





















4	Tr Q6HIZ4 Q6HIZ	Model#4	0.4373	0.3124
5	Tr Q6HIZ4 Q6HIZ	Model#5	0.3501	0.2472
6	Tr Q6HIZ4 Q6HIZ	Model#6	0.4175	0.3058
7	Tr Q6HIZ4 Q6HIZ	Model#7	0.3668	0.2575
8	Tr Q6HIZ4 Q6HIZ	Model#8	0.3732	0.2630
9	Tr Q6HIZ4 Q6HIZ	Model#9	0.3882	0.2753
10	Tr Q6HIZ4 Q6HIZ	Model#10	0.3780	0.2763
11	Tr Q6HIZ4 Q6HIZ	Homology model	-----	-----

The final refined model structure of alkaline protease was considered for the evaluation of homology model, which revealed that 90.61% of the residues were in the core region in the Ramachandran map performed by MOE for the analysis of protein geometry structure. It contains the core score > 0.02 and the outlier score < 0.0005. However, only 1.39% of amino acid residues were revealed outside the allowed region (Figure 11).

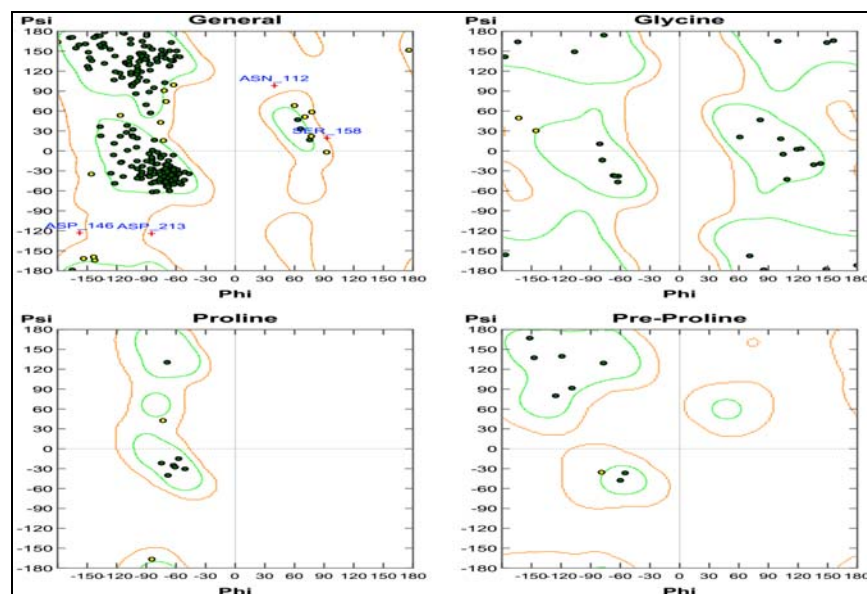


Figure 11. Ramachandran map of the alkaline protease generated by MOE (Version 2008.10).

Further, the results obtained from Verify 3D showed that about 96.40% of the residues had an average 3D-1D score  $\geq 0.2$ , which revealed that the protein structure is acceptable. Besides, the ERRAT2 score values were used to check the quality of the modulated protein, which estimated the overall quality factor of about 86.617 (Figure 12). The overall quality factor is expressed as the percentage of protein for which the calculated error value falls below 95% rejection limit. The good high resolution structures generally produce the values around 95% or higher. For lower resolution (2.5 to 3Å), the average overall quality factor is around 91%. In the present study, the ERRAT score of 86.617% revealed that the predicted protein structure of alkaline protease is well accepted. Similarly, more than 96.4% of the amino acids with the Verify 3D score  $\geq 0.2$  revealed that the modeled structure is highly accurate and could be used for the molecular docking experiment.

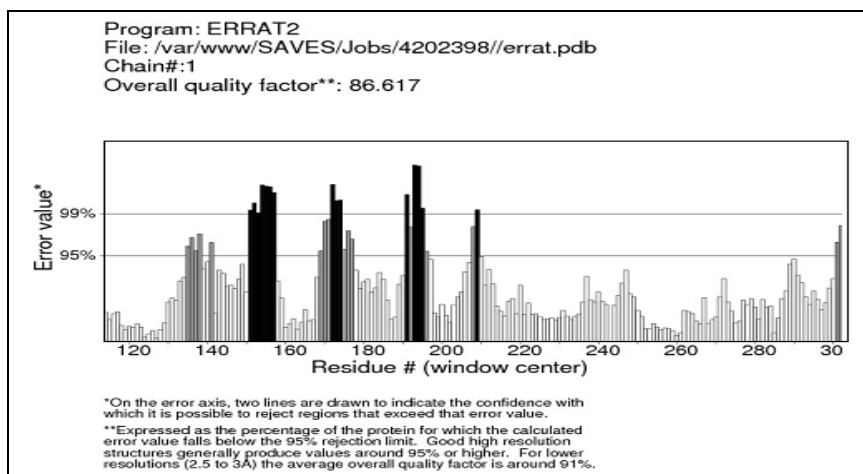


Figure 12. Overall quality factor of the protein generated by the ERRAT server.

The recent advances in bioinformatics with the rapid accumulation of 3D structures of protein complexes through X-ray crystallization provide insights into the protein-protein interactions facilitating rational drug design and treatment of diseases. However, all the protein complexes have not been crystallized and therefore various computational techniques have been developed to address such situation.

One of the promising approaches is protein docking where the structure of a complex between two proteins is predicted based on the independently crystallized structures of the components. The Z-DOCK program performs full rigid-body search of the docking orientations between the two proteins, which includes performance optimization and a novel pair-wise statistical energy potential. The ZDOCK searches all the possible binding modes in the translational and rotational space between the two proteins and evaluates each pose using energy based scoring function. The result of the ZDOCK server was then put into PatchDock to prognosticate the binding affinity with casein protein using PatchDock. PatchDock is geometry based molecular docking based on the shape complementarity principles, which aimed at finding the docking transformations that yield good molecular shape complementarity [55]. Such transformations when applied induce both wide interface areas and small amounts of steric clashes. A wide interface is ascertained to include several matched local features of the docked molecules that have complementary characteristics (Figure 13).

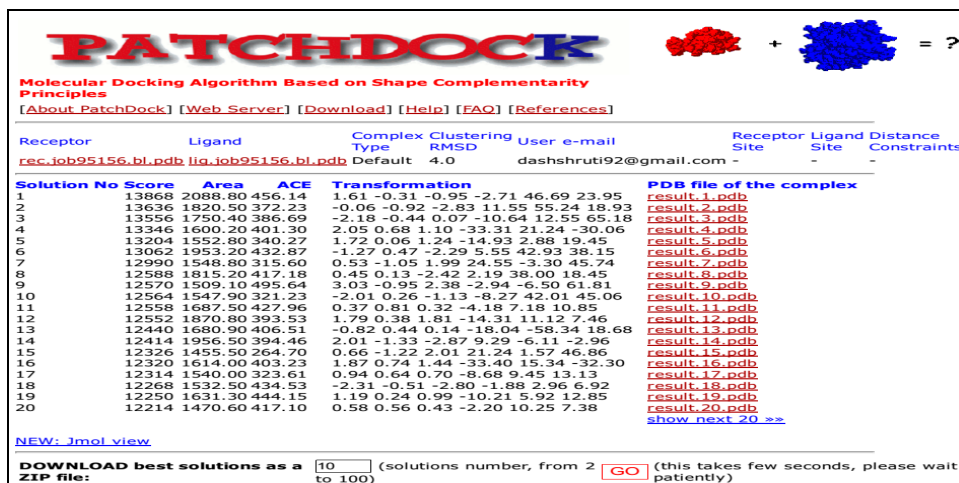


Figure 13. PATCHDOCK representing the geometric score, interface area size and desolvation energy of the top 20 scoring solutions.

PatchDock algorithm divides the Connolly dot surface representation of the molecules into concave, convex and flat patches [56]. Then, the complementary patches are matched in order to generate candidate transformations. Each candidate transformation is further evaluated by scoring function that considers both geometric fit and atomic desolvation energy [57]. Finally, RMSD (root mean square deviation) clustering is applied to candidate solutions to discard the redundant solutions. The main reason behind the PatchDock's high efficiency is its expeditious transformational search, which is driven by the local features matching rather than the brute force probing of the six-dimensional transformation space. Further, it expedites the computational processing time by utilizing the advanced data structures and spatial pattern detection techniques such as geometric hashing and pose clustering that were originally developed in the field of computer vision [55].

The protein complex with PatchDock score of 13868 with interface area of 2088.8 Å was considered as the probable site of interaction in absence of co-crystal structure between alkaline protease and casein (Figure 15). Conclusively, a complex structure was found, which showed that the alkaline protease binds with the casein protein to facilitate its degradation (Figure 14).

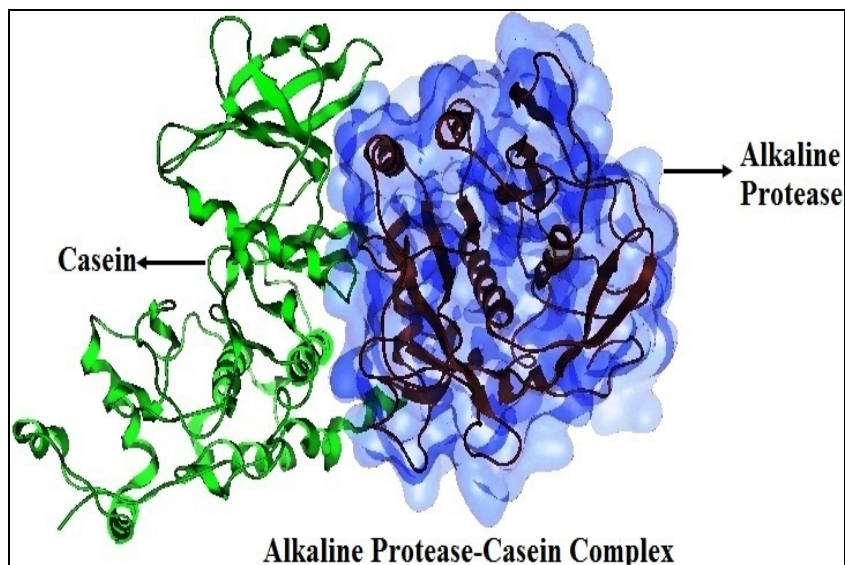


Figure 14. Molecular structure of alkaline protease-casein complex after PATCHDOCK.

### III. CONCLUSION

It is evident from the study that the bacterium isolated from milkshed soil sample is considered to be the good source of alkaline protease production. The isolated bacterium is identified as *Bacillus* sp. based on the morphological, biochemical and molecular characterization. The isolated bacterium was found to exhibit closer resemblance with *Bacillus thuringiensis* based on 16S rDNA sequence comparison. The study revealed that the isolated bacterium exhibited relatively higher growth rate in alkaline medium (pH 9) at 37°C, which was considered to be the favorable conditions for higher protease activity. The sensitivity of bacterium was analyzed through antibiotics sensitivity test. Besides, the thermal death time of the bacterium was determined to be 4 hr at 60°C. Moreover, the molecular modeling suggested that the alkaline protease was being produced by the isolated bacterium has higher binding affinity with casein protein, which facilitate its degradation in soil. The study suggested that the isolated bacterium could be used as an alternative source for the commercial production of alkaline protease for industrial use.

### V. ACKNOWLEDGEMENTS

The authors were thankful to the Head, P.G. Department of Biotechnology and Bioinformatics, Sambalpur University, Odisha for providing the necessary laboratory facilities during the study. Further, the help rendered by several persons during sampling, data analysis and interpretation of data in several counts were duly acknowledged.

### REFERENCES

- [1] B. K. Bajaj and G. Jamwal. "Thermostable alkaline protease production from *Bacillus pumilus* D-6 by using agro residues as substrates", *Advances in enzyme research*, vol. 1(2), pp. 30-36, 2013.
- [2] P. Patil, S. Sabale and A. Devale. "Isolation and characterization of protease producing bacteria from rhizosphere soil and optimization of protease production parameters", *International Journal of Current Microbiology and Applied Sciences*, vol. 2, pp. 58-64, 2015.
- [3] P. Singhal, V. K. Nigam and A. S. "Vidyarthi. Studies on production, characterization and applications of microbial alkaline proteases", *International Journal of Advanced Biotechnology and Research*, vol. 3(3), pp. 653-669, 2012.
- [4] S. Radha, V. J. Nithya, H. R. Babu, A. Sridevi, N. B. L. Prasad and G. Narasimha. "Production and optimization of acid protease by *Aspergillus spp* under submerged fermentation", *Archives of Applied Science Research*, vol. 3(2), pp. 155-163, 2011.
- [5] D. J. Mukesh Kumar, V. Premavathi, N. Govindarajan, M. D. Balakumaran and P. T. Kalaichelvan. "Production and purification of alkaline protease from *Bacillus* sp. MPTK 712 isolated from dairy sludge", *Global Veterinaria*, vol. 8(5), pp. 433-439, 2012.
- [6] J. K. Yang, I. L. Shih, Y. M. Tzeng and S. L. Wang. "Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes", *Enzyme and Microbial Technology*, vol. 26, pp. 406-413, 2000.
- [7] H. M. Kalisz. "Microbial proteinases", *Advances in Biochemical Engineering / Biotechnology*, ol. 36, pp. 1-65, 1988.
- [8] R. Gupta, Q. K. Beg and P. Lorenz. "Bacterial alkaline proteases: molecular approaches and industrial applications", *Applied Microbiology and Biotechnology*, vol. 59(1), pp. 15-32, 2002.

- [9] G. C. Kumar and H. Takagi. "Microbial alkaline proteases: From a bio industrial view point", *Biotechnology Advances*, vol. 17, pp. 561-594, 1999.
- [10] A. Bayouhdh, K. Gharsallah, M. Chamkha, A. Dhouib, S. Ammar and M. Asri. "Purification and characterization of an alkaline protease from *Pseudomonas aeruginosa* MNI", *Journal of Industrial Microbiology and Biotechnology*, vol. 24, pp. 291-295, 2000.
- [11] H. Ogino, F. Watanabe, M. Yamada, S. Nakagawa, T. Hirose, A. Noguchi, M. Yasuda and H. Ishikawa. "Purification and characterization of organic solvent stable protease from organic solvent tolerant *Pseudomonas aeruginosa* PST-01", *Journal of Bioscience and Bioengineering*, vol. 87, pp. 61-68, 1999.
- [12] D. G. Petinate, R. M. Martins, R. R. Coelho, M. N. L. Meirelles, M. H. Branquinham and A. B. Vermelho. "Influence of growth medium in protease and pigment production by *Streptomyces cyanens*". *Mem Inst. Oswaldo Cruz, Rio de Janeiro*, vol. 94, pp. 173-177, 1999.
- [13] N. Akcan and F. Uyar. "Production of extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation", *EurAsian Journal of Biosciences*, vol. 5, pp. 64-72, 2011.
- [14] S. K. Chakrabarti, N. Matsumura and R. S. Ranu. "Purification and characterization of an extracellular alkaline serine protease from *A. terreus*", *Current Microbiology*, vol. 40, pp. 239-244, 2000.
- [15] R. C. Kasana, R. Salwan and S. K. Yadav. "Microbial proteases: detection, production, and genetic improvement", *Critical Reviews of Microbiology*, vol. 37(3), pp. 262-276, 2011.
- [16] S. Saxena, J. Verma, Sikha and D. R. Modi. "RAPD-PCR and 16S rDNA phylogenetic analysis of alkaline protease producing bacteria isolated from soil of India: identification and detection of genetic variability", *Journal of Genetic Engineering and Biotechnology*, vol. 12, pp. 27-35, 2014.
- [17] M. A. Mehmood, U. Sehar and N. Ahmad. "Use of bioinformatics tools in different spheres of life sciences", *Data Mining in Genomics and Proteomics*, vol. 5(2), pp. 158-171, 2014.
- [18] P. Zakeri, B. Jeuris, R. Vandebriel and Y. Moreau. "Protein folds recognition using geometric kernel data fusion", *Bioinformatics*, vol. 30, pp. 1850-1857, 2014.
- [19] E. Gasteiger, A. Gattiker, C. Hoogland, I. Ivanyi and R. D. Appel. "ExpPASy: The proteomics server for in depth protein knowledge and analysis", *Nucleic Acids Research*, vol. 31, pp. 3784-3788, 2003.
- [20] K. R. Aneja. "Microbiology: A laboratory manual". 5<sup>th</sup> (Eds) Wishwa Prakashan, New Age International Pvt Ltd., New Delhi, pp. 268-452, 1996.
- [21] M. T. Madigan and J. M. Martinko. "In: Brock biology of microorganisms". 11<sup>th</sup> ed. New Jersey: Pearson Education, Upper Saddle River, pp. 834, 2006.
- [22] K. G. Chan, S. Z. Tiew and C. C. Ng. "Rapid isolation method of soil *Bacilli* and screening of their quorum quenching activity", *Asia Pacific Journal of Molecular Biology and Biotechnology*, vol. 15(3), pp. 153-156, 2007.
- [23] J. F. Imhoff, H. Sahling, J. Suling and T. Kath. "16S rDNA based phylogeny of sulfur oxidizing bacterial endosymbionts in marine bivalves from cold seep habitats", *Marine Ecology Progress Series*, vol. 249, pp. 39-51, 2003.
- [24] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman. "Gapped BLAST and PSI-BLAST: A new generation of protein database search programs", *Nucleic Acids Research*, vol. 25, pp. 3389-3402, 1997.
- [25] S. Kumar, G. Stecher and K. Tamura. "MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets", *Molecular Biology and Evolution*, vol. 33(3), pp. 2628-2639, 2016.
- [26] N. Saitou and M. Nei. "The neighbor joining method: A new method for reconstructing phylogenetic trees", *Molecular Biology and Evolution*, vol. 4, pp. 406-425, 1987.
- [27] J. Felsenstein. "Confidence limits on phylogenetics: An approach using the bootstrap", *Evolution*, vol. 39, pp. 783-791, 1985.
- [28] M. L. Anson. "The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin", *The Journal of General Physiology*, vol. 22, pp. 79-89, 1938.
- [29] O. Folin and V. Cioalteau. "Enzymatic assay of protease using casein as a substrate", *The Journal of Biological Chemistry*, vol. 73, pp. 627, 1929.
- [30] S. K. Singh, V. R. Tripathi, R. K. Jain, S. Vikram and S. K. Garg. "An antibiotic, heavy metal resistant and halotolerant *Bacillus cereus* SIU1 and its thermoalkaline protease", *Microbial Cell Factories*, vol. 9, pp. 59-65, 2010.
- [31] U. Patil and A. Chaudhari. "Production of alkaline protease by solvent-tolerant alkaliphilic *Bacillus circulans* MTCC 7942 isolated from hydrocarbon contaminated habitat: process parameters optimization", *ISRN Biochemistry*, pp.1-10, 2013.
- [32] A. S. S. Ibrahim, A. Al-Salamah, B. Yahya, Y. B. Elbadawi, M. A. El-Tayeb and S. S. S. Ibrahim. "Production of extracellular alkaline protease by new halotolerant alkaliphilic *Bacillus* sp. NPST-AK15 isolated from hyper saline soda lakes", *Electronic Journal of Biotechnology*, vol. 18, pp. 236-243, 2015.
- [33] C. T. Shivasharana and G. R. Naik. "Ecofriendly applications of thermostable alkaline protease produced from a *Bacillus* sp. JB-99 under solid state fermentation", *International Journal of Environmental Sciences*, vol. 3(3), pp. 956-964, 2012.
- [34] A. Khuro. "One Factor at a time based optimization of protease from poultry associated *Bacillus licheniformis*", *Journal of Applied Pharmaceutical Science*, vol. 6(3), pp. 88-95, 2016.
- [35] M. A. Whooley, J. A. O'Callaghan and A. J. I. McLoughlin. "Effect of substrate on the regulation of exoprotease production by *Pseudomonas aeruginosa* ATCC 10145", *Journal of General Microbiology*, vol. 129, pp. 981-988, 1983.
- [36] M. Folasade, J. Olajuyigbe and O. A. Joshua. "Some properties of extracellular protease from *Bacillus licheniformis* LBBL-11 isolated from "iru", a traditionally fermented African locust bean condiment", *African Journal of Biochemistry Research*, vol. 2(10), pp. 206-210, 2008.
- [37] M. K. Swamy, K. M. Sudipta, K. C. Rohit, B. Purushotham and G. R. Rudramurthy. "Isolation, screening and optimization of factors effecting protease production from *Comomonas kerstersii* KSM7", *International Journal of PharmTech Research*, vol. 6(2), pp. 858-867, 2014.
- [38] O. N. Tiwari, T. B. Devi, K. S. Devi, G. Oinam, T. Indrama, K. Ojit, O. Avijeet and L. Ningshen. "Isolation and optimization of alkaline protease producing bacteria from undisturbed soil of NE region of India falling under Indo-Burma biodiversity hotspots", *Journal of Applied Biology and Biotechnology*, vol. 3(4), pp. 25-31, 2015.
- [39] A. R. Shah and D. Madamwar. "Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization", *Process Biochemistry*, vol. 40, pp. 1763-1771, 2005.
- [40] H. Genckal and C. Tari. "Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats", *Enzyme and Microbial Technology*, vol. 39, pp. 703-710, 2005.
- [41] A. K. Lawal, S. O. Olatope, Y. L. Majolagbe, F. A. Alebiosu, J. B. Bashar, O. F. Kayode, E. N. Dike, S. O. Akinola and G. N. Elemo. "Microbial production of alkaline protease", *Prime Journal of Microbiology Research*, vol. 1(2), pp. 27-37, 2011.
- [42] F. Uyar, I. Porsuk, G. Kizil and E. I. Yilmaz. "Optimal conditions for production of extracellular protease from newly isolated *Bacillus cereus* strain CA15", *European and Asian Journal of BioSciences*, vol. 5, pp. 1-9, 2011.

- [43] M. K. Swamy, S. S. N. Kashyap, R. Vijay, R. Tiwari and M. Anuradha. "Production and optimization of extra cellular protease from *Bacillus* sp. isolated from soil", *International Journal of Advanced Biotechnology and Research*, vol. 3(2), pp. 564-569, 2012.
- [44] N. Tekin, A. C. Cihan, Z. S. Takac, C. Y. Tuzun, K. Tunc and C. Cokmus. "Alkaline protease production of *Bacillus cohnii* APT5", *Turkish Journal of Biology*, vol. 36, pp. 430-440, 2012.
- [45] B. K. M. Lakshmi, P. V. Ratnasri, K. A. Devi and K. P. J. Hemalatha. "Screening, optimization of production and partial characterization of alkaline protease from haloalkaliphilic *Bacillus* sp.", *International Journal of Research in Engineering and Technology*, vol. 3(2), pp. 435-443; 2014.
- [46] P. Potier, P. Drevet, A. M. Gounot and A. R. Hip kiss. "Temperature dependent changes in proteolytic activities and protein composition in the psychotropic bacterium *Arthrobacter gzbiformis* s155", *Journal of General Microbiology*, vol. 136, pp. 283-291, 1990.
- [47] C. S. Rao, T. Sathish, M. Mahalaxmi, G. S. Laxmi, R. S. Rao and R. S. Prakasham. "Modelling and optimization of fermentation factors for enhancement of alkaline protease production by isolated *Bacillus circulans* using feed forward neural network and genetic algorithm", *Journal of Applied Microbiology*, pp. 889-898, 2007.
- [48] M. A. Yusuf and T. A. T. Abdul Hamid. „Optimization of temperature and pH for the growth and bacteriocin production of *Enterococcus faecium*. B3L3", *IOSR Journal of Pharmacy*, vol. 2(6), pp. 49-59, 2012.
- [49] B. Bhunia, B. Basak and A. Dey. "A review on production of serine alkaline protease by *Bacillus* sp.", *Journal of Biochemical Technology*, vol. 3(4), pp. 448-457, 2012.
- [50] P. Palsaniya, R. Mishra, N. Beejawat, S. Sethi and B. L. Gupta. "Optimization of alkaline protease production from bacteria isolated from soil", *Journal of Microbiology and Biotechnology*, vol. 2(6), pp. 858-865, 2012.
- [51] P. M. Rodrigues, V. V. Andrade and M. L. L. Martins. "Stability and activity of the partially purified spray dried protease from bacillus sp smia-2 and its characterization as a laundry detergent additive", *International Journal of Bioassays*, vol. 2(3), pp. 562-567, 2013.
- [52] D. Revathi and A. Palanisamy. "Production, purification and characterization of protease from *Yersinia* sp and *Staphylococcus* sp.", *International Journal of Advanced Research*, vol. 3(7), pp. 424-434, 2015.
- [53] O. P. Verma, P. Kumari, S. Shukla and A. Singh. "Production of alkaline protease by *Bacillus subtilis* (MTCC7312) using submerged fermentation and optimization of process parameters", *European Journal of Experimental Biology*, vol. 1(3), pp. 124-129, 2011.
- [54] M. Nei and S. Kumar. "Molecular evolution and phylogenetics", Oxford University Press, New York, pp. 788-1034, 2000.
- [55] S. D. Duhovny, Y. Inbar, R. Nussinov and H. J. Wolfson. "PatchDock and SymmDock: servers for rigid and symmetric docking", *Nucleic Acids Research*, vol. 33, pp. 363-367, 2005.
- [56] M. L. Connolly. "Solvent accessible surfaces of proteins and nucleic acids", *Science*, vol. 221, pp. 709-713; 1983.
- [57] C. Zhang, G. Vasmatzis, J. L. Cornette and C. DeLisi. "Determination of atomic desolvation energies from the structures of crystallized proteins", *Journal of Molecular Biology*, vol. 267, pp. 707-726, 1997.