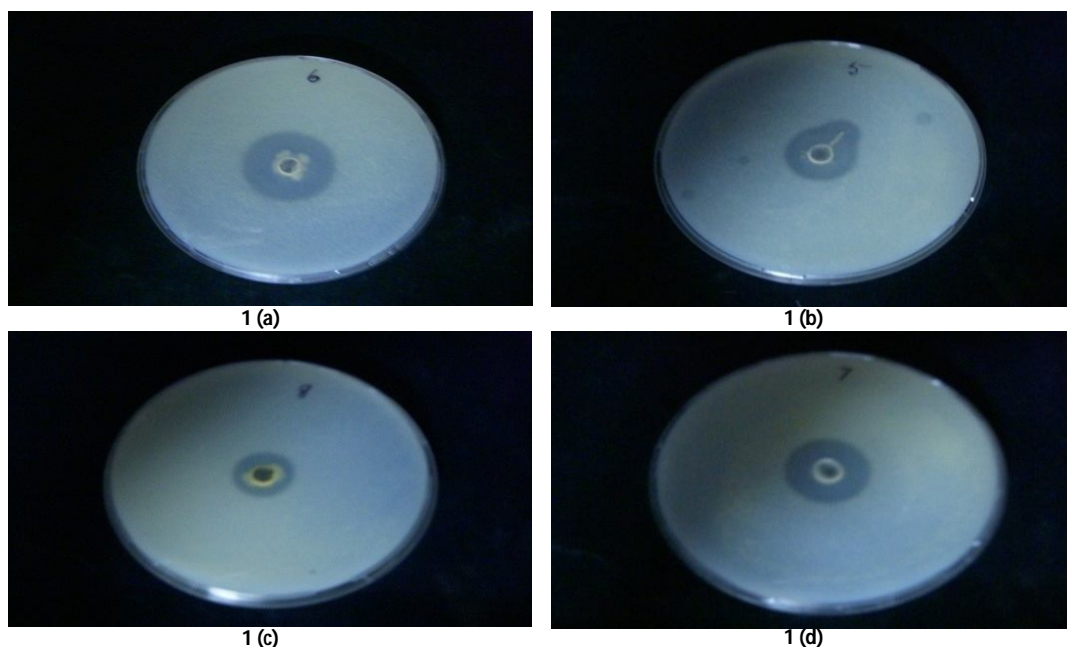


Fig. 1. Zone diffusion was measured after 24 h incubation: 1(a) Zone diffusion of strain GTCR407 was 3 cm; 1(b) Zone diffusion of strain GTCR207 was 2.4 cm; 1(c) Zone diffusion of strain GTCR307 was 0.9 cm; 1(d) Zone diffusion of strain GTCR107 was 2 cm.

3.2 Qualitative detection of protease and growth in various salt concentrations

All the isolates were screened for the ability of protease production in Norberg-casein agar plate containing different concentration of NaCl and the strain, GTCR407, showed high level of protease production which was detected by the clear zone of casein hydrolysis that measured 3 cm in diameter. On the other hand, isolates GTCR107, GTCR207 and GTCR307 showed comparatively smaller hydrolytic zones measuring 2, 2.4 and 0.9 cm in diameter respectively (Fig. 1). These differences in the casein hydrolysis may be attributed to the ability of protease production by each organism to hydrolyze the substrate as well as the organism's obvious signal response to

available complex nutrition in the environment. The density of colony formation of all the isolates was varied in various NaCl concentrations and the maximum growth of all the isolates was found in the range from 2M to 3.3M of NaCl (*data not shown*). Based on these decisive factors, the halotolerant organism, isolate GTCR407, was chosen for further evaluation.

3.3 Biochemical characteristics of the organism

The result of biochemical characterization for the isolate GTCR407 closely matches with the common characters of the organism *Acinetobacter* Sp. and that was also confirmed by various morphological features and chemical reactivity tests (Table 1). In addition, the

isolate showing catalase positive and oxidase negative confirm the genera. According to the procedure for determination of phenotypic properties recommended by the international committee on systematic bacteriology (ICSB), the isolate was tested for acid production from glucose and lactose which showed positive. The isolate also showed positive for acid production from xylose and exhibits few other characters of genera *Neisseria* and *Moraxella*. Moreover, the isolate showing hydrolysis positive for starch and casein, negative for gelatin demonstrates that it possesses variant characters of the genera *Acinetobacter*.

3.4 Molecular taxonomy

Molecular analysis of 16S rRNA amplification for the isolate was carried out by Solgent Co., Korea, and it provided the nucleotide sequence of 1365bp units. Further, we BLAST the sequence, and it confirmed the molecular phylogenetic position of the isolate in the hierarchy of microbial taxonomy and the sequence was found 97% homology with *Acinetobacter baumannii*, and the strain GTCR407 was identified to be novel.

3.5 Optimization of the substrate and salt concentration for growth and protease production

The optimization of culture medium with different combination of substrates and salt concentrations (0 to 3.5 M) was evaluated for cell growth and production of protease. The optimum value of cell growth and production of protease was observed at 48 h of culture as shown in Table 2 & 3 [19, 20].

Table 1. Main characteristics of the genera *Acinetobacter* Sp. Strain.

Feature	Inference
Gram stain	-
Morphology	Coccobacilli
Pigmentation	White
Motility	Non-motile
Cell arrangement	Pairs/Short chains
Facultative anaerobe	-
Fermentative	+
Catalase test	+
Oxidase test	-
Decomposition of: Methyl red	-
Voges Proskauer	-
Production of: Nitrate	+
Nitrite	-
Utilization of Citrate	+
Urease	+
Acid production from Arabinose	-
Glucose	+
Lactose	+
Sucrose	+
Maltose	-
Galactose	+
Trehalose	-
Mannitol	-
Xylose	+
Hydrogen sulfide (H ₂ S) production	-
Hydrolysis of: Starch	+
Casein	+
Tween 80	-
Esculin	-
Gelatin	-
Phosphatase	ND
Growth on Mac Conkey agar	+
Habitat	Putrefied salt cured hide

Table 2. Optimization of culture medium with various salt concentrations and its effect on growth of the genera *Acinetobacter* Sp.

Medium Type [¶]	Reference [19]	Medium composition [‡]	Cell density [†] measurement by Spectrophotometer			
			3.5M	3M	2M	Control
1	Van Niel's (ATCC Medium 1370)	Yeast extract, K ₂ HPO ₄ , MgSO ₄ , NaCl	0.047	ND	ND	0.314
2	Halophilic nutrient (LMG Medium 220)	Yeast extract, Glucose, Peptone, NaCl	2.377	2.409	2.715 (1:1)	3.476 (1:3)
3	Halophilic Halobacterium medium (Modified)	Beef extract, Na ₃ C ₆ H ₅ O ₇ , MgSO ₄ , KCl, NaCl	0.486	0.753	0.935	1.468 (1:1)
4	Nicholson <i>et al.</i> , 2004	K ₂ HPO ₄ , KH ₂ PO ₄ , KCl NH ₄ Cl ₂ , MgCl ₂ , NaCl	0.027	ND	ND	0.289
5	Nutrient broth Modified	Peptone, Gelatin, NaCl	0.254	ND	ND	0.432
6	<i>Bacillus polymyxa</i> (ATCC Medium 455)	Yeast extract, Peptone, Starch, NaCl	1.89	2.156 (1:1)	2.868(1:2)	3.895 (1:3)
7	<i>Limnobacter</i> medium Modified (DSMZ medium 919)	Yeast extract, Peptone, Glucose, Starch, NaCl	2.448 (1:1)	2.737 (1:2)	3.321(1:3)	6.890 (1:6)

*Control, Medium devoid of NaCl; ‡ Cell density (OD₆₀₀), values are shown as samples were withdrawn at the exponential phase of cell growth after 48 h of incubation at 37°C; † Optimization, done on constant volume of 100ml in 250ml Erlenmeyer flask kept in an orbital shaker at 150 RPM; ¶ Medium Type, Specified for Reference ease; ND, Not Determined. All the experiments are done in triplicate. All the medium compositions were referred from Handbook of media for environmental microbiology except medium type 4 [20].

Table 3. Optimization of culture medium with various salt concentrations and its effect on extracellular protease production of the genera *Acinetobacter* Sp.

Medium Type †	Reference [19]	Medium composition ‡	Enzyme activity [‡] (µg/ml/min)			
			3.5M	3M	2M	Control [‡]
1	Van Niel's (ATCC Medium 1370)	Yeast extracts NaCl MgSO ₄ , K ₂ HPO ₄	6.97	ND	ND	15.32
2	Halophilic nutrient (LMG Medium 220)	Yeast extract, Glucose Peptone, NaCl	20.35	34.79	52.86	86.58
3	Halophilic Halobacterium medium (Modified)	Beef extract, KCl Na ₃ C ₆ H ₅ O ₇ , MgSO ₄ , NaCl	16.28	23.53	29.87	38.62
4	Nicholson <i>et al.</i> , 2004	K ₂ HPO ₄ , KH ₂ PO ₄ , NH ₄ Cl ₂ , MgCl ₂ , KCl, NaCl	4.64	ND	ND	10.89
5	Nutrient broth Modified <i>Bacillus polymyxa</i>	Peptone, Gelatin, NaCl Yeast extract, Peptone, Starch, NaCl	14.53	ND	ND	28.18
6	(ATCC Medium 455)		32.43	48.52	98.65	128.89
7	Limnobacter medium modified (DSMZ medium 919)	Yeast extract, Peptone, Glucose, Starch, NaCl	44.27	68.58	118.93	167.65

*Control, medium devoid of NaCl; ‡ Enzyme activity, was measured by tyrosine release (µg/ml/min) by Anson-Hagihara method and the protein were estimated by Lowry's method using BSA as standard at (OD₆₄₀), values are shown as samples were withdrawn after 48 h of incubation at 37°C; † Optimization, done on constant volume of 100ml in 250ml Erlenmeyer flask kept in an orbital shaker at 150 rpm; ‡ Medium Type, Specified for Reference ease; ND, Not Determined. All the experiments are done in triplicate. All the medium compositions were referred from Handbook of media for environmental microbiology except medium type 4 [20].

The culture in the medium types 1, 3, 4 and 5 in all NaCl concentrations and their respective controls showed significantly less growth (Table 2) and enzyme activity (Table 3). Therefore, the cell densities and enzyme activities at 3 M and 2 M were not determined in these mediums except medium type 3. These results may attribute to the presence of incompatible carbon and nitrogen sources and the presence of inorganic sources alone in certain mediums. The medium types 2, 6 and 7 were found as appropriate types showing appreciable cell density at 2 M with the values of 2.715, 2.868, and 3.321 respectively and the organism was able to grow in other NaCl concentrations as well. However, the significant cell growth was present in these mediums containing without salt (Table 2). Though the medium type 2 and 6 showed appreciable production of protease in all salt concentrations, the medium type 7 containing complex organic carbon and nitrogen sources without NaCl showed the optimum

value of cell density and production of protease as 6.890 and 167.65 µg/ml/min respectively. The result instigates that the production of protease depends upon cell growth. Moreover, regardless of the medium type's cell growth and production of protease decreased when NaCl concentration increased in the culture medium.

3.6 Temperature and pH influence on cellular growth

In the substrate, cell growth in the optimized medium increased with the increase of initial pH of the medium. The cell growth was evaluated at different initial pH values ranging from 6 to 9 and the appreciable growth was observed from pH 7.0 to 8.5, whereas optimum growth was at pH 8 with cell density of 6.486. The influence of temperature on cell growth was also determined within the temperature range of 25°C-45°C and the optimum growth was observed at 37°C with cell density of 6.816.

Table 4: Effect of various pH and Temperature on growth of the isolate in the optimized culture medium.

Day*	Cell density [‡] measurement by Spectrophotometer									
	Medium pH ^a					Medium Temperature ^b				
	6	7	8	8.5	9	25	30	37	40	45
1	0.256	2.141	2.564	2.338	0.753	0.363	0.896	2.984	2.685	0.838
2	0.613	5.229	6.486	4.369	1.563	0.549	1.625	6.816	4.619	1.137
3	0.534	4.386	5.236	3.643	0.882	0.426	0.976	5.762	3.427	0.682
4	0.428	3.628	4.842	3.216	0.685	0.392	0.888	4.446	2.966	0.358

‡ Cell density (OD₆₀₀), values is shown as samples were withdrawn at each interval of 24 h cell growth from the culture medium devoid of NaCl; a, pH of the medium was maintained by adding 20% Na₂CO₃ in the culture medium; b, The culture medium temperature is maintained in different degree Celsius individually in an incubator orbital shaker at 150 rpm; *Day, 24 h intervals (OD₆₀₀), values of cell growth in the culture correspondingly relative to extracellular protease secretion in the medium; All the experiments are done in triplicate.

4. Discussion

The evolution of the living organism in the universe is a continuous process, and it is well known that the organisms are being classified according to their phylogenetic trait, but the extent of our knowledge

about the range of metabolic and organismal diversity is limited. Furthermore, the traits are exhibited on its measure which is based on the need of the organism to fit in the environment they live. The behavior of one such organism *Acinetobacter baumannii*, earlier studied only from the clinical environments, has been reported

here and for the first time, the organism was isolated from the salt-cured putrefied skin.

In all moderately halophilic bacteria, the common factors are their ability to fit and grow within a limited range of salt concentrations and these factors are highly variable according to the growth temperature, pH and the nature of nutrient availability. It is evident from the previous reports of *Natronococcus occultus*, *Marinococcus halophilus* and *Salinicoccus alkaliphiles* [21, 22 and 23]. Similarly, in our studies, all the isolates from the sample of salt-cured putrefied skin were screened based on the substrate-induced gene expression for catabolic genes, which link their functional process in the environment [24] and the experiment conducted for potential growth and efficient secretion of extracellular protease in various salt concentrations showed that each isolate has different optimum concentration of salt for the growth (*data not shown*) and levels of protease secretion (Fig. 1). This result implies that analysis of specific functions across all the members of the community can give the portrait on nutrients and energy maintenance of the organisms in the environment [25, 26]. The isolate GTCR407 chosen for further investigations showed optimum growth at 1.8M of NaCl concentration and large zone of protease hydrolysis. This clearly shows that the isolate is halotolerant with

Complex nutrient requirements for growth and they are maintaining the ability to grow in the homogenous saline environment by osmotic regulation [27].

The biochemical characterization and 16S rRNA studies of the isolate GTCR407 identified that the isolate belongs to the genus *Acinetobacter baumannii*. Though these studies confirm the phylogeny of isolate, biochemical characteristics such as positive for acid production from glucose, lactose and xylose, hydrolysis positive for starch and casein, negative for gelatin are of delineating values of phylogenetic classification (Table 1). These characters instigate that isolate is a variant form of the genus itself. Moreover, our isolate appears to be a transitional form possessing various characteristics in common with both genus *Neisseria* and *Moraxella* of the same family. The occurrence of such forms would be the result of genome mosaicity which depends on the habitat of the species in such a way that the free-living species can incorporate large pieces of DNA from the environment [28]. The reason for the non-isolation of this organism from the habitat so far could be attributed to its recalcitrant nature to culture and as reported earlier, the organisms isolated from an environment with the highest frequency are not necessarily those that are present in the highest numbers [27].

In addition with results based on biochemical characterization, the growth and production of extracellular protease by *A. baumannii* in salt containing mediums can be taken into account for delineation of this species because these are

characteristic feature of ecological adaptation and metabolic diversity of the species and this feature is not represented in the species *A. baumannii* yet. This clearly indicates that the *A. baumannii* found in the new free-living habitat would have incorporated the required genes for metabolism and biosynthesis of protease as different selection constraints in the new environment through facilitation of LGT [29, 30]. The metabolically diverse group of bacteria is most prone to LGT to acquire operational genes (code for central metabolism, energy conversion and biosynthesis) than informational genes for novel metabolic functions and they often group the co-regulated genes in operons which would co-inherit by LGT and leads to delineation in microbial taxonomy [14,31,32].

The previous studies state that the production of protease was dependent on the strain and media compositions [33, 34]. This agrees well with our findings and the different levels of production of extracellular protease in the different culture mediums with various salt concentrations by the reported isolate were observed (Table 2 and 3) because some microbial dissimilatory process was found to occur in low and high salinity environments [27] also might be due to the activation of catabolic gene on restricted stress condition to produce extracellular protease where the starvation of nutrients could be the limiting condition and could be supported from earlier study that the regulation of polyphosphate kinase gene expression on the level of phosphate availability in the medium [35]. Alkaliphiles grow at above pH 9 whereas haloalkaliphiles require below pH 9 in addition to high salinity [36]. Similarly, the optimum growth was obtained with cell density value of 6.486 when the medium pH was increased to 8 in our experiments. The culture result shown on different temperatures clearly indicates that the optimum temperature (37°C) induces high growth with a cell density value of 6.816 (Table 4). The influence of physical parameters on the growth of the isolate can also be correlated well with their product formation and reaction rate to production of extracellular protease as well as in other biochemical processes. As evidenced from the previous results, either specifically or non-specifically, extracellular pH controls many enzymatic processes and transport of molecules through porter/antiporter systems [37, 38] and temperature regulate energy metabolism of the organism by transcription and translation [39, 40, 41].

5. Conclusion

Our aim of this report on identifying microorganism was to link the process they carry out within a habitat to the delineation of the genus because the process has potential phylogenetic values. As a result, the existence of this species in the defined environment is due to ecological adaptation which indicates the tendency of the genus for ecological

diversification and these above facts imply that the strain with maximum homology to *A. baumannii* can be delineated into new species within the genus. If the oxidative negative and fermentative and hydrolysis positive types should be separated into subgenus, the species of the genus based on ecology and nutritive forms have to be placed separately. This, however, would necessitate modification within the genus. Further confirmation on the identification of the isolate using intergenic spacer (ITS) region for a more discriminating target sequence of 16S rRNA and 23S rRNA genes, in addition to, nucleic acid hybridization and genetic transformation studies will delineate the isolate into new species as indicated from our results of biochemical characterization and 16S rRNA study. We suggest that *A. baumannii* would have gained extracellular protease secreting metabolic function through LGT from an unrelated species. The investigation of isolate in this ecological niche has impelled the dimension of study which had revealed functional characters of the organism living in the chemical ecology as well as the study stresses the importance of metagenomics evaluation in salt-cured skins and hides of tannery environment in the future.

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