Journal of Genetic Engineering and Biotechnology (2017) **15**, 115–126

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Journal of Genetic Engineering and Biotechnology

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Microbial alkaline proteases: Optimization of production parameters and their properties

Kanupriya Miglani Sharma [a,1], **Rajesh Kumar** [a,*,1], **Surbhi Panwar** [b,1], **Ashwani Kumar** [c,1]

[a] *Department of Biotechnology Engineering, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra 136119, India*
[b] *Department of Genetics and Plant Breeding, Chaudhary Charan Singh University, Meerut 250004, Uttar Pradesh, India*
[c] *Department of Nutrition Biology, Central University of Haryana, Mahendergarh 123029, Haryana, India*

Received 15 October 2016; revised 25 December 2016; accepted 5 February 2017
Available online 27 February 2017

KEYWORDS

Alkaline protease;
Optimization;
Submerged fermentation;
Solid state fermentation

Abstract Proteases are hydrolytic enzymes capable of degrading proteins into small peptides and amino acids. They account for nearly 60% of the total industrial enzyme market. Proteases are extensively exploited commercially, in food, pharmaceutical, leather and detergent industry. Given their potential use, there has been renewed interest in the discovery of proteases with novel properties and a constant thrust to optimize the enzyme production. This review summarizes a fraction of the enormous reports available on various aspects of alkaline proteases. Diverse sources for isolation of alkaline protease producing microorganisms are reported. The various nutritional and environmental parameters affecting the production of alkaline proteases in submerged and solid state fermentation are described. The enzymatic and physicochemical properties of alkaline proteases from several microorganisms are discussed which can help to identify enzymes with high activity and stability over extreme pH and temperature, so that they can be developed for industrial applications.

Contents

* Corresponding author at: Department of Biotechnology Engineering, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra 136119, Haryana, India.
E-mail addresses: rkumar2015@kuk.ac.in, dahiya76@gmail.com (R. Kumar).
[1] All the authors contributed equally.

Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2017.02.001
1687-157X

1. Introduction

The use of enzymes in ancient fermentation processes has been known since antiquity. Their existence was associated with the history of ancient Greece where enzymes from microorganisms were used in baking, brewing, alcohol production, cheese making, etc and with better knowledge and advances in analytical techniques have demonstrated that can perform a variety of functions such as selective modification of protein and lysis of fibroin clot etc. [9]. According to the market research report on world enzymes published in 2014, the world market for enzymes is expected to record compound annual growth rate (CAGR) of approximately 7.8% during the forecast period of 2015–2020 and reach USD 6.30 Billion in terms of value [54]. This is driven by continued robust growth in pharmaceutical enzyme demand, fine chemical production, bioethanol production and detergent industries [35]. Proteases are important industrial enzymes accounting for 60% of total global enzyme sales and represent one of the three largest groups of industrial enzymes [88].

Proteases are enzymes of ubiquitous nature which catalyse hydrolytic reactions resulting in breakdown of protein molecules into peptides and amino acids [126]. As compared to plants and animals, microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods, and they produce an abundant, regular supply of the desired product. Also, microbial proteins have a longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals [70].

In early days, proteases were classified according to their source (animal, plant or microbial), catalytic action (endo or exopeptidases), the molecular size, charge or substrate specificity, however a more rational system was recommended by Enzyme Commission (EC) which provides six major classes for all the enzymes. According to the Enzyme Commission (EC) classification, proteases belong to class three (hydrolases), and sub-group four (which hydrolyse peptide bonds) [126]. Four mechanistic classes are recognized by the Enzyme Commission and within these classes, six families of proteases are recognized till date: serine proteases (EC 3.4.21), serine carboxy proteases (EC 3.4.16), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23), metallo proteases I (EC 3.4.24) and metallo carboxy proteases (EC 3.4.17) [137]. Alkaline proteases (EC.3.4.21-24, 99) are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre or are of metallo-type and they are the largely studied group of enzymes because of their wide use in detergent, food, pharmaceutical and leather industries. [47]. The performance of protease is influenced by several factors, such as pH of industrial process, ionic strength, temperature and mechanical handling. Newer enzymes with novel properties that can further enhance the industrial process are always in demand.

Owing to their huge demand in industries, researchers are continuously exploring different aspects of proteases [64,57,34,61]. Therefore, keeping this in view, the review will focus on isolation of alkaline protease producing microorganisms along with production and properties of microbial alkaline proteases. Strategies for exploitation of protease resources and improvement of enzymes to obtain more robust proteases have also been discussed.

2. Isolation of alkaline protease producing microorganisms

Alkaline proteases are indispensable enzymes hence ubiquitous in nature. They are isolated from various sources by surface plating on an alkaline medium and subsequent screening for the desired characteristics. Table 1 shows some protease producing microorganisms isolated from different sources. Natural habitats such as dumping site at Langol, Manipur, India [66]; Egyptian soda lake [53]; Alkaline lonar lake Maharastra, [81]; hot springs, Jordan [10] have been explored for isolation of protease producing microorganisms. Soil samples from leather factories [100,20], detergent industry [86], wood factory [106], milk processing plant [23] and industrial waste such as tannery waste [37,11,123], food processing industrial effluent [104] have also been used. Protease producers have been isolated from soil samples collected from Kurukshetra university campus [70] and sugarcane molasses [141], Thai fish sauce [140], thua nao [26] vegetable waste [55], animal dung [110,12,108], degraded and fresh meat [46] and buffalo hide [143]. Other sources used for isolation of alkaline protease producers include mangrove sediment sample [133], compost containing dead animal's remnants [85], vermicompost pit soil sample [144], sewage sludge sample [22], meat waste contaminated soil [60] and soil from poultry waste site [39]. Alkaline protease has been isolated from fresh fruiting body of the edible mushroom *Pleurotus citrinopileatus* [24]. Similarly, protease

Table 1 Different sources of isolation of alkaline protease producing microorganisms.

Organism	Source of isolation	Reference
Bacteria		
Bacillus sp. JB 99	Sugarcane molasses	Johnvesly et al. [58]
Pseudomonas aeruginosa MN7	Tannery wastewater	Ellouz et al. [37]
Listeria monocytogenes	Degraded meat of cow	Shumi et al. [115]
Geobacillus caldoproteolyticus, sp. nov	Sewage sludge	Chen et al. [22]
Bacillus cereus 146	Soil of a wood factory	Shafee et al. [106]
Bacillus amovivorus	Degraded pulse sample	Sharmin et al. [111]
Bacillus megaterium	Thai fish sauce	Yossan et al. [140]
Xenorhabdus nematophila BA2	Nematode *Steinernema carpocapsae*	Mohamed [79]
Bacillus circulans BM15	Mangrove sediment	Venugopal and Saramma [133]
Bacillus sp.	Dog dung	[110]
Bacillus lincheniformis N-2	Compost containing dead animal's remnants	Nadeem et al. [85]
Bacillus firmus MTCC 7728	Soil from leather factories	Rao and Narasu [100]
Bacillus cereus MCM B-326	Buffalo hide	Zambare et al. [143]
Bacillus proteolyticus CFR3001	Fish processing waste	Bhaskar et al. [18]
Bacillus laterosporus-AK1	Agro waste storage compost	Arulmani et al. [13]
Bacillus circulans	Vegetable waste	Jaswal et al. [55]
Bacillus sp.	Alkaline salty soil	Darani et al. [28]
Bacillus sp. HUTBS71	Water from hot spring	Akel et al. [10]
Bacillus sp. SAL 1	Tannery waste	Almas et al. [11]
Bacillus sp.	Tannery industry effluent	Srinivasan et al. [123]
Bacillus subtilis KO	Molasses	Younis et al. [141]
Pseudomonas fluorescens	Meat waste contaminated soil	Kalaiarasi and Sunitha [60]
Pseudomonas thermareum GW1	Soil from poultry waste site	Gaur et al. [39]
Bacillus pumilus SG2	Food processing industrial effluent	Sangeetha et al. [104]
Pseudomonas aeruginosa MCM B-327	Vermicompost pit soil	Zambare et al. [144]
Geomicrobium sp. EMB2	Sambhar Salt Lake	Karan et al. [63]
Bacillus sp. AS-S20-I	Soil	Mukherjee and Rai [82]
Fungi		
Penicillium sp.	Soil from soy meal manufacturing industry	Agrawal et al. [5]
Beauveria felina	Soil from soy meal manufacturing industry	Agrawal et al. [6]
Mucor sp.	Herbivorous dung	Alves et al. [12]
Engyodontium album BTMFS10	Marine sediment	Chellappan et al. [21]
Aspergillus ustus	Sediment samples from Central Indian Basin	Damare et al. [27]
Penicillium chrysogenum IHH5	Soil	Haq et al. [50]
Aspergillus clavatus ES1	Wastewater	Hajji et al. [48]
Penicillium godlewskii SBSS 25	Soil	Sindhu et al. [117]
Penicillium sp.	Soil	Hamzah et al. [49]
Aspergillus flavus and *Aspergillus terreus*	Soil around leather industry	Chellapandi [20]
Beauveria sp. MTCC 5184	Rabbit dung	Shankar et al. [108]
Actinomycetes		
Streptomyces nogalator Ac 80	Soil from leather and hair dumping areas	Mitra and Chakrabartty [78]
Streptomyces sp.	Soil	Mehta et al. [76]
Streptomyces roseiscleroticus	Sediment sample of an estuarine shrimp pond	Vonothini et al. [135]
Nocardiopsis prasina HA-4	Limestone quarry	Ningthoujam et al. [88]
Streptomyces sp. A6	Intertidal zone 2 km way from the sea coast	Singh and Chhatpar [118]
Streptomyces ambofaciens	Soil	Bajaj and Sharma [15]

production by mushroom *Laccocephalum mylittae* has been reported [145]. Thirty-nine, protease producing, *Streptomycetes* were isolated from sewage of tanneries and soil around it, agricultural soil, agricultural fields, water sources from a fish farm, sediments with neutral and alkaline pH and Qaroun lake [102].

3. Alkaline protease production under submerged fermentation

The nature of the fermentation, solid or submerged, influences various aspects of the growth of the microorganism as well as enzyme production [49]. The use of the submerged culture is advantageous because of the ease of sterilization and process control is easier to engineer in these systems [134]. Proteases are generally produced using submerged fermentation due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream in spite of the cost-intensiveness for medium components [97].

3.1. Optimization of fermentation conditions under SmF

Media composition plays a significant role in enzyme production by microorganisms. Apart from this environmental

factors such as temperature, pH, incubation time also influence microbial metabolism up to a large extent [1]. These factors are important to promote, stimulate, enhance and optimize the production of proteases [99]. In order to obtain high and commercially viable yields of protease it is essential to optimize fermentation media for the growth and production of protease [109]. No defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production.

3.1.1. Source of growth media

The carbon and nitrogen are major media components which act as essential stimulant for growth of microorganism and enzyme production as well. Maximum enzyme production by a bacterial strain AKS-4 was observed when glucose at a concentration of 1% (w/v) was used as carbon source in the growth media. Protease production was 59.10 U/ml when glucose was used [109]. An increased level of protease production by *Bacillus pseudofirmus* AL-89 has been observed upon addition of glucose whereas for *Nesterenkonia* sp. AL-20 protease production was suppressed in the presence of glucose. In the latter case, the glucose may act as catabolic inhibitor [41]. Protease production by *Aspergillus oryzae* 637 increased steeply with increase in glucose concentration up to 0.6% (w/v) and further increase up to 1.2% (w/v) showed only marginal increase [124]. The maximal production of alkaline protease (2450 U/ml) by *Bacillus licheniformis* was obtained in medium supplemented with 60 g/l glucose and further increase in glucose concentration resulted in slight reduction in enzyme production [35]. Glucose at high concentration inhibited the enzyme production by *Streptomyces* sp. and 0.5% (w/v) concentration was optimum for enzyme production while growth was optimum at 1% (w/v) concentration [76]. This is probably due to the catabolic repression mechanism suggesting that in the absence of glucose; the protease plays a role in supplying peptides or amino acids as the carbon or energy source in addition to being a nitrogen source. Consequently, protease synthesis could be repressed when the energy status of the cells is high in the presence of glucose. It is now known that the catabolite control protein (CcpA) is responsible for the regulatory mechanisms of glucose catabolism, and acts as a signal for the repression in protease synthesis [128]. Protease production by *Geobacillus caldoproteolyticus* sp. nov. was repressed by glucose but enhanced by supplementation of basal media with sucrose [22]. Protease production by *Pseudomonas aeruginosa* MCM B-327 in soyabean-tryptone media was suppressed by 95% and 60%, when supplemented with glucose and fructose respectively [144]. On the other hand, fructose and glucose proved to the best carbon sources for improving the productivity of protease from *Aspergillus flavus* and *Aspergillus terreus* respectively [20]. Kumar et al. [70] used various carbon sources like glucose, lactose, galactose and starch for protease production by *Bacillus aryabhattai* K3 and reported maximum protease production (622.64 U/ml) with lactose (10 g/l) as carbon source in the medium. Similarly Dodia et al. [33] while studying eight isolates for protease production found that for most of the isolates enzyme secretion was optimum with lactose. *B. licheniformis* BBRC 100053 exhibited higher productivity of protease in culture media containing lactose as carbon source [42]. Use of 5% (w/v) starch as carbon source led to maximum protease production by *Bacillus* sp. 2–5 [28]. The *Bacillus clausii* strain No. 58 grew well on various starch-based carbon sources. Corn starch at a concentration of 0.5% (w/v) gave the highest productivity of protease, followed by wheat flour and wheat bran. However, supplementation of potato starch caused a decrease in protease titre, which possibly may be due to the presence of protease inhibitors in potato [67]. The highest protease production by *S. roseiscleroticus* was obtained when starch was used as the carbon source while it was minimum with dextrose [135], whereas, Jaswal et al. [55] reported that maltose and glucose were equivalent and significantly better than starch and fructose for production of protease by *Bacillus circulans*. Sucrose at 5% (w/v) was found optimal for enzyme production by actinomycete *Nocardiopsis prasina* HA4 [88]. Dextrose and sucrose were found as the best two sources for both biomass and protease production by *L. mylittae* especially when these two were combined together in a ratio of 1:1 in the medium [145]. Dextrose was also reported to be the suitable carbon source for protease producing *Bacillus subtilis* strain [29]. Among carbon sources used for protease production by *Pseudomonas fluorescens*, wheat bran and maltose were found to support protease production [60]. Wheat flour was observed to be most effective for protease production by a *Bacillus* sp. [23]. Usharani and Muthuraj [131] reported *Bacillus laterosporus* to be capable of utilizing a wide range of carbon sources; the best carbon sources for protease secretion were soluble starch, trisodium citrate, citric acid and glycerol.

The requirement for a specific nitrogen supplement differs from organism to organism and different workers have used different organic nitrogen sources (simple or complex), inorganic nitrogen sources and amino acids for enhancing protease production. The effect of different nitrogen sources, such as peptone, tryptone, potassium nitrate and yeast extract phosphate at a concentration of 1%, on the production of protease using Rheinheimera strain (KM459533) was studied and the enzyme production was reported to be 433.63 U/ml [128]. $(NH_4)_2HPO_4$ was also found to be the best nitrogen source for protease production by *A.oryzae* 637 [124]. Ammonium sulphate has been reported to be the best nitrogen source for protease production by *Bacillus* sp. strain AS-S20-I [82]. On contrary, ammonium chloride and ammonium sulphate have been reported to suppress alkaline protease production by *Bacillus* sp. 2–5 [28]. Neither ammonium chloride nor urea was beneficial for biomass or protease production by *L. mylittae;* however corn steep liquor enhanced the production of protease [145].

The highest level of protease production by *Bacillus cereus* strain 146 was observed in presence of beef extract as nitrogen source in the growth media. The presence of yeast extract, peptone and tryptone increased growth but resulted in low protease production in this case [106]. The best nitrogen source for protease production by another *Bacillus* sp. was beef extract while yeast extract and tryptone were also comparable [86]. Also, the highest protease production by *Streptomyces roseiscleroticus* [135], *A. flavus* [20] and *A. terreus* [20] was obtained when beef extract was used as nitrogen source. Tryptone was found to increase protease production for a *Bacillus* strain [123]. Among nitrogen sources (beef extract, tryptone, peptone, glycine, casein) used for protease production by *Pseudomonas fluorescens*, maximum titre was reported with peptone [60]. Peptone was also found optimal for protease production

by *N. prasina* HA4 [88] and *B. licheniformis* BBRC 100053 [42]. Yeast extract showed maximum influence in enhancement of enzyme production by *Bacillus* sp. [97]. For *Streptomyces* sp. growth and enzyme production was optimum with yeast extract [76]. A number of workers [95,1,113,42] have reported a combination of peptone and yeast extract to be optimum for protease production. In case of *Bacillus* sp. APP1, among all the organic nitrogen sources used, soyabean meal had a significant effect on the production of the extracellular protease [23]. Jaswal et al. [55] also reported that soyabean meal was better than casein, gelatine and peptone for production of protease by *B. circulans*. Maximum protease production by *P. aeruginosa* MCM B-327 was obtained with a combination of soybean meal and tryptone [144]. Sharma et al. (2005) reported use of casein, peptone, yeast extract and beef extract as nitrogen source for protease production by bacterial strain AKS-4 s and observed high protease production (49.77 U/ml) in the presence of casein. For protease production by *Microbacterium sp*. kr10, feather meal was observed to be optimal nitrogen source [129]. Among different organic nitrogen sources, skim milk gave maximum protease yield in case of *Bacillus caseinilyticus* followed by malt extract, peptone and yeast extract. Ammonium chloride as inorganic nitrogen source was found to inhibit the production. $MgCl_2$ and $CaCl_2$ induced protease production [81] Mustard cake has been reported as the ideal nitrogen source for protease production by *Streptomyces ambofaciens* [15].

3.1.2. Environmental conditions

3.1.2.1. Incubation period. Incubation period affects the enzyme production significantly and it varies from 24 h to a week depending upon type of microorganism and other culture conditions such as inoculum size, metabolic state of cell pH and temperature. Protease production by *Bacillus pumilus* UN-31-C-42 started 16 h after incubation, increased gradually and reached a maximum at about 28 h [52]. For *B. subtilis* PE-11 [3] and *B. licheniformis* LBBL-11 [90] maximum growth and enzyme production was observed after 2 days. *Bacillus* sp. APP1 [23] produced maximum titre of protease after an incubation period of 2 days though the highest biomass yield was recorded after 4 days of incubation period. *B. subtilis* KO strain exhibited its maximum production of protease within 48 h incubation period [141]. *Pseudomonas fluorescens* produced maximum protease after 24 h of incubation, the enzyme activity gradually decreased from 48 to 168 h [60]. The maximum protease production from *V. pantothenticus* [46], *B. subtilis* [69] and *B. licheniformis* [69] was recorded after 72 h. Optimum incubation time for protease production by *B. licheniformis and Bacillus coagulans* has been reported as 96 h [14]. *B. subtilis* ATCC 633 and *Bacillus* sp. UFLA 817 CF have been reported to show maximum protease activity after 24 h growth, a period that coincided with the end of the exponential phase [31]. Protease secretion by *B. cereus* VITSN04 followed a similar pattern as for growth, with the highest enzyme activity in the exponential phase [127]. The protease production by *G. caldoproteolyticus* sp. nov. [22], *B. clausii* No. 58 [67], *Bacillus brevis* SSA1 [4], *Bacillus firmus* MTCC 7728 [100], *Xenorhabdus nematophila* BA2 [79] and *Geomicrobium* sp. EMB2 [63] have been reported to reach maximum level in the stationary phase, after 9, 48, 72, 48, 48 and 72 h of incubation respectively.

The enzyme production by *Penicillium chrysogenum* gradually increased with the passage of time and the highest enzyme activity was obtained after 72 h of incubation, prolonged incubation decreased the enzyme activity; however the growth of the microorganism was not significantly affected [50]. Also, maximum protease production by *A. flavus* and *Aspergillus terreus* was reported after 72 h [20]. Enzyme synthesis by *Mucor* sp. started in the first 24 h when nutrient consumption was high and there was reduction in the enzyme activity after 120 h of incubation [12]. The synthesis and secretion of the protease by *A. terreus* IMI 282743 was initiated during the exponential growth phase (7 days after incubation) with a substantial increase near the end of the growth phase towards maximum amounts of protease produced during the stationary growth phase (9 days after incubation) [138]. The optimum culture period for the production of proteases by *Botrytis cinerea* was 9 days [1]. *N. prasina* HA4 has been reported to achieve maximal enzyme production in 5 days [88]. Maximum protease production by *S. ambofaciens* was after 48 h of incubation [15].

3.1.2.2. Medium pH. The pH of culture affects all enzymatic processes and transportation of various components across the cell membrane. However, the molecular basis of pH affecting bacterial metabolism in culture broth is obscure. Since proton motive force in chemiosmosis is affected by the medium pH value, it is possible that under optimum pH range, the relative metabolic efficiency is high [119] Hence it is an important parameter to optimize. A medium with neutral initial pH has been reported for alkaline protease production by *P. chrysogenum* IHH5 [50], *S. roseiscleroticus* [135], *B. cereus* [72] *Bacillus polymyxa* [72], *Bacillus aquimaris* VITP4 [113] and *P. aeruginosa* MCM B-327 [144]. While slightly acidic medium (pH 6.3–6.5) have been reported as optimum for protease production by *Bacillus* sp. MIG [43] and *B. cereus* SIU1 [119], slightly alkaline medium (pH 8.0–8.5) has been reported to be optimum for protease production by *B. licheniformis* IKBC-17[89], *B. subtilis* IKBS 10 [89], *Bacillus macerans* IKBM-11 [89], *Bacillus amovivorus* [111] and *Aspergillus niger* [30]. The reason behind this kind of observation is not yet very clear. For eight isolates studied for protease production, it was found that the optimum pH for growth was 9.0 for the majority of the isolates, while the optimum pH with regard to enzyme secretion varied between pH 8.0–10.0 [33]. A pH 9 has been reported as optimal for protease production by *Bacillus* sp. [97], *Bacillus* sp. strain APP1 [23], *Bacillus proteolyticus* CFR3001 [18], *V. pantothenticus* [46] and *Pseudomonas fluorescens* [60]. Higher initial pH, 10.0 for *A. oryzae* 637 [124] and *B. licheniformis* TISTR 1010 [132], 10.5 for *B. circulans* [55] and 10.7 for *Bacillus* sp. 2–5 [28] have also been reported for maximum protease production.

3.1.2.3. Incubation temperature. Temperature is a critical parameter that has to be controlled and varied from organism to organism for maximum cell growth and enzyme production. The optimum temperature requirement reported for alkaline protease production by different microorganisms differs widely. The optimum temperature for protease production by *P. aeruginosa* PseA [45], *B. licheniformis* [14], *B. coagulans* [14], *B. cereus* [65], *P. aeruginosa* MCM B-327[144], *P. chrysogenum* IHH5 [50] and *A. oryzae* 637 [124] has been reported to be 30 °C. Lower optimum temperature of 25 °C has been

reported for *B. circulans* [55], *Microbacterium* sp. [129] and 28 °C for *B. cinerea* [1]. *P. fluorescens* was capable of producing protease in the range of 27–57 °C with production maximum at 37 °C [60]. A temperature of 37 °C has been reported as optimal temperature for protease production by a number of *Bacillus* species such as *B. amovivorus* [111], *B. proteolyticus* CFR3001 [18], *B. aquimaris* VITP4 [113] and *B. subtilis* strain Rand [2]. In contrast to that a temperature of 40 °C has been reported to be best for production of protease by *Bacillus* sp. 2–5 [28], *B. licheniformis* GUS1 [105], *V. pantothenticus* [46] and *S. roseiscleroticus* [135]. Maximum production of protease by *A. niger* was obtained at 45 °C [30]. High optimum temperature of 50 °C has been reported for *Bacillus* sp. strain APP1 [23] and *B. subtilis* BS1 [107]. *B. licheniformis* IKBC-17, *B. subtilis* IKBS-10. The optimum temperature of alkaline protease production by *B. cereus and B. polymyxa* has also been reported as 60 °C [72]. The production of protease using statistical method was studied using *B. subtilis K-1* strain. The highest protease activity was detected at 60 °C (312.6 U/ml); however, substantial activity was observed at 70 °C (306.2 U/ml) and 80 °C (301.6 U/ml) [120].

3.2. Alkaline protease production under solid-state fermentation (SSF)

Solid state fermentation (SSF) is defined as the fermentation process which occurs in the absence or near absence of free water [38]. In this process, the solid substrate not only supplies the nutrient to the culture but also serves as an anchorage for the microbial cells. SSF processes are of special economic interest for countries with an abundance of biomass and agroindustrial residues, as these can be used as cheap raw materials. [36]. With regard to cost economics, SSF has been proved to be more efficient than SmF. The product can be recovered in highly concentrated form as compared to those obtained by submerged fermentations [126]. Other advantages include superior volumetric productivity, simpler machinery, use of inexpensive substrate, low energy requirements and low waste water output, simple technique, low capital investment, lower levels of catabolite repression and better product recovery. It therefore becomes very important to determine the environmental conditions of the microorganism for maximum production [139]. However, SSF processes present some limitations, such as the SSF technique is mainly confined to process involving fungi, the restricted range of microorganisms that are able to grow under reduced moisture levels, control of conditions is again very difficult and there is no defined concentration of media components [121]. Also, SSF is usually slower because of the diffusion barriers imposed by the solid nature of the fermented mass [36].

3.2.1. Optimization of fermentation conditions under solid state fermentation

The major factors that affect microbial synthesis of enzymes in a SSF system include: selection of a suitable substrate and microorganism; pre-treatment of the substrate; particle size of the substrate; water content and a_w of the substrate; relative humidity; type and size of the inoculum; control of temperature of fermenting matter/removal of metabolic heat; period of cultivation; maintenance of uniformity in the environment of SSF system, and the gaseous atmosphere, i.e. oxygen consumption rate and carbon dioxide evolution rate. For optimization of these factors new statistical techniques are now used which are more scientific and less labour intensive [62] Effect of some of these factors on alkaline protease production is reviewed below.

3.2.1.1. Substrate. Substrates from agricultural or industrial wastes (wheat straw or barley, sugar cane bagasse, coffee pulp, grape wastes, copra pasta, among other) or inert materials (as resins of ionic exchange, acrolite or polyurethane foam) can be used. The pre-treatments of these materials are required and generally include milling and washing [7]. Govarthanan et al. [44] used horse gram husk as substrate while producing protease using *Bacillus* sp. SKK11 and reported maximum protease production (240 U/ml) by using maltose as a source of carbon. Sometimes, a combination of two or even three different substrates gives higher enzyme yields than each of the substrates used individually [126]. There are several reports describing use of agro-industrial residues for the production of alkaline protease, by bacteria [25,142,17,28,38] and fungus [73,94,117,84]. Cost and availability are important considerations, and therefore the selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes [36]. Pigeon pea waste, pineapple waste, orange peel waste, sugarcane bagasse, wheat bran, rice bran, raw potato starch and raw sweet potato starch were tested for protease production by *Bacillus* sp. JB-99 and maximum protease activity was obtained with pigeon pea waste [58]. Different agro-industrial waste materials (green gram, chick pea, red gram, black gram husks and wheat bran) were tested for protease production by *Bacillus* sp. and maximum enzyme production was observed with green gram husk, while minimum protease production was reported with red gram husk [97]. Green gram husk has also been reported to support maximum protease production by *B. circulans* [96]. Coffee wastes such as pressed coffee pulp, coffee cherry husk, coffee parchment husk, silver skin and coffee spent wastes were tested for protease production by *A. oryzae* CFR305 and coffee cherry husk was found to be most suitable [84]. Maximum protease production was observed in watermelon rind among five substrates (melon rind, watermelon rind, rice, lentil and corn husks) used for protease production by *Bacillus* sp. [38]. High titre of protease activity by *T. thalpophilus* PEE 14 was obtained in medium containing wheat bran as substrate [32]. Wheat bran has also been used for protease production by *Beauveria feline* [6] and *Engyodontium album* BTMFS10 [21]. Rice bran was reported to be the best substrate for protease production by a *Bacillus* sp. [116]. Broken rice of different varieties i.e. PONNI, IR-20, CR-1009, ADT-36 and ADT-66 was used for protease production by *A. niger* MTCC 281 and maximum protease production was observed in PONNI [94]. Deoiled Jatropha seed cake was reported to support good bacterial growth and enzyme production when assessed for its suitability as substrate for enzyme production by *P. aeruginosa* PseA [75]. Defatted soybean cake was used for protease production by a *Penicillium* sp. [40]. A *B. subtilis* isolate was shown to be able to produce extracellular protease in solid-state fermentation using soy cake as culture medium [121]. Soybean was used for production of alkaline protease by *Teredinobacter turnirae* [36]. Among the different agro-industrial waste products and kitchen waste materials, viz. mustered oil cake, wheat bran, rice bran, *Imperata cylindrica*

grass, banana leaves, potato peels and used tea leaves screened as substrates/solid supports for the production of alkaline protease by *B. subtilis* DM-04, potato peel followed by *I. cylindrica* grass supported maximum protease production. Potato peel and *I. cylindrica* grass mixed in a ratio of 1:1 (w/w) significantly enhanced the protease production as compared to individual substrate [83]. *A. oryzae* NRRL 2217 was capable of producing maximum protease on mixed substrate coconut oil cake: wheat bran in mass ratio of 1:3 [125]. Wheat bran enriched with fish scales and egg shell in a ratio of 1:2:0.005 (w/w) was used for protease production by *Penicillium* sp. [49].

3.2.1.2. Particle size of substrate. In solid-state fermentation process, the availability of surface area play a vital role for microbial attachment, mass transfer of various nutrients and substrates and subsequent growth of microbial strain and product production [97]. Smaller substrate particles provide a larger surface area for microbial attack, however, too small particles may result in substrate agglomeration which may interfere with aeration and may thus result in poor growth. At the same time, larger particles provide better aeration efficiency but provide limited surface for microbial attack. Therefore, it may be necessary to provide compromised particle size [93]. The coarse size (2 mm) of soybean was found to be optimal size of the substrate for higher protease production by *T. turnirae* [36]. Maximum enzyme production by *Bacillus* sp. was noticed with 1.4–1.0 mm particle size green gram husk material [97]. Wheat bran of particle size < 425 μm was found optimal for protease production by *E. album* BTMFS10 [21]. Protease production by *P. aeruginosa* growing on animal fleshing size of 0.25 cm was higher than all other substrate sizes [68]. Protease production by *A. oryzae* CFR305 was found to be high with 1 mm particle size of coffee cherry husk as compared to 0.5 mm and 2 mm [84].

3.2.1.3. Moisture content. An increase in moisture level is believed to reduce the porosity of the substrate such as wheat bran, thus limiting oxygen transfer, while lower moisture content causes reduction in solubility of nutrients of substrate, lower degree of swelling. Hence, an optimal level of moisture is required for maximum enzyme productivity. High enzymatic titre (240 U/g) was attained when the initial moisture level was 22.4% [112]. It is known that water content of a medium has a profound influence on the production of products by microorganisms. In a solid state fermentation the water content of the substrate is greatly influenced by the absorbing capacity and capillary forces of the substrate, the growth temperature, the amount of metabolic heat generated the quantity of moisture evolved and growth requirement of organisms. The moisture level in SSF has a great impact on the physical properties of the substrate and this factor makes it different from Smf. It is crucial to provide optimized water level that controls the water activity (aw) of the fermenting substrate for achieving maximum product [97]. An increase in moisture content causes a decrease in the porosity of the substrate, thereby decreasing the gas exchange. Low moisture content leads to sub-optimal growth and a lower degree of substrate swelling which also decreases enzyme production [74,92]. Agrawal et al. [5] reported 50% of initial moisture content to be best for protease production by *Penicillium* sp. Similar observation has been reported by other workers for *A. oryzae* NRRL 2217 [125], *A. oryzae* CFR305 [84] and *P. aeruginosa* PseA [75]. In case of *E. album* BTMFS10 [21] and *Penicillium godlewskii* SBSS 25 [117] a moisture content of 60% was found to be optimal for protease production. A high moisture content of 80% for *T. thalpophilus* PEE 14 [32], 120% for *Beauveria felina* [6], 90–170% for *B. circulans* [96] and 100% for *B. subtilis* DM-04 [83] have also been reported.

3.2.1.4. Incubation time. Generally, the time required for the optimum protease production by bacteria or fungus may be as long as 48 h–9 days [8]; [98]. Incubation time of 20 h for *P. aeruginosa* [68], 24 h for *B. subtilis* DM-04 [83], 60 h for *B. circulans* [96], 60 h for *Bacillus* sp. [97], 63 h for *T. turnirae* [36], 72 h for *Bacillus* sp. [38], 72 h for *P. aeruginosa* PseA [75] have been reported for maximum protease production. The incubation time for maximum protease production in SSF by different fungal species have been reported as, 72 h for *Penicillium* sp. [5], 168 h for *B. felina* [6], 48 h for *A. oryzae* NRRL 2217 [125], 120 h for *E. album* BTMFS10 [21], 72 h for *A. niger* MTCC 281[94], 96 h for by *P. godlewskii* SBSS 25 [117], 120 h for *A. oryzae* CFR305 [84]. For actinomycete, *T. thalpophilus* PEE 14, the highest titre of enzyme was attained after 72 h [32]. Protease production by an organism can be both growth and non-growth associated. Protease production profile of *B. subtilis* was found to be associated with growth, the enzyme production peak occurred at the mid exponential phase (9 h), but enzyme denaturation began to occur before biomass reached its maximum level [121].

3.2.1.5. pH. A number of reports suggest pH 7.0–7.5 to be optimal for protease production by bacteria and fungi. They include *Teredinobacter turnirae* [36], *B. subtilis* DM-04 [83], *A. oryzae* [103], *B. felina* [6], *A. niger* MTCC 218 [94] and *A. oryzae* CFR305 [84]. It has been reported that *E. album* BTMFS10 prefers both acidic (pH 4.0 and 5.0) and alkaline pHs (pH 10.0) for protease production [21]. A little acidic pH of 6.0 was found to be optimum for protease production by *P. aeruginosa* PseA [75]. For *B. circulans*, pH 10 has been reported to be suitable for protease production [96]. Protease production by *B. subtilis* remained approximately the same irrespective of the initial pH in the whole range tested (5.0–10.0) [121].

3.2.1.6. Incubation temperature. For most of the fungi an optimum temperature range of 28–30 °C has been reported for protease production under SSF condition. They include *Penicillium* sp. [5], *A. oryzae* NRRL 2217 [125], *B. felina* [6], 30 °C *A. oryzae* CFR305 [84]. While a little lower temperature of 25 °C has been reported for *E. album* BTMFS10 [21], a little higher temperature of 35 °C has been reported for *P. godlewskii* SBSS 25 [117] and *A. niger* MTCC 218 [94]. In case of bacteria, temperature of 36 °C for *B. circulans* [96], 37 °C for *B. subtilis* [121], 30 °C for *T. turnirae* [36], 30 °C *P. aeruginosa* PseA [75] and 50 °C for *B. subtilis* DM-04 [83] has been used for protease production under SSF condition.

4. Properties of alkaline proteases

Alkaline proteases from several microorganisms have been studied extensively so that they can be used for specific applications based on their properties. For industrial applications, proteases must possess activity and stability under relatively hostile conditions, often comprising extremes in temperature,

Table 2 Properties of some alkaline proteases from different microbial sources.

Microorganism	pH optima	Temperature optima (°C)	pI	Molecular weight	Other properties	Reference
Nocardiopsis sp.	10.5	50	–	–	Stable in the presence of oxidants and surfactants	Moreira et al. [80]
Bacillus pumilus UN-31-C-42	10.0	55	9	32 kDa	–	Huang et al. [52]
Bacillus pseudofirmus AL-89	11.0	60	–	24 kDa	–	Gessesse et al. [41]
Nesterenkonia halobia AL-20	10.0	70	–	23 kDa	Ca^{+2} not required for activity and thermal stability	Gessesse et al. [41]
Bacillus subtilis PE-11	10.0	60	–	15 kDa	Thermostable, strongly activated by metal ions	Adinarayana et al. [3]
Bacillus clausii	11.5	80	–	–	Thermostable, oxidant, SDS-stable	Kumar et al. [67]
Vibrio metschnikovii DL 33–51	12.0	60	–	29.5 kDa	Half-life of 7.5 h at 50 °C, Stable in the presence of oxidants and surfactants	Mei and Jiang [77]
Pseudomonas aeruginosa PD100	8.0	60	6.2	38 kDa	Stable in the presence of SDS and Tween 80	Najafi et al. [87]
Bacillus megaterium	10.0	50	–	27 kDa	Strongly activated by metal ions	Yossan et al. [140]
Bacillus patagoniensis PAT05	9.0–12.0	60	> 10.3	29.4 kDa	Stable in the presence of oxidants, surfactants and chelating agents	Olivera et al. [91]
Bacillus laterosporus-AK1	9.0	75	–	86.29 kDa	Strongly activated by metal ions	Arulmani et al. [13]
Bacillus proteolyticus CFR3001	8.0	40–50	–	29 kDa	Antibacterial	Bhaskar et al. [18]
Bacillus circulans BM15	7.0	40	–	30 kDa	Stable in the presence of oxidants and surfactants	Venugopal and Saramma [133]
Pseudomonas aeruginosa PseA	9.0	55	–	–	Solvent tolerant	Gupta and Khare [45]
Xenorhabdus nematophila	8.5.	30	–	39 kDa	–	Mohamed [79]
Bacillus subtilis	8.0–9.0	37–45	–	–	Thermostable	Mukherjee et al. [83]
Bacillus species HUTBS71	7.8	65	–	49 kDa	Half life of 8 h at 60 °C	Akel et al. [10]
Bacillus subtilis SAL 1	9.0	60	–	27 kDa	–	Almas et al. [11]
Bacillus sp.	8.0	65	–	68 kDa	Thermostable	Srinivasan et al. [123]
Bacillus circulans	11.0	70	–	39.5 kDa	Thermostable, Detergent stable	Rao et al. [101]
Pseudomonas thermaerum GW1	8.0	60	–	43 kDa	Solvent-stable	Gaur et al. [39]
Bacillus firmus Tap5	9.0	60	–	34 kDa	Thermostable	Joshi [59]
Serratia marcescens subsp. Sakuensis TKU019	10.0	50	–	58 kDa	Tween 40 has stimulatory effect on activity	Liang et al. [71]
Bacillus cereus VITSN04	8.0	30	–	32 kDa	–	Sundararajan et al. [127]
Pseudomonas aeruginosa MCM B-327	8.0	35	–	56 kDa	Novel nature with non-collagenase, non-keratinase but strong dehairing activities	Zambare et al. [144]
Bacillus licheniformis MP1	10.0	70	–	30 kDa	Stable in the presence of surfactants	Jellouli et al. [56]
Bacillus halodurans JB 99	11.0	70	–	29 kDa	Half life of 4 h at 70 °C	Shrinivas and Naik [114]
Fungi						
Aspergillus fumigatus TKU003	8.0	40	~8	124 kDa	–	Wang et al. [136]
Engyodontium album BTMFS10	11.0	60	–	38 kDa	–	Chellappan et al. [21]
Aspergillus ustus	9.0	45	6.6, 6.9	32 kDa	Cold tolerant	Damare et al. [27]
Aspergillus flavus AP_2	8.0	45	–	46 kDa	–	Hossain et al. [51]
Aspergillus clavatus	8.5	50	–	32 kDa	Activated by divalent cations, Stable in the	Hajji et al. [48]

Table 2 (*continued*)

Microorganism	pH optima	Temperature optima (°C)	pI	Molecular weight	Other properties	Reference
ES1					presence of non-ionic surfactants	
Aspergillus clavatus	9.5	40	–	35 kDa	Ca^{+2} increased thermal stability	Tremacoldi et al. [130]
Aspergillus nidulans HA-10	8.0	35	–	42 kDa	–	Charles et al. [19]
Aspergillus niger	10.0	40	–	38 kDa	Stable for 1 h at 40 °C	Devi et al. [30]
Aspergillus oryzae	10.0	60	–	35 kDa	–	Murthy and Naidu [84]
Beauveria sp. MTCC 5184	9.0	50	9.3	29 kDa	Able to separate the endothelial cells and can be used in animal cell culture	Shankar et al. [108]
Actinomycetes						
Streptomyces nogalator Ac 80	7.5–8.5	28	–	66	–	Mitra and Chakrabartty [78]
Nocardiopsis prasina HA-4	7.0, 10.0	55	–	–	Thermostable, Alkalitolerant	Ningthoujam et al. [88]
Streptomyces ambofaciens	8.0	60	–	–	Thermostable, Alkalitolerant	Bajaj and Sharma [15]

pH and presence of inhibitors and oxidizing agents. Thus, candidate enzymes must have both process suitability and prolonged stability if intended for industrial applications. The ultimate aim of any research activity is to find out an enzyme which is robust in nature and versatile in applications. The important properties are summarized in Table 2. Some properties are discussed briefly in the following section.

4.1. Substrate specificity

The important feature of alkaline proteases is their ability to discriminate among competing substrates and the utility of these enzymes often depends on their substrate specificity [108]. Proteases can hydrolyse natural substrates like casein, bovine serum albumin, gelatin, elastin-orcein, keratin, azure, and collagen as well as on synthetic substrates like *N*-benzoyl-L-tyrosine ethyl ester (BTEE), *N*-benzoyl-L-arginine ethyl ester (BAEE), *N*-succi nyl-Ala-Ala-Ala-p-nitroanilide (SAAAPNA), *N*-succinyl-Ala-A la-Pro-Phe-p-nitroanilide (SAAPPPNA), *N*-benzoyl-DL-arginine-p-nitroanilide (BAPNA), *N*-benzoyl-Pro-Phe-Arg-p-nitroanilide (BPPAPNA) and *N*-[3-(2-furyl) acryloy]-Leu-Gly-Pro-Ala (FALGPA). Cytochrome C was found to be the best substrate specific to the protease enzyme secreted by *Bacillus megaterium*, followed by soyabean protein isolate, casein [140]. For protease produced by a thermophilic strain of *B. subtilis* DM-04, casein served as the most preferred substrate, followed by gelatin, whereas bovine serum globulin and fibrinogen were least hydrolysed by this enzyme [83]. Alkaline protease produced by *Bacillus brevis* SSA1 was found to be active over a broad range of substrates like casein, BSA, gelatin and haemoglobin [4]. The enzyme activity of protease produced by *A. flavus* AP_2 was the highest with gelatin [51]. The alkaline protease from *Beauveria* sp. (BAP) was more active against casein compared to haemoglobin and bovine serum albumin [108].

4.2. Effect of metal ions and stabilizers/additives

Various metal ions and reagents have been reported to influence the activity of proteases. Calcium ions are known as inducers and stabilizers of many enzymes and protect them from conformational changes [122]. Ca^{+2}, Mg^{+2} and Mn^{+2} ions positively regulated the enzyme activity of alkaline protease from *B. circulans* [101]. Mn^{+2} activated enzyme activity of protease from *Pseudomonas thermaerum* GW1 by fivefold, while Cu^{+2}, Mg^{+2} and Ca^{+2} moderately activated enzyme activity [39]. The addition of Mg^{+2} and Ca^{+2} increased the enzyme activity of protease from *B. licheniformis* MP1 by about 13% and 15%, respectively [56]. Ca^{+2} had no stimulatory effect on activity of protease produced by actinomycete, *Nocardiopsis prasina* unlike the case of several bacterial proteases [88]. Addition of 5 mM $CaCl_2$ enhanced the activity 105.3% of alkaline protease enzyme produced by *A. niger*. [30]. Basu et al. [16] reported activation of protease from *A. niger* AB100 by metal ions such as Ca^{+2}, Fe^{+2}, Zn^{+2} and Mg^{+2}. Isopropanol, methanol and benzene increased activity of protease from *Pseudomonas thermaerum* GW1 [39]. Gupta and Khare [45] reported that crude *P. aeruginosa* PseA protease showed a remarkable stability in the presence of most solvents, having the logarithm of the partition coefficient (log *P*) above 2.0, but was less stable in the presence of hydrophilic solvents. The protease from *B. subtilis* strain Rand was not only stable in the presence of organic solvents, but it also exhibited a higher activity than in the absence of organic solvent [2].

5. Conclusion

With increasing emphasis on the environmental protection, the use of biocatalysts gained considerable attention in this biotechnological era. There is a need to explore new microorganisms for production of newer enzymes which have versatile ability to fulfil industrial demand. The challenges which demand attention include loss of enzyme activity over a period of time, higher cost of production and more precise control over process used for production particularly SSF. For production of enzyme for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process. The performance of

protease is influenced by several factors, such as pH of production medium, ionic strength, temperature and mechanical handling. Newer enzymes with novel properties that can further enhance the industrial process using the current enzyme is always in demand. Further, the genetic and protein engineering can play a big role for the large scale production as well as for the alteration of different properties of proteases, keeping in view the harsh conditions during industrial processes.

Conflict of interest

The authors declare that they have no conflict of interest.

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