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MINI-REVIEW

Trends in extracellular serine proteases of bacteria as detergent bioadditive: alternate and environmental friendly tool for detergent industry

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Abstract

Proteases, one of the largest groups of industrial enzymes occupy a major share in detergent industry. To meet the existing demands, proteases with efficient catalytic properties are being explored from bacteria residing in extreme habitats. Alkaline proteases are also considered as promising candidates for industrial sectors due to the activity and stability under alkaline and harsh environment. Therefore, a systematic review on experimental studies of bacterial proteases was conducted with emphasis on purification, characterization, cloning and expression and their suitability as detergent additive. Relevant searches using a combination of filters/keywords were performed in the online databases; PubMed, Science Direct, Scopus and Web of Science. Over thousands of research papers, 71 articles in Scopus, 48 articles in Science Direct, 18 articles in PubMed and 8 articles in Web of Science were selected with regard to bacterial extracellular proteases till date. Selected articles revealed majority of the studies conducted between the years 2015 and 17 and were focused on purification of proteases from bacteria. Among microbes, a total of 41 bacterial genera have been explored with limited studies from extreme habitats. Majority of the studies have reported the involvement of subtilisin-like serine proteases with effective properties for detergent industries. The studies revealed shifting of trend from purification to cloning to genetic engineering to meet the industrial demands. The present systematic review describes the proteases from extremophilic bacteria and use of biotechnological techniques such as site-directed mutagenesis and codon optimization to engineer enzymes with better hot spots in the active sites to meet industrial challenges.

Keywords Proteases/peptidases/proteolytic · Serine · Cloning · Expression · Extremophiles

Introduction

Global market share of industrial enzymes was expected approximately $4.4 billion in 2015 http://bccresearch.blogspot). Among these industrial enzymes, proteases with over 60% share of worldwide market are being used as bioadditives in laundry, pharmaceutical, leather, food, agriculture and other industries (Gonzalez-Rabade et al. 2011; Sharma et al. 2016). Microbes from extreme environments in past decades serve as a centre of scientific spotlight which are being explored to meet existing industrial demands. The microbial enzymes from different habitats are continuously explored due to their better catalytic potential at low or moderate temperatures (Haki and Rakshit 2003). In present scenario and thrust to explore energy efficient industrial processes, attention on the use of enzymes from extremophiles has gained rapid attention keeping in view the importance of green processes. In comparison to mesophilic counterparts, extremophiles such as thermophiles and psychrophiles are being explored due to their better structural adaptability and enhanced specific activity of their enzymes at low temperature (Cowan et al. 1985; De Azeredo et al. 2003; Hajji et al. 2008; Zaferanloo et al. 2014; Boulkour et al. 2015). Bioadditives reported from psychrotrophs can interact with water,

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hydrophobic compounds, ice and gases at low temperature and hence offers a green sustainable and energy efficient processes and other biotechnological applications (Wu et al. 2004; Zhang et al. 2016; Yildirim et al. 2017).

Microbes from Arctic, Antarctic, and alpine zone containing ~ 6500 microbial strains have been explored for cold-active extracellular proteases, lipases, and exopolysaccharides production (Perfumo et al. 2018). Among proteases, alkaline proteases are the leading contributors and account for ~ 40% of the total global enzyme for industry (Kirk et al. 2002). Screening microbes from extreme environments can facilitate our thrust for energy efficient enzymes (Raval et al. 2014). Since the discovery of subtilisin Carlsberg, a first alkaline protease from *Bacillus licheniformis* for detergent industry (Lesney 2003), a number of alkaline proteases from *Aeribacillus pallidus* (Yildirim et al. 2017; Mechri et al. 2017), *Bacillus circulans* DZ100 (Benkiar et al. 2013), *Bacillus pseudofirmus* (Raval et al. 2014), *Bacillus pumilus* NRC21 (Tork et al. 2016), *Bacillus sphaericus* (Wati et al. 1997a, b), *Bacillus* sp. EMB9 (Sinha and Khare 2013) and WF146 (Wu et al. 2004), *Bacillus subtilis* APMSU6 (Maruthiah et al. 2013), *Caldicoprobacter guelmensis* (Bouacem et al. 2015), *Geobacillus caldoproteolyticus* (Chen et al. 2004), *Geobacillus toebii* Strain LBT 77 (Thebti et al. 2016), *Planococcus* sp. (Zhang et al. 2016), *Pseudoalteromonas haloplanktis* (dePascale et al. 2010), *Serratia proteamaculans* Gulmez et al. 2018), *Shewanella arcticai* (Gulmez et al. 2018), *Stenotrophomonas maltophilia* (Ribitsch et al. 2012), *Streptomyces* sp. AB1 (Jaouadi et al. 2010) and *Streptomyces* sp. strain AH4 (Touioui et al. 2015) have been characterized, cloned and explored for various biotechnological applications such as detergent, leather and feather degradation. Proteases from microbes isolated from extreme habitats with different features such as hot springs (Thebti et al. 2016), halophilic area, arctic or polar regions, deep sea, glacier ice and cold desert soils can be harnessed for efficient enzymes for various applications (Zhang et al. 2016). Limited studies has been done on the exploration of proteases for industry using culture independent techniques from extreme habitats (Qoura et al. 2015; Singh et al. 2015).

Application of proteases in detergents as green process with high activity at low temperature has brought a major shift from being minor additives to key ingredients. Proteases are considered as one of the most important industrial enzymes with applications in detergents, foods, textiles and leather, pharmaceuticals, silvery recovery from X-ray films and bioremediation processes. Presently, a number of *Bacillus* strains from alkalophilic habitats have been explored for detergents applications and first study was reported from *Bacillus alcalophilus* in 1934 by Vedder (Fritze et al. 1990; Gupta et al. 2002). Use of proteases in detergents constitutes ~ 25% of the total worldwide enzymes sales (Godfrey and West 1996). Among them, alkaline proteases or subtilisins of *Bacillus* sp. contribute to ~ 89% share of detergent enzymes and Novo Nordisk and Genencor International are the two major distributors covering ~ 95% global market of proteases (Gupta et al. 2002). A number of proteases with trademark such as Alcalase, BIO-40, and BIOTEX have been explored from *Bacillus* spp. (Lesney 2003). Industrial demands for proteases rely on a broad specificity for substrates, wide temperature and pH range and stability, and compatibility in chelating and oxidizing environments for the effective removal of stains. Proteases like kannases have been commercialized by Novo Nordisk Bioindustry, Japan.

Exploration of microbes from these extreme habitats offers access to novel microbes and robust enzymes which can meet industrial demands. Keeping in view the importance of proteases from extremophiles, an attempt has been made to summarise the literature published between 2000 till present based on the information collected from different databases.

Database search and selection criteria

PubMed https://www.ncbi.nlm.nih.gov/pubmed/), ScienceDirect https://www.sciencedirect.com/), SCOPUS https://www.scopus.com/search/form) and Web of Science Clarivate Analytics version 1.0 were selected for online retrieval of the literature searches using a combination of keywords as filters, i.e. Protease) AND Peptidase) AND Proteolytic) AND Serine) AND Cloning, expression) AND Bacteria/bacterial/bacterium) AND Detergent/laundry). To limit the search, further terms like extracellular, purification, and recombinant were also included. Search was performed by the authors independently and data published till date was used for the present review. The literature included in the present study involved research articles exclusively and reviews articles were only studied and cited for supporting information on microbial proteases. Data extraction was conducted to establish reliability and avoiding any data entry errors.

A total of 4,97,201 articles were found with the keyword proteases in PubMed, 2,53,165, articles in Science Direct, 39,400 in Web of Science and 1, 82,895 in SCOPUS between the years 2000 to till present Fig. 1a). After applying all the keywords, a comprehensive list of abstracts was prepared to meet the inclusion criteria. Relevant studies based on titles and abstract were screened and selected for full-text evaluation. Duplicate entries from all the databases were screened and removed manually as well as by Mendeley. Studies highlighting purification and characterization, cloning and expression in heterologous host and application of purified protease as detergent additive and other industrial aspects were selected. After exclusion of irrelevant data, only 18

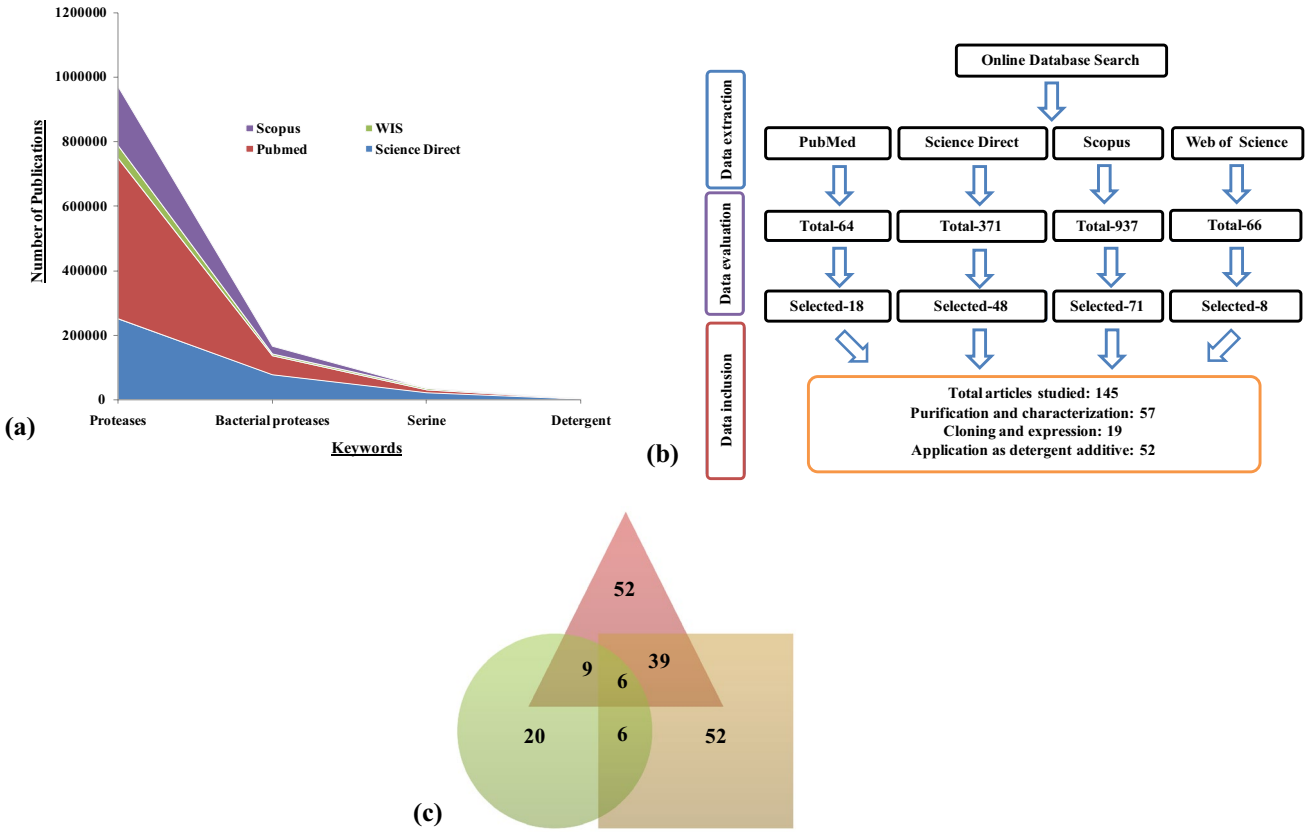

Fig. 1 **a** Publications of research articles in (2000–till present) Scopus, Web of sciences, Pubmed and Science Direct using keywords (Protease) AND (Peptidase) AND (Proteolytic) AND (Serine) AND (Bacteria) AND (Detergent/laundry); **b** publications of research articles in Science Direct, Pubmed, Web of sciences and Scopus, using a keywords (Protease) AND (Peptidase) AND (Proteolytic) AND (Serine) AND (Detergent/laundry) from 2000 onwards to till present. The research articles evaluated for removal of duplicates, studies exactly not following the inclusion criteria and incomplete experimental research were removed; and **c** Venn diagram showing experimental studies on purification, expression and application of bacterial proteases as detergent additive based on research articles shared among Science Direct Scopus, PubMed and Web of Science. Triangle shows number of research articles on purification of proteases, circle shows number of research articles on expression of proteases in heterologous host and square shows number of articles on proteases with application as detergent additive

articles from PubMed, 48 articles from Science Direct, 8 articles from Web of Science and 537 from SCOPUS were retrieved and evaluated for the desired inclusion criteria (Fig. 1b). The full-text of selected research articles were selected and imported into Mendeley reference manager.

The experimental studies on proteases from extremophilic bacteria revealed 52 articles on purification and characterization of proteases, 19 articles on cloning and expression in heterologous host and 52 articles showing suitability of protease as detergent additive (Fig. 1b). Further analysis revealed 9 articles on purification of protease and expression in heterologous host, 6 articles on purification, expression and application as detergent additive, 39 articles on purification and application as detergent additive and only 6 articles on expression of protease in heterologous host with application as detergent additive (Fig. 1c).

Diversity of proteases producing bacteria residing in extreme environments

Evaluation of these research articles revealed 62% proteases from mesophiles, 13% from thermophiles, 11% from halophiles, 7% from psychrophiles and only 6% from alkaliphiles. Moreover, a total of 41 genera have been reported for protease production. Based on selected data used for comprehensive and systematic data analysis, 145 articles belonging to 41 bacterial genera were reported for purification, cloning and expression of proteases in heterologous host with majority of the studies

Table 1 Extracellular bacterial proteases explored as additives in industrial processes

S. No	Bacteria	Type of protease	Purification	pH	Temperature (°C)	Metal	Application	References
1.	*Acinetobacter* sp. IHB B 5011	Subtilisin	Ni–NTA	9	40	–	Laundry	Salwan et al. (2018)
2.	*Bacillus halotolerans*	Serine	Sephacryl S200, SP-Sepharose	9	50	Ca^{2+}, Mn^{2+}	Industrial	Dorra et al. (2018)
3.	*Bacillus subtilis CFR5*	Peptidase	–	8	40	Ca^{2+}	Deactivates trypsin inhibitor	Sharmila et al. (2018)
4.	*Lysinibacillus fusiformisC250R*	Serine	–	10	70	–	Detergent	Mechri et al. (2017)
5.	*Bacillus subtilis* PTTC 1023	Subtilisin	–	10.5	50	–	Detergent	Gulmez et al. (2017)
6.	*Lysobacter* sp. A03	Peptidase	–	9	40	Ca^{2+}	Industrial	Pereira et al. (2017)
7.	oil-polluted Mud flat metagenomic	Peptidase M48	–	8	50	–	Detergents	Gong et al. (2017)
8.	*Pseudomonas fluorescens* strain TBS09	Metalloprotease	–	7	60	–	–	Boulkour et al. (2015)
9.	*Sphaerobacter thermophiles*	Prolyl endoprotease	–	6.6	63	Co^{2+}	Gluten degradation	Shetty et al. (2017)
10.	*Aeribacillus pallidus* strain VP3	Serine	FPLC anion exchange	10	60	–	Detergent	Mechri et al. (2017)
11.	*Stenotrophomonas maltophilia* N4	Serine	–	10.5	50	Ca^{2+}	Biocidal	Jankiewicz et al. (2016)
12.	Metagenome from tannery activated sludge	Serine	Ni–NTA	11	55	Ca^{2+}	Detergent	Devi et al. (2016)
13.	*Dichelobacter nodosus*	Subtilisin-like serine	Ni–NTA	7	35	Ca^{2+}	Pharmaceuticals	Wani et al. (2016)
14.	Metagenom of Yucatán underground water	Serine	–	–	–	–	–	Apolinar–Hernández et al. (2016a, b)
15.	*Actinomadura viridilutea* DZ50	Serine keratinase	FPLC anion exchange, HPLC	11	80	–	Industrial	Elhoul et al. (2016)
16.	*Bacillus Pumilus* NRC21	Metallokeratinase	Cation exchange, gel filtration	8.5	50	Na^{+}, K^{+}, Mg^{2+}	–	Tork et al. (2016)
17.	*Shewanella arctica*	Subtilisin-like serine	Ni–NTA	8	60		Detergent	Qoura et al. (2015)
18.	Chumathang hot spring sediment	Serine	–	11	80	–	Biotechnological	Singh et al. (2015)
19.	*Bacillus* sp.	Serine protease	–	6.5	54	Ca^{2+}, Mg^{2+} Zn^{2+} and Ba^{2+}	Fibrinolytic activity	Cheng et al. (2015)
20.	*Streptomyces koyangensis* strain TN650	Serine thiol	–	10	70	–	detergent	Elhoul et al. (2015)
21.	*Streptomyces flavogriseus*	–	–	7	50	–	Detergent	Ghorbel et al. (2014)

Table 1 (continued)

S. No	Bacteria	Type of protease	Purification	pH	Temperature (°C)	Metal	Application	References
22.	*Actinomadura keratinilytica* strain Cpt29	Keratinase		10	70	Mn^{2+}	Feather-biodegradation	Habbeche et al. (2014)
23.	*Bacillus gibsonii*	Subtilisin	–	–	60	–	Detergents	Martinez et al. (2013)
24.	*Bacillus circulans* strain DZ100	Serine	Ni–NTA	12.5	85		Detergent, biodegradation of feather	Benkiar et al. (2013)
25.	*Metagenome from saline habitat*	Serine	–	–	–	–	–	Purohit and Singh (2013)
26.	*Acinetobacter sp. IHB B 5011*	Serine	–	9	40	Na^{+}, Mn^{2+}, Ca^{2+}, Zn^{2+}	Detergents	Salwan and Kasana (2013)
27.	*Serratia proteamaculans* (PSP)	–	–	–	–	–	–	Mikhailova et al. (2014)
28.	*Stenotrophomonas maltophilia*	–	–	–	–	–	–	Ribitsch et al. (2012)
29.	*Pseudoalteromonas haloplanktis* TAC125	Zn-dependent aminopeptidase	–	–	–	–	Biotechnological	dePascale et al. (2010)
30.	*Aeromonas veronii* PG01	Metalloprotease	GPC, HPLC	7.5	60	–	–	Divakar et al. (2010)
31.	Metagenome	–	–	9.5	40	–	Detergent	Ribitsch et al. (2010a, b)
32.	*Bacillus pumilus* CBS	–	–	12	75	–	–	Jaouadi et al. (2010)
33.	*Streptomyces* sp. strain AB1	–	–	11.5	75	Mg^{2+}	Detergent formulations, dehairing	Jaouadi et al. (2010)
34..	*Mycobacterium leprae*	–	–		40	–	Therapeutic strategies	Ribeiro-Guimarães et al. (2009)
35.	*Bacillus* sp. HR-08	Serine alkaline protease	DEAE-Sepharose ionic exchange and Sephacryl S-200	10	60	Ca^{2+}	detergents	Moradian et al. (2009)
36.	*Oenococcus oeni*	–	–	7	45	–	Wine protein hydrolysis	Folio et al. (2008)
37.	*Streptomyces* sp	Serine metalloprotease/ keratinase	Sephacryl S200	11	45	Ca^{2+}	Detergent and leather	Tatineni et al. (2008)
38.	*Burkholderia pseudomallei*	Serine metalloprotease	Ni–NTA	5, 11	4, 68	–	–	Chin et al. (2007)
39.	*Chryseobacterium* sp. kr6	Zn-metalloprotease/M14	Phenyl Sepharose and Superose 12HR	8.5	50	Ca^{2+}	Feather-degrading and de-hairing activities	Riffel and Brandelli (2007)
40.	*Streptococcus pneumoniae*	–	–	–	–	–	Candidate vaccine component	Romanello et al. (2006)
41.	Pseu*domonas aeruginosa* strains PA103	–	Affinity	–	–	–	Virulence-promoting factor	Marquart et al. (2005)
42.	*Streptomyces septatus* TH-2	Metalloendopeptidase	–	–	–	Zn^{2+}	–	Hatanaka et al. (2005)

Table 1 (continued)

S. No	Bacteria	Type of protease	Purification	pH	Temperature (°C)	Metal	Application	References
43.	*Bacillus cereus*	Subtilisin	Anion-exchange chromatography, and gel filtration	10.5	50	–	–	Prakash et al. (2005)
44.	*Bacillus licheniformis*	Glutamyl-specific endopeptidase	Superdex 75 column	8.5	4	–	–	Trachuk et al. (2005)
45.	*Archaeoglobus fulgidus*	–	–	–	–	–	–	Botos et al. (2005)
46.	*Bacillus* sp. WF146	Subtilisin-like	–	–	85	Ca^{2+}	–	Wu et al. (2004)

from *Bacillus* spp. (Table 1). Different bacteria explored for industrial applications included *Acinetobacter*, *Actinomadura*, *Aeribacillus*, *Aeromonas*, *Aeropyrum*, *Alcaligenes*, *Archaeoglobus*, *Bacillus*, *Brevibacillus*, *Burkholderia*, *Caldicoprobacter*, *Chryseobacterium*, *Dermatophilus*, *Dichelobacter*, *Fibrobacter*, *Lactobacillus*, *Lysinibacillus*, *Lysobacter*, *Micromonospora*, *Mycobacterium*, *Nocardiopsis*, *Oenococcus*, *Paenibacillus*, *Planococcus*, *Prevotella*, *Pseudoalteromonas*, *Pseudomonas*, *Salinicoccus*, *Serratia*, *Shewanella*, *Sphaerobacter*, *Stenotrophomonas*, *Streptococcus*, *Streptomyces*, *Teredinobacter*, *Thermoactinomyces*, *Thermoanaerobacter*, *Thermus*, *Treponema*, *Virgibacillus*, *Xanthomonas*. Among different bacterial proteases, *Bacillus* and *Streptomyces* constituted the major part of literature (Table 1).

Among all publications, majority of the reported proteases have been reported as subtilisin-like serine proteases with limited reports on glutamyl-endopeptidase, Zn-metalloprotease/M14, proline specific prolyl endoprotease and kumamolysin. These proteases have been reported for effective wash performance in combination with detergents for suitability as additive in laundry (Table 1). Few reports on application of proteases in feather degradation, leather, fibrinolytic, pharmaceutical and biocidal activity have also been recorded. The research articles revealed purification of proteases using anion exchange, cation exchange, gel filtration, hydrophobic interaction and affinity, as well as FPLC and HPLC chromatography. The chromatographic based methods involve use of SP Sepharose, DEAE-cellulose, DEAE-Sepharose, Phenyl-Sepharose and superose, Sephadex G-100, Superdex-75, Sephacryl S-200, Superose and Ni–NTA column (Table 1). The molecular weight of the purified proteases ranged from 19.5 to 250 kDa and have optimum temperature range of 4–100 °C and pH range of 5–13 (Gabdrakhmanova et al. 2002, Trachuk et al. 2005; Snajder et al. 2015; Chin et al. 2007; Thebti et al. 2016). The presence of metal ions like Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Ba^{2+}, Mn^{2+}, Na^{+} and K^{+} stimulated enzyme activity (Kasana 2010). Regarding inhibition effect, majority of the proteases are inhibited in the presence of inhibitors PMSF and DFP, thus identified as subtilisin-like serine proteases. Other proteases were inhibited in the presence of inhibitors aprotinin, EDTA, EGTA, 1, 10-phenanthroline and phosphoramidon.

Structural analysis of bacterial extracellular S8 serine proteases

Representative proteases from thermophilic, mesophilic and psychrophilic bacteria were selected to compare their structural variation and evolutionary history. The gene sequences of serine proteases ranged from 1239 to 2371 bp have been identified as subtilisin like serine proteases belonging to

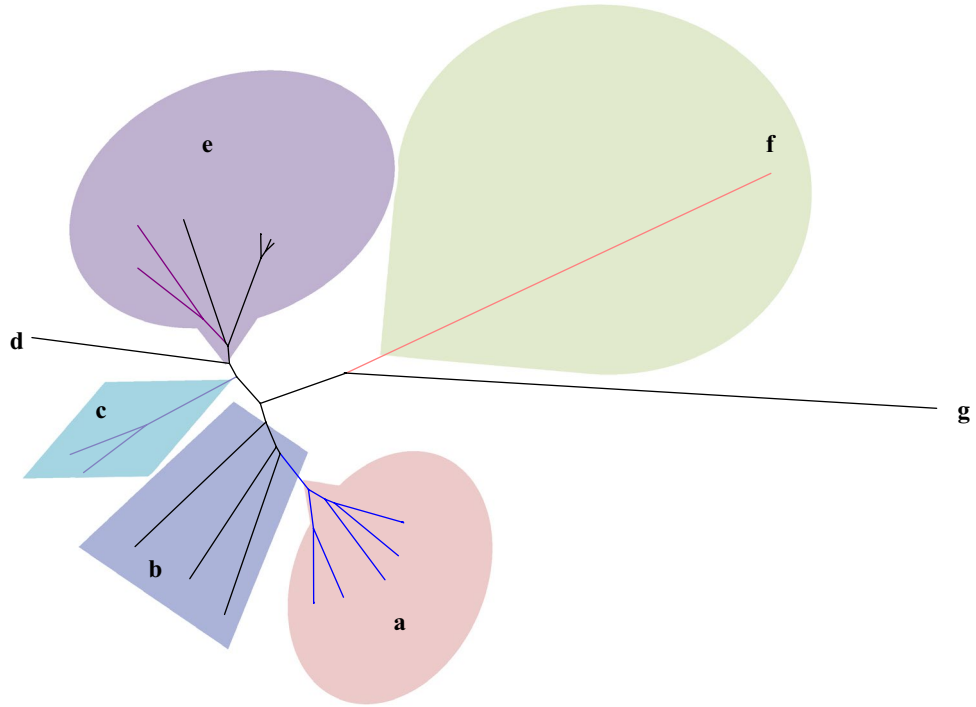

Fig. 2 Phylogenetic analysis of S8 family alkaline serine proteases showing clustering of psychrophilic and thermophilic proteases as different branches **a** WP 010369807 *Pseudoalteromonas piscicida,* WP 063374258; *Pseudoalteromonas luteoviolacea,* WP013051407; *Shewanella violacea* BAB61726, *Pseudoalteromonas* sp. AS-11, ABA60899 *Serratia* sp. GF96, WP 012197479 *Shewanella baltica*; **b** WP 094058666 *Bacterioplanes sanyensis,* WP 017819842 *Vibrio alginolyticus,* WP 015781271 *Kangiella koreensis*; **c** Thermophilic proteases WP 01083250 *Nocardioides* sp. CF8, WP 084100044 *Knoellia flava*; and **d** WP 027481108 *Deinococcus pimensis*; **e** WP 013704806 *Marinithermus hydrothermalis,* WP 013456966 *Oceanithermus profundus,* WP 082381964 *Ardenticatena maritima*; P08594 aqualysin-1, WP 053768483 *Thermus aquaticus,* WP 038035270 *Thermus parvatiensis,* WP 093005298 *Thermus arciformis*; **f** WP 010376775 *Pseudoalteromonas piscicida*, WP 039492850 *Pseudoalteromonas elyakovii,* WP 010605882 *Pseudoalteromonas flavipulchra* **g** AGV12715 *Acinetobacter* sp. IHB B 5011 (MN12). The phylogenetic tree was prepared in MEGA 7.0 using N–J method

family peptidase S8. Nine studies reported identification of proteases based on MALDI/TOF and N-terminal sequencing. Phylogenetic analysis of extracellular serine proteases was conducted using MEGA7 to analyse their association among each other. The analysis revealed close relatedness and clustering of proteases from thermophiles and psychrophiles as separate branches (Fig. 2).

The N-terminal signal peptide was predicted to determine the extracellular nature of proteases using SignalP Server 4.0 http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011). The size of signal peptide is variable and ranges from 21 to 29 amino acid long among different proteases reported from extremophiles which facilitates secretion of prosubtilisin across cytoplasmic membrane. The structural analysis of representative proteases from thermophilic, mesophilic and psychrophilic bacteria was done using NCBI-CDD (http://www.ncbi.nlm.nih.gov/cdd) to predict active site residues, calcium binding sites and catalytic triad (Marchler-Bauer et al. 2005). The amino acid sequences of these proteases were also compared to predict the location of domains and inhibitor sites in SMART (Letunic et al. 2006). The domain analysis of representative proteases of psychrophilic *Shewanella arctica* revealed presence of inhibitor (41–120 aa), peptidase S8 (156–402 aa) and PPC domain (436–505 aa and 506–619 aa), thermophilic revealed the presence of signal peptide, preprosequence, peptidase S8 domain, inhibitors and PPC domain with variation in amino acid residues (Fig. 3). The domain analysis of thermophilic *Thermus aquaticus* revealed presence of signal peptide (1–25 aa), inhibitor (54–126 aa) and peptidase S8 domain (157–399 aa). The domain analysis of *Pseudoalteromonas flavipulchra* revealed signal peptide (1–27 aa), peptidase S8 (182–499 aa) and two PPC domain (524–594 aa and 632–701 aa). PPC domains are present only in psychrophilic bacteria and absent in thermophilic and mesophilic bacteria. The amino acid sequence of mesophilic *B. clausii* showed presence of signal peptide (1–29 aa), inhibitor (30–108) and peptidase S8 domain (131–374 aa). The domain analysis was also compared with previously reported alkaline serine protease of *Acinetobacter* sp. IHB B 5011 that showed the presence

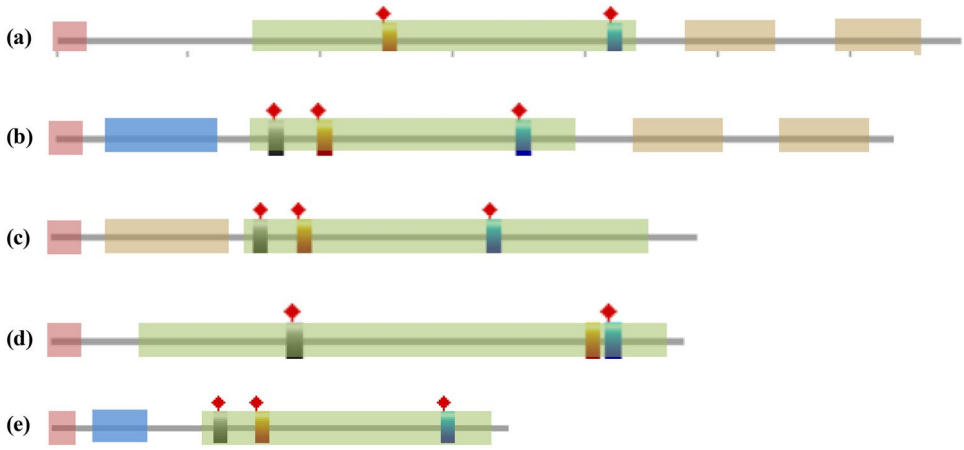

Fig. 3 Domain analysis showing variation in position of signal peptide, inhibitor (if present) peptidase S8 and PPC domain in S8 proteases of **a** WP_010605882 *Pseudoalteromonas flavipulchra*; **b** WP_013051407 *Shewanella violacea*; **c** WP_053768483 *Thermus aquaticus*; **d** AGV12715 *Acinetobacter* sp. IHB B 5011 (MN12); **e** AGN91700 *Bacillus circulans* DZ100. The scanning of active sites was performed in ScanProsite tool in ExPASy

of signal peptide, pre-prosequence which guides correct folding of mature peptidase S8 domain (Salwan et al. 2018). No inhibitor and PPC domain were reported here. The present data revealed limited studies involving maturation of active proteases after N and C-terminal processing. Such autoprocessing has been reported in proteases of *Bacillus* spp., *Burkholderia pseudomallei* (Chin et al. 2007), *Lysobacter* sp. (Pereira et al. 2017), *Streptococcus pneumonia* (Romanello et al. 2006), *Stenotrophomonas maltophilia* Ribitsch et al. 2012) and *Thermus aquaticus* (Terada et al. 1990;). Ribitsch et al. 2012 reported truncation of the C-terminal domain of StmPr1 protease of *Stenotrophomonas maltophilia* which caused enhanced processing of N-terminal prosequences and hence production of active enzyme.

Homology modelling of peptidase S8 domain was done using Swiss-Model (Fig. 4) (http://swissmodel.expasy.org/) based on the template with PDB code 3TI7 *Dichelobacter nodosus*, 2B6N *Serratia* sp., 4DZT *Thermus aquaticus* and 3WHI *Bacillus subtilis*. The structure of peptidase S8 domain showing 66.48% sequence identity were prepared and aligned with the templates for quality assessment. All the model proteins showed root mean square deviation RMSD value below 1.5 Å which validates. All the model proteins were submitted to VERIFY-3D server http://servicesn.mbi.ucla.edu/Verify3d/) for validation and showed the acceptance of models with 95–100% passing score. PROCHECK analysis was done to check stereo-chemical quality properties of the model proteins which revealed over 90% of the residues in the most favourable regions with only 0.4–0.7% of the residues in disallowed regions.

Engineering methods for enhanced production and effective catalytic behaviour

According to the literature, research articles were also searched for characterization of recombinant proteins, optimised expression of recombinant proteins and methods for engineering enzymes. Only 20 studies revealed the cloning and expression of proteases in heterologous host (Fig. 1b). The genes of varying size were successfully cloned in vectors like pHY300PLK, pMS470Δ8, pET28a), pET19-b, pET32a-c +), pWEB-TNC, pBR322, pET22b, pET-30, pTrc99A, pTrc99A, pMCSGx, pWH1520, pD441-NH, pET-5a, pIJT02, pGEX-4T-2 and expressed in heterologous host such as *Bacillus megaterium, B. subtilis* AJ73, *B. thuringiensis*, *E. coli* BL21DE3, *E. coli* rosetta-gami DE3 and *Streptomyces lividans*. The recombinant proteins have also been purified using Ni–NTA affinity chromatography and characterized for substrate specificity, temperature and pH profile and utility in industrial applications.

Biotechnological techniques have seen evolutionary and cutting edge change in recent time. Systematic data analysis revealed emergence of alternative approaches from mining microbial diversity to recombinant protein engineering techniques such as site-directed mutagenesis involving substitution of one amino acid by other naturally occurring amino acids, codon optimisation and recombinant protein production by cloning gene in suitable vector and expression in heterologous systems to meet industrial demands. Engineering enzymes with residue-specific approach such as replacement of methionine by seleno-methionine residues is a common method of enzyme engineering for better catalytic properties. Various reports on modification of subtilisin type of proteases from *Bacillus* spp. using site-directed mutagenesis, DNA shuffling, cassette mutagenesis, error-prone PCR and loop removal are available (Gupta et al. 2002). However, engineering enzymes with modified unnatural amino acids and hot spots in the active sites has also been reported and continuously explored for improving enzymes with enhanced biocatalytic properties (Ravikumar et al. 2015; Balke et al. 2017). This strategy relies on the modification of active site residues (Bryan 2000). Heterologous expression of genes offers several advantages being fast to grow, higher and regulated protein production, easy to handle, and high versatility. However, expression of foreign genes of distant origin has several problems including

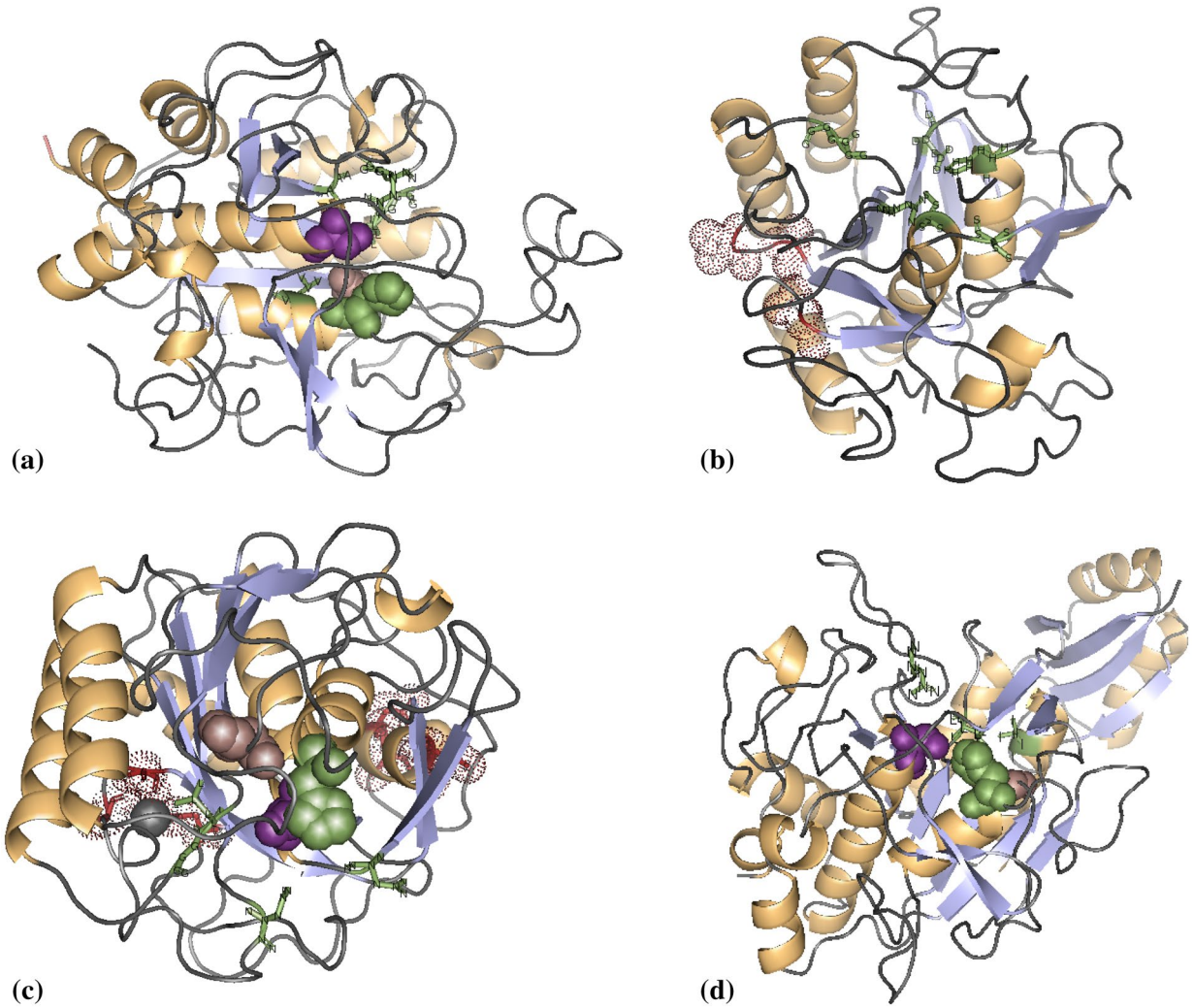

Fig. 4 Structural modeling of S8 alkaline proteases of psychrophilic **a** *Pseudoalteromonas flavipulchra* using template 3TI7 of *Dichelobacter nodosus*); and **b** *Shewanella violacea* using 2b6n of *Serratia* sp.); thermophilic, **c** *Thermus aquaticus* using template 4dzt of *Thermus aquaticus* and mesophilic, **d** *Bacillus circulans* DZ100 using template 3WHI of *Bacillus subtilis.* The graphics were prepared in PYMOL 2.1 for comparison. Catalytic triad (DHS) aspartate, histidine and serine residues are shown as spheres, active site residues as green sticks with labelled one letter code and calcium binding sites as sphere mesh in red

modifications at posttranslational level, toxic or presence of rare codons in the host (Snajder et al. 2015). The associated challenges of expression in heterologous hosts limits the use in commercial scale preparation of recombinant proteins but can be addressed with development of engineered system (Wacker et al. 2002) and better regulation of expression host through promoters (Terpe 2006). Synthetic oligonucleotide shuffling is another method to facilitate the recombination of lower homology genes. Various wild-type protease genes were shuffled and this synthetic shuffling yielded diverse recombinants that displayed superior properties for use as additive in detergents (Yuan et al. 2005; Ness et al. 2002). Alternative to these methods, codon optimisation based on the modification of target gene sequence is a promising tool which has been explored for enhanced expression of serine protease of *Aeropyrum pernix* K1, human genes, and nucleopolyhedrovirus DNA polymerase of *Bombyx mori* in *E. coli* (Burgess-Brown et al. 2008; Song et al. 2014; Snajder et al. 2015). The study has reported codon optimisation as a key step for the enhanced expression of pernisine in *E. coli* by replacing the rare codons with the more frequently occurring amino acids while loss of activity occurs by doing changes in the active site residues (Mechri et al. 2017). Use of DNA sequence manipulation using codon-optimisation has resulted in optimised expression of proteins in heterologous hosts with considerably reduced production cost (Elena et al. 2014; Snajder et al. 2015).

Genomic and metagenomic approaches for discovering new proteases

Development in genome sequencing and annotation is rapidly growing but its application as an alternative approach for mining candidate genes for industrial application is in early stage. A comparative study of mesophiles, thermophiles, halophiles and psychrophiles provided an overview of richness of protease diversity and their organization in genomic map (Fig. 5; Table 2). Complete genome of representative mesophilic *Bacillus clausii*, psychrophilic *Pseudoalteromonas flavipulchra* and thermophilic *Thermus aquaticus* were retrieved from NCBI database and submitted to RAST annotation server. The genomes were analyzed and compared for genome size, GC content, presence of number of serine proteases (Table 2). The circular genome map of these selected bacteria was prepared in GView web server based on reference sequence of *Pseudomonas aeruginosa* PA103. The CDS were analysed according to the COGs functional classification categories. The comparative genomic analysis provides promising incidences in harnessing microbes for better and efficient protease production.

Metagenomic based studies are useful and attractive alternate tools to discover uncharacterized enzymes from

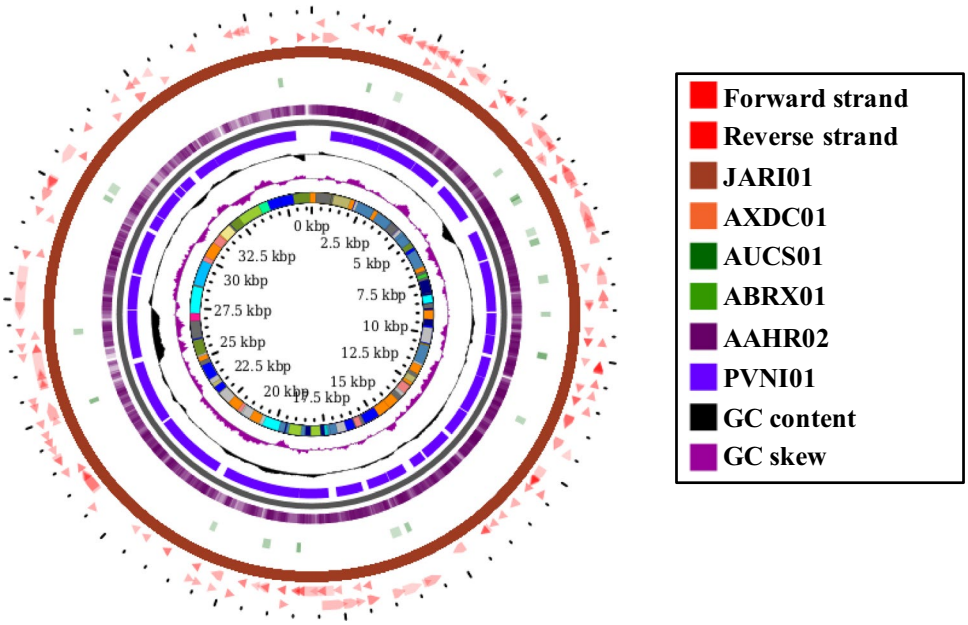

Fig. 5 Circular genome map of extremophilic bacteria showing organization of various genes on forward and reverse strand, reference sequence of *Pseudomonas aeruginosa* PA103 (JARI01), genomes of mesophiles—*Burkholderia pseudomallei* AAHR02, *Bacillus pumilus* ATCC 7061 (ABRX01), genomes of thermophiles—*Dermatophilus congolensis* DSM 44180 (AUCS01), *Caldanaerobacter subterraneus* subsp. *yonseiensis* KB-1 (AXDC01), and archeal genome—*Actinomadura viridilutea* strain DSM 44433 (PVNI01) in different colors

Table 2 Genomic features of bacterial genomes and extracellular serine proteases

Bacteria	Category	Accession no.	Size (Mb)	Total proteases	Serine protease	Status
Actinomadura viridilutea	Thermophile	NZ_PVNI01000001:NZ_PVNI01000062	6.4	64	11	Complete
Bacillus pumilus ATCC 7061	Mesophile	NZ_ABRX01000001-NZ_ABRX01000016	3.7	36	10	Scaffold
Burkholderia pseudomallei	Mesophile	CH899711-CH899721, DS981341-DS981409	6.7		7	Scaffold
Caldanaerobacter subterraneus KB-1	Thermophile	NZ_AXDC01000001-NZ_AXDC01000102	2.6	38	6	Scaffold
Dermatophilus congolensis DSM 44180	Mesophile	KE386981-KE386981	2.5	26	5	Scaffold
Pseudoalteromonas flavipulchra	Psychrophile	JH650741-JH650756	5.2	53	8	Scaffold
Pseudomonas aeruginosa	Mesophile	KK111587-KK111849	6.4	56	3	Complete
Thermus aquaticus	Thermophile	ABVK02000001:ABVK02000022	2.2	26	5	Contig

diverse ecological niches particularly for non-culturable microbes (Amann et al. 1995). Successful discoveries have been obtained using primers targeting conserved protein sequences (Morimoto and Fujii 2009; Fieseler et al. 2006), while activity-based screening is a more direct way to discover new enzymes. A variety of libraries have been reported for enzyme activity including lipases, β-lactamases, proteases, nitrilases, polysaccharide-modifying enzymes, oxidoreductases, and dehydrogenases (Ferrer et al. 2005; Hu et al. 2009; Jeon et al. 2009). Besides this, metagenomic approach not only provide information regarding single gene encoding activity but the entire operons can also be identified (Suenaga 2012; Uchiyama et al. 2005; Chung et al. 2008). The clones expressing a certain function are identified and indicating the genomic information and phylogeny of the genes (Handelsman 2005; Riesenfeld et al. 2004). This approach has recovered novel biocatalysts and expanded our knowledge on uncultured microorganisms (Daniel 2004, Riesenfeld et al. 2004). Despite the abundance of new enzymes isolated by metagenomic approaches and the industrial potential of proteases, only limited reports have been published concerning metagenome-derived proteases. Metalloproteases have been reported from a deep sea sediment metagenomic library (Lee et al. 2007) and soil metagenomic libraries (Waschkowitz et al. 2009). Similarly, serine proteases have been reported from metagenomic library of tannery activated sludge, saline habitat, yucatan underground water and oil-polluted mud-flat metagenome with suitability of protease as laundry additive (Apolinar-Hernández et al. 2016a, b; Ribitsch et al. 2010a, b; Purohit and Singh 2013; Devi et al. 2016; Gong et al. 2017). Several metagenomic studies have also reported screening of false-positive clones for proteolytic activities and has remained unsuccessful (Rondon et al. 2000; Jones et al. 2007).

Discussion

Proteases from diverse sources are extensively explored for various biotechnological and industrial applications as bio-additives in laundry, food, leather, textiles, pharmaceuticals and bioremediation processes (Kasana 2010), as well as in agricultural and basic research related to enzyme modification, photogenicity, complement system and apoptosis pathways (Rao et al. 1998; Mahajan and Badgujar 2010). Proteases are ubiquitous and are reported from a wide diversity of biological sources such as microorganisms, plants and animals. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Proteases of bacterial origin are the most significant because of their wide biotechnological potential. Diverse species *B. cereus*, *B. mojavensis*, *B. megaterium, B. stearothermophilus* and *B. subtilis* have been exploited for extracellular production of proteases (Beg et al. 2002; Shumi et al. 2004; Ravishankar et al. 2012). Many proteases have been commercialized from these species and active under neutral and alkaline conditions. Many bacteria having ability to secrete serine, cysteine, metallo, glutamic and aspartic type of proteases have still remained uncharacterized.

We have found hundreds of research articles related to purification and characterization of proteases as additive in laundry. Studies on cloning of candidate genes are limited and only 6 papers were found in which all the three aspects were discussed. Although a number of review articles are available on microbial proteases still systematic review are available only for proteases. Present review also highlights the scope of alterative tools for developing or engineering efficient enzymes. Although efforts have been placed for protein engineering, the recent advances are still not fully explored for developing efficient proteases. In an attempt to review serine proteases of microbes which is a major class of microbial proteases for detergent applications, systematic and meta-analysis was conducted. Present study revealed that majority of bacteria explored to industrial demands belongs to *Bacillus* spp. Most of the research has been done on the purification of enzymes with limited experimental efforts on cloning and exploration of candidate genes. Both the exploration of extremophiles for protease production is an attractive alternative and coupling of emerging techniques such as active hotspot manipulation of enzymes for broad substrates with enhanced properties can play big role in enzyme engineering. Studies using metagenomic approaches have shown potential alternate for mining unculturable diversity of microbes. Further studies on codon optimisation and use of unnatural amino acids for efficient enzymes engineering can be a boom to detergent industry. Advent of genomic approaches such as Hi seq 2500 Illumina and availability of complete genome of several bacteria from extreme habitat can help in developing environmental friendly and green sustainable processes. There are few studies that report identification of bacteria for protease activity, purification and characterization, cloning and expression in heterologous host with limited studies from extremophiles. Still exploring microbial systems for the protease production with enhanced efficacy, optimised expression system and higher production can ensure a boom in the enzymes.

Conclusions

Use of modern biotechnological techniques such as codon optimisation and other enzyme engineering tools can be promising for developing efficient enzymes to meet the demands for green and sustainable industrial processes. On the other hand, extremophiles such as halophiles,

thermophiles and psychrophiles offer vast potential to tap their diversity to meet industrial demands. The present review article highlights the paradigmatic shift in trends of protein purification from native microbes to enzyme engineering and exploring whole genomes for mining protease diversity. Sequencing of complete genomes with cutting edge genomics techniques will speed-up the genome mining of proteases from bacteria residing in extreme habitats with potential industrial applications. Coupling of codon optimisation and site-directed mutagenesis can fasten our search and will contribute significantly to the next green revolution.

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