The ability of extremophilic micro-organisms to withstand harsh conditions highlights their great potential in industrial biotechnology. These microorganisms are of particular interest for environmental biotechnologists as they can serve to degrade toxic components present in the environment under extreme conditions. A large number of industries including beverage, pulp and paper and cement industries, release wastewater of highly alkaline nature. The potential of extremophilic bacteria growing at such high pH (Alkaliphiles) could be exploited for treatment of this type of wastewater. In addition, these extremophilic organisms also provide experimental opportunities for examination of physiological processes under conditions in which the stress of the extreme environment brings issues of general biological importance into special focus. The aim of this study was to characterize a novel alkaliphilic bacterium at molecular level in order to gain insight into the basic physiology of alkaliphiles with a goal to develop more robust bioremediation applications using alkaliphilic bacteria.

Highly alkaline waters are a multifarious source of pollution. The pH of this type of wastewater has to be neutralized before it can be released into the environment otherwise it may pose serious threat to the aquatic flora and fauna. The potential of alkaliphilic bacteria to grow in alkaline conditions and to alter the pH of the medium was utilized in this study for the neutralization of alkaline wastewater, taking beverage industry wastewater as a case study. As the organisms isolated from man-made artificial high pH environments containing industrial discharges are expected to be better equipped for the bio-utilization of resources available there, the autochthonous alkaliphilic bacteria were isolated which might have a potential to neutralize alkaline wastewater from beverage industry followed by identification and characterization of the selected bacterium. The parameters for biological neutralization were optimized and the metabolic product(s) released by the bacterium in order to neutralize the medium pH were also identified.

In addition to the applied aspects of the alkaliphilic bacteria, their basic physiology has also fascinated researchers. Since the selected bacterium is a facultative
alkaliphile, it provides us an interesting opportunity to study the adaptation of this bacterium in alkaline vs. neutral conditions. Till date there are only a few alkaliphile genomes available, mostly from the genus *Bacillus* and no alkaliphilic member of genus *Exiguobacterium* has been sequenced yet. Therefore, the genome sequencing of the strain 12/1 was initiated in order to study the niche specific adaptations at the genome level. Once the genome sequence was available, the changes in transcriptome as well as the proteome in response to high pH were followed in order to get a holistic picture of the alkali adaptation of this organism.

A number of alkaliphilic bacteria (21) were isolated from highly alkaline sludge obtained from a beverage industry located near Delhi. The isolated strains were then screened for their maximum growth pH and their potential to neutralize high pH wastewater obtained from beverage industry. On the basis of maximum growth pH and neutralization ability, isolate 12/1 was further characterized and studied for its potential application in neutralization of alkaline wastewater. The strain was identified as a member of genus *Exiguobacterium* by 16S rRNA gene sequencing. A polyphasic taxonomic approach was used to further characterize this bacterium and it was found that the strain differed from the most closely related species (*E. aurantiacum* DSM 6208<sup>T</sup>, *E. aestuarii* DSM 16306<sup>T</sup>, *E. marinus* DSM 16307<sup>T</sup>, *E. mexicanum* DSM 16483<sup>T</sup> and *E. profundum* DSM 17289<sup>T</sup>) in a number of phenotypic and chemotaxonomic properties. Finally DNA-DNA hybridization was carried out to establish that the strain represents a novel species within the genus *Exiguobacterium* for which the name *Exiguobacterium alkaliphilum* is proposed.

Following the preliminary observations on the neutralization potential of isolate 12/1, the neutralization conditions were optimized using Taguchi design of experiment. Experiment with parameters - temperature 35 °C, shaking speed 200 rpm and inoculum size 1:1 showed the best result (least negative S/N value). The parameter - inoculum size had the maximum effect on the neutralization time followed by incubation temperature and agitation speed. The neutralization experiments of beverage industry wastewater were repeated both with wet culture pellet and lyophilized culture which showed promising results. The bacterial neutralization was finally compared with conventional acid neutralization. While the treatment with acid
increased the Total Dissolved Solids (TDS) in the wastewater by 3.5%, the biological neutralization, in contrast, reduced the TDS content by 0.6% suggesting the superiority of biological neutralization over the conventional neutralization.

The type of metabolic products released by the bacterium during the course of neutralization was studied using infrared spectroscopy. It was found that the neutralization is brought about by the production of carboxylic acid which was identified as formic acid by HPLC analysis.

The morphologies of bacterial cells subjected to different pH were studied by electron microscopy. No marked differences in the cell surface properties were found in the scanning electron micrographs. Likewise, in the transmission electron micrographs also, no specialized internal structures or membrane vesicles were observed. Therefore, it was concluded that the alkaliphile adapts to alkaline pH without gross changes in cellular morphology and the adaptation probably occurs by modulation of cellular properties at the transcriptome or proteome level.

In order to understand the basic metabolic pathways in the bacterium and to further study the effect of external medium pH on the bacterium, the whole genome sequencing was performed. The sequencing was carried out using two complementary sequencing technologies – 454 sequencing which produces long reads, and, Illumina sequencing which produces short reads. Following the hybrid assembly of the contigs obtained from both the sequencing platforms, around 2.93 MB of draft genome was generated. The draft genome is similar in length to the two sequenced Exiguobacterium genomes – *Exiguobacterium sibiricum* and *E. sp.* AT1b. The transcriptome of the bacterium grown under high and neutral pH conditions was also sequenced and was mapped onto the draft genome in order to reveal the differential expression. The relevant genes found to be over-expressed at alkaline pH values include those coding for multisubunit cation proton antiporter subunits (involved in maintaining pH homeostasis), solute sodium symporters (involved in sodium coupled solute transport which becomes important in proton limiting alkaline conditions), formate efflux pump (involved in pumping formic acid out of the cell and might aid in reducing pH in the immediate vicinity of the cell) and cardiolipin synthase (involved
in synthesis of cardiolipin which has proton capture type of function around the cell membrane). Similarly, the proteome of the bacterium grown under high and neutral pH values was also subjected to two dimensional SDS-PAGE. The proteins over-expressed at pH 10 include those involved in amino acid and pyruvate metabolism whereas those over-expressed at pH 7 include chaperonins, proteins involved in salvage pathway of purine synthesis and carbonic anhydrase which might be involved in bicarbonate dependent de novo synthesis of unsaturated fatty acids and might have a role in modulating fatty acid composition in response to external pH.

The study provides a strong foundation for the utilization of this bacterium for neutralization of alkaline wastewater from beverage industry at the commercial level. In addition, the basic research conducted here on alkaliophiles is of significance for the understanding of adaptation of microorganisms to high pH environments which is expected to be of wide commercial value.