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MICROBIAL SERINE ALKALINE PROTEASES; PRODUCTION AND APPLICATIONS

Shameen Aslam and Hamid Mukhtar

Institute of Industrial Biotechnology, Government College University, Lahore-54000, Pakistan
Email:hamidwaseer@yahoo.com

ABSTRACT

Proteases belonging to class 3 of hydrolases are very important enzymes produced by many living organisms. These enzymes perform many biological and physiological functions. Serine alkaline proteases (SAPs) are proteases that consist of serine residue in their catalytic triad. Both submerged and solid state fermentation can be used for their production. There are certain parameters that need to be optimized for successful and increased production of enzymes. The optimum pH range for production of SAP is 7-10 but some microorganisms require highly alkaline pH (greater than 10). However, the incubation temperature used for production of SAP can vary form organism to organism. An incubation period of 24-72 hours is usually required by microorganisms for production of SAP, but some microorganisms also have been reported to produce SAPs after incubation of 7-10 days. Agro-industrial wastes such as wheat bran, soybean meal, sugarcane bagasse and molasses etc. can be used as cheap sources of carbon and nitrogen for production of SAPs. These alkaline proteases are environmentally friendly as compared to chemical methods and thus have a wide variety of industrial applications that include leather industry, silk degumming, detergent industry, medical industry, food industry, recovery of silver from photographic films and waste management etc.

Key words: serine alkaline protease, production, fermentation, *Bacillus* sp., parameters, industrial applications

INTRODUCTION

Proteases also called as proteinases or peptidases are very important enzymes with different physiological functions and commercial applications, which is evident by the fact that approximately 60% of the enzyme market is accounted by them (Hajji *et al.,* 2007; Saurabh *et al.,* 2007). According to enzyme classification number, they belong to class 3 of hydrolases and its subclass 4. Their main function is hydrolysing proteins into peptides and amino acids by cleaving the peptide bonds between amino acids, a process called as proteolytic cleavage (Mehtani *et al.,* 2013; Varia *et al.,* 2019). Due to their wide applications in different industries, there is increase in the study of proteolytic enzymes.

There are different types of proteases that utilize different catalytic mechanisms to carry out the same reaction of proteolytic cleavage. According to IUBMB Nomenclature Committee, proteases are classified into two major categories on the basis of the types of reaction they catalyze. The two major groups of proteases are exopeptidases and endopeptidases which are further classified into classes as shown in Figure 1. Exopeptidases hydrolyse the peptide bonds that are adjacent to either carboxyl or amino terminal of the substrate. They are classified into carboxy-peptidases and amino-peptidases on the basis of their action at carbon and nitrogen terminals of the substrate, respectively (Naveed *et al.,* 2020). On the basis of chemical group involved in catalytic activity, endo-peptidases are classified into six further classes which are serine, cysteine, aspartic acid, metallo, glutamic acid and threonine proteases (Verma *et al.,* 2011).

Serine alkaline proteases

Serine proteases are an ancient and a large group of proteases that use serine residue for catalyzing hydrolytic reactions involving esters, amides and peptides. The name came from Ser residue which is present at the active site. Among all types of proteases, one-third are serine proteases. The catalytic centre of serine proteases consist of histidine, aspartic acid and serine residues, among which serine acts as a nucleophile, aspartate as an electrophile and histidine as a base (Hedstorm, 2002; Toth *et al*., 2007).

The catalytic mechanism of serine proteases depend on the hydroxyl group of serine residue. In the reaction mechanism, first of all, the reaction is started by the attack at the carbonyl moiety of the scissile bond by the nucleophilic serine residue of the active site, and as a result, a proton is transferred from the serine hydroxyl group to the active site histidine (Hedstorm, 2002; Di Cera, 2009). The catalytic triad facilitates the transfer of proton while aspartate helps in stabilizing the developing positive charge. Then, there is cleavage of peptide bond by the donation of proton to the nitrogen atom as described in Figure 3. After that, there is esterification of amino-terminal

part of the substrate to the enzyme serine in covalent acyl intermediate, and the carboxy-terminal part of substrate is released that leads to the completion of the acylation process of reaction. After that, there is a decylation process which is opposite of acylation, in which there is a substitution of H_2O with amine component of the substrate.

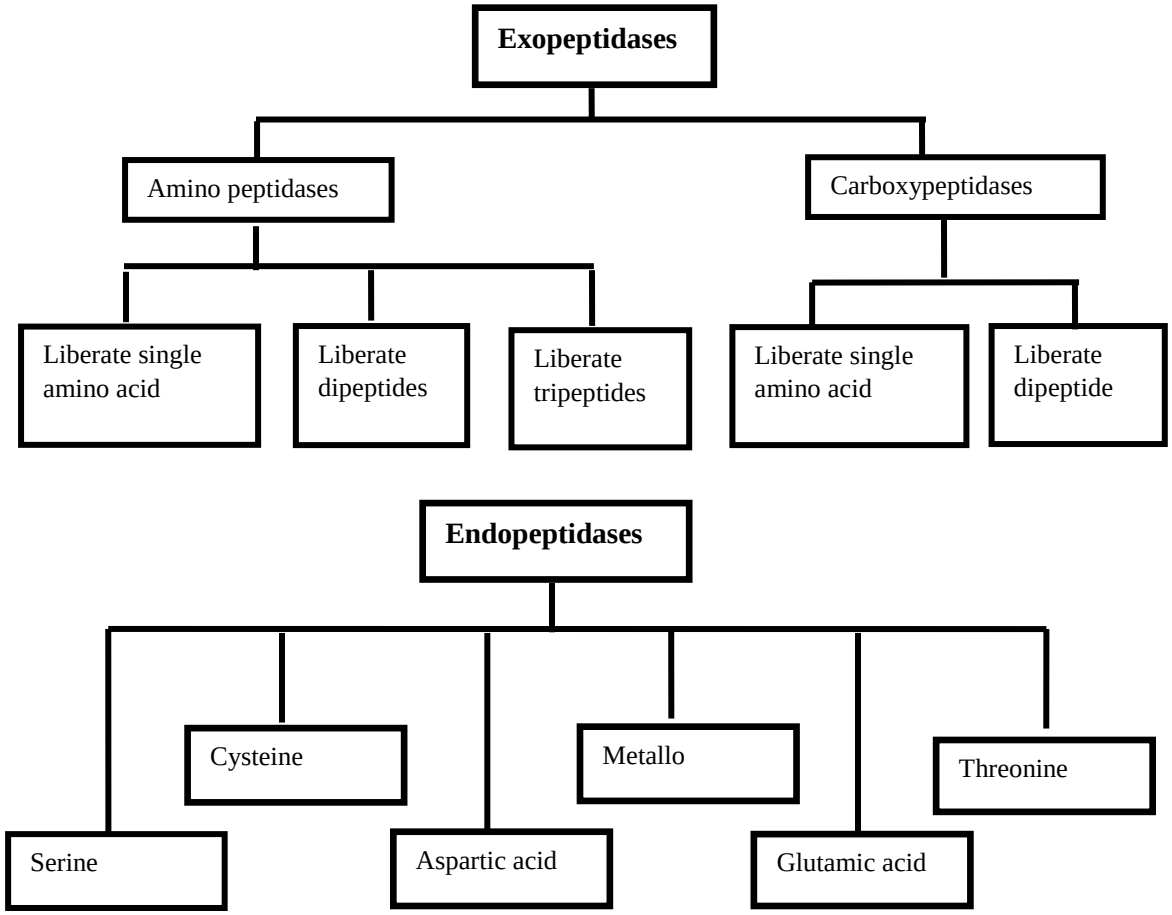

Fig. 1. Classification of proteases.

Most of serine alkaline proteases show optimal activity at pH 8-10. Most of them are thermostable and remain active and stable at a high temperature of 50-70°C. The molecular weight of these proteases are found in the range of 20 to 30 kDa but some also have molecular weight greater than 40 kDa. These enzymes show irreversible inhibition by Phenyl-methyl-sulfonyl fluoride (PMSF), di-isopropyl-flouro-phosphate (DFP) and tosyl-L-lysine Chloro-methyl ketone (TLCK). The PMSF inhibits the activity of serine alkaline protease by sulfonating the serine residue present in the active site. Metal ions are generally required for their activity and stability. Some common examples include Ca^{2+} , Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} ions etc. (Gupta *et al.*, 2002). The cations play a vital role in maintaining the active conformation of the enzyme at high temperatures by protecting the enzyme structure from thermal denaturation. Among the metal ions, Ca^{2+} ions play an essential role in maintaining stability and activity of alkaline proteases at high temperature (Jisha *et al.*, 2013).

Production of serine alkaline proteases

Proteases produced by microorganisms can be both extracellular and intracellular. Established fermentation methods can be used for the production of large quantities of proteases in a relatively short interval of time. Mostly, proteases produced by microorganisms are extracellular in nature and thus the microbial producer secretes them in the fermentation broth medium. This simplifies their downstream processing (Souza *et al*., 2015). Mostly serine alkaline proteases are produced and purified from different species of *Bacillus.* The crystal structure of alkaline

serine protease as shown in Figure 2 is produced by *Bacillus sp.* KSM-KP43. The examples of other microbes that produce these types of proteases include species of *Arthrobacter*, *Streptomyces*, *Flavobacterium*, *Aspergillus*, *Serratia* and *Aeribacillus etc.* (Mienda *et al.*, 2014).

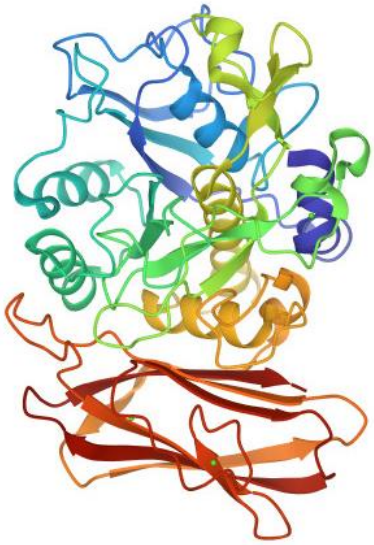

Fig. 2. Crystal Structure of alkaline serine protease KP-43 from *Bacillus sp.* KSM-KP43 (1.50 angstrom, 293 K)(Nonaka *et al.*,2004).

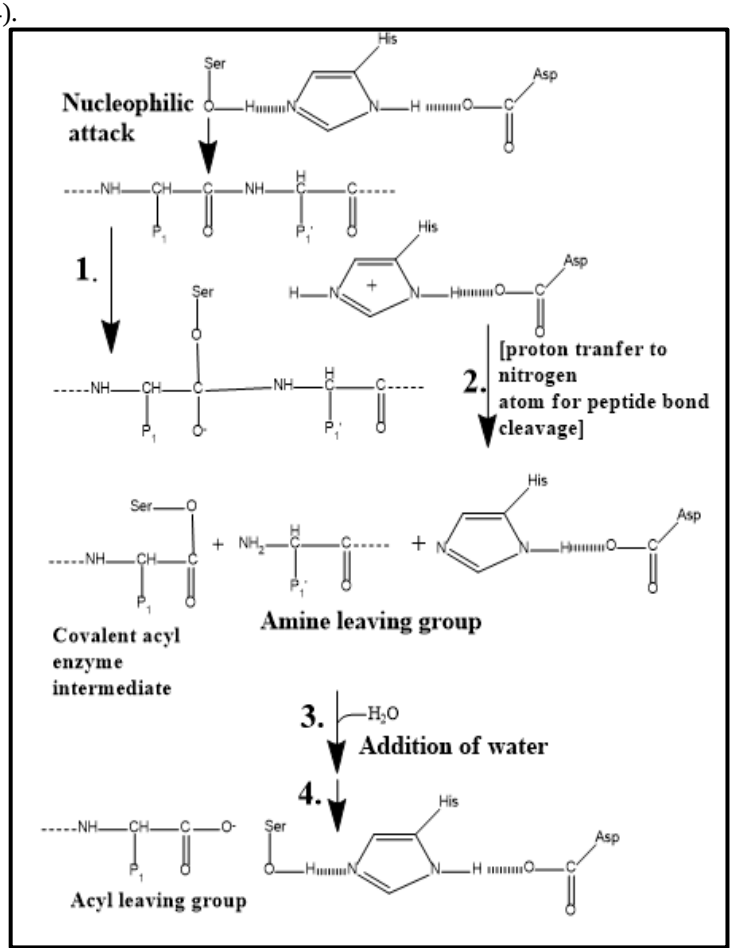

Fig. 3. Reaction mechanism of serine alkaline proteases

Table 1. Biochemical characterization of some serine alkaline proteases produced by microorganisms

Organism	**Temp (°C)**	**pH**	**MW (kDa)**	**Metal Ion (Divalent)**	***Km***	**V*max***	**Inhibitors**	**References**
Aspergillus tamarii URM4634	50	9	49.3	Mg^{2+} and Ca^{2+} ions	0.434 mg/mL	7.739 mg/mL/min	PMSF	(Da silva *et al.*, 2018)
Bacillus safensis strain RH12	60	9	~28	Ca^{2+} , Co^{2+} , and Mg^{2+} Ions	-	-	PMSF, DFP	(Rekik *et al.*, 2019)
Haloalkaliphilic *Bacillus lehensis* JO-26	50	10	34.6	Ca^{2+} Ions	1.38 mg/ml	27.14 µmol mg^{-1} min^{-1}	PMSF	(Bhatt and Singh, 2020)
Bacillus velezensis SW5	40	8	33.9	Ca^{2+} and Zn^{2+} Ions	-	-	PMSF, EDTA	(Yang *et al.*, 2020)
Bacillus subtilis M33	55	10	39	-	0.706 mg/ml	3000 µM.min^{-1}	PMSF	(Karaboga andLogoglu, 2019)
Gracilibacillus boraciitolerans strain LO15	65	10	~ 30	-	-	-	DFP and PMSF	(Ouelhadj *et al.*, 2020)
Bacillus sp. DEM07	50	10	27.5	Na^{+} and Ca^{2+} Ions	0.06 mg/ml	1.25 µmol/min	PMSF	(Nazari and Mehrabi, 2019)
Bacillus atrophaeus NIJ	70	11	~28	Ca^{2+} , Mn^{2+} and Mg^{2+} ions	-	-	PMSF and DFP	(Rahem *et al.*, 2021)
Bacillus cereus	50	10	38	Mn^{2+} ions	0.64mM	420 µmol/mL min	PMSF and EDTA	(Gurunathan *et al.*, 2021)
Streptomyces sp. GS-1	45	8.5	30	Mn^{2+} ions	-	32.25 µmol l^{-1} min^{-1} mg^{-1}	PMSF	(Sarkar andSuthindhiran,2020)

Both submerged (SmF) and solid state fermentation (SSF) can be utilized for the production of these serine alkaline proteases. In submerged fermentation, the microbes utilize the nutrients present in the liquid production medium and in return release the enzymes in the solution (Renge *et al.*, 2012). It can be carried out in cotton plugged Erlenmeyer shaker flasks. However, for more control and good performance of the process, bioreactors or fermentors are also used. In solid state fermentation, the microorganisms are cultivated on solid substrates such as wheat bran, rice bran, rice husk, corn flour, sugar beet pulp, grains etc. The solid substrates are responsible for supplying nutrients to the culture and also serve as anchorage for the growth of microorganisms. The summary of some bacteria and fungi that produce serine alkaline proteases along with their mode of fermentation is shown in Table 2.

Parameters affecting the production of SAPs

There are some important parameters that need to be optimized for the maximum growth of the organism along with the increased and successful production of enzymes. These parameters include cultural and nutritional parameters. The summary of these parameters is depicted in Figure 4.

Cultural parameters

Cultural parameters are those that play a vital role in the cultivation of microorganisms and these include the pH of the medium, incubation temperature, inoculum, incubation time and agitation etc. These parameters are described below.

Table 2. Some bacteria and fungi that produce serine alkaline proteases along with the mode of fermentation used.

Major group	**Species**	**Mode of fermentation**	**References**
Bacteria	*B. thuringinsis-SH*-II-1A	SmF	(Harer *et al.*, 2018)
	Bacillus subtilis DM-04	SmF	(Rai and Mukherjee, 2010)
	Bacillus pumilus SG2	SmF	(Sangeetha and Arulpandi, 2019)
	Bacillus pumilus MCAS8	SmF	(Renganathan *et al.* ,2012)
	B. horikoshii	SmF	(Joo *et al.*, 2002)
	Gracilibacillus boraciitolerans strain LO15	SmF	(Ouelhadj *et al.*, 2020)
Fungi	*Aspergillus flavus* MTCC 9952	SSF	(Yadev *et al.*, 2015)
	Myceliophthora sp.	SSF	(Zanphorlin *et al.*, 2011)
	Aspergillus flavus	SSF	(Damare *et al.*, 2020)
	Streptomyces koyangensis TN650	SmF	(Elhoul *et al.*, 2015)
	Aspergillus clavatus ES1	SSF	(Hajji *et al.*, 2007)
	Trametes cingulata strain CTM10101	SmF	(Benmrad *et al.*, 2016)
	Streptomyces sp.	SSF	(Sarkar and Suthindhiran, 2020)
	Aspergillus tamarii URM4634	SSF	(De silva *et al.*, 2018)
	Geotrichum candidum QAUGC01	SmF	(Muhammad *et al.*, 2019)
	Neocosmospora sp. N1	SSF	(Matkawala *et al.*, 2019)

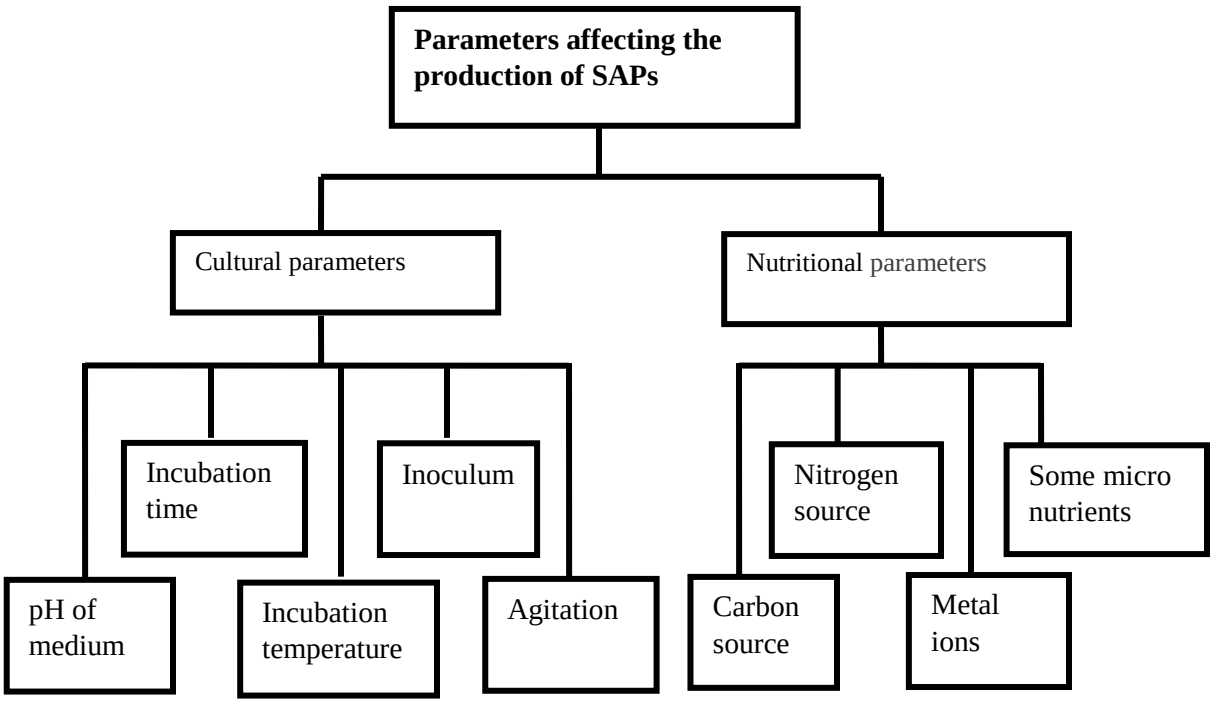

Fig. 4 . Summary of some parameters affecting the growth of microorganisms and production of enzymes.

pH of medium

An important parameter for the production of enzymes from microorganisms through fermentation is pH of the medium. It greatly influences the metabolic activities carried out by microorganisms and also affects the transportation of components through the plasma membranes (Calik *et al.*, 2002). Alkaliphilic microorganisms require alkaline pH for their growth and production of enzymes. The optimum pH for the production of serine alkaline proteases has been reported in the range of 7-10. However, some microbes have been reported to produce SAP at pH greater than 10 e.g. serine alkaline proteases were produced from halo-tolerant *Bacillus sp.*,

Salipaludibacillus agaradhaerens strain AK-Rat a pH of 10.5 (Ibrahim *et al.,* 2015; Ibrahim *et al.,* 2019). Some fungal species require low pH for production of proteases such as *Penicillium chrysogenium* X5 produced SAP at pH of 5.6 (Benmrad *et al.,* 2018) while *Geotrichum candidum* QAUGC01 produced SAP at pH 4 (Muhammad *et al.,* 2019).

Incubation temperature

Temperature is a very important parameter that affects the production of enzymes by microorganisms. The temperature required by microorganisms for the production of serine alkaline protease is diverse and can vary from organism to organism. Mostly the mesophilic bacteria require 37°C for growth and production of enzymes. However, the fungal species are reported to grow and produce enzymes in range from 28-30°C. Some thermophilic and hyper-thermophilic species have been reported to produce SAPs at a temperature range of 40-70°C. For psychrophiles, low temperature is required for this purpose e.g. a psychrophilic bacterium *Pseudomonas* strain DY-A produced serine alkaline protease at 10°C (Zeng *et al.,* 2003).

Incubation period

The incubation period significantly affects the production of enzymes and it can vary from 24 hours to one week depending upon the type of microorganism, and other environmental conditions of culture, such as temperature and pH etc. (Sharma *et al.,* 2017). The production of enzymes is directly proportional to the incubation period but up to a certain limit. The enzymes can lose their activity if incubated for a prolonged period of time. Mostly, bacterial species require an incubation period of 24-72 hours for production of serine alkaline proteases. However, some fungal species require more incubation period (up to 7-10 days) e.g. 10 days incubation has been reported for production of SAP by *Streptomyces sp.* GS-1 (Sarkar and Suthindhiran, 2020). The incubation period used for different microorganisms for production of serine alkaline proteases is listed in Table 3.

Inoculum

The amount, age, type and concentration of inoculum added in the fermentation medium governs the growth of the microorganism. Different studies have shown that low inoculum level shows a lower yield and high inoculum level shows a higher yield of serine alkaline proteases. So, the inoculum level has to be optimized for maximum protease production (Bhunia *et al.,* 2012). The bacterial inoculum in the range 1-10% has been reported for the production of serine alkaline proteases. On the other hand, for fungi, the spore or conidial suspension is used. And in the suspension, the number of spores or conidia/ml can vary. Mostly, 10^7-10^8 spore/ ml have been reported for production of SAPs from fungi. The age of inoculum is also very important. Some researchers have reported the use of fresh culture e.g. 2% (v/v) fresh culture was used for production of SAP by *Bacillus licheniformis* NCIM-2042 (Bhunia *et al*., 2013) while some have reported to use 4-20 hours old culture for production of serine alkaline proteases e.g. 5% (v/v) of a 20 h old culture was used for production of SAPs from *Bacillus sp.* HR-08 and KR-8102 (Moradian *et al.,* 2006).

Agitation

Agitation plays an important mixing and shearing role in the fermentation media. It ensures a uniform suspension of microbial cells in the homogenous nutrient medium. It also helps in increasing the rate of oxygen transfer from air bubbles to the liquid medium. Different researchers have reported the use of 150-200 rpm for the production of serine alkaline proteases. However, the agitation of 50 rpm has been reported for the production of serine alkaline protease from *Pediococcus acidilactici NCDC* 252 (Bansal *et al.,* 2021).

Nutritional parameters

Nutritional parameters are those that provide nutrients to microorganisms and greatly influence the production of enzymes. These parameters include nitrogen source, carbon source, metal ions and some micro nutrients etc. The concentration of components of the media is very important as some microbes can synthesize all the components of cells form nitrogen and carbon sources. However, some require micro nutrients such as amino acids, trace elements and vitamins etc. (Bhunia *et al.,* 2012). These parameters for serine alkaline proteases are discussed below.

Carbon sources

Carbon is a very important component of the fermentation media for the optimum growth of micro organisms and for production of enzymes. Microorganisms require carbon as an energy source for the growth of their cells and for biosynthesis of different compounds. Agro-industrial wastes such as sugarcane bagasse, wheat bran, hulled grain of wheat, wheat straw and molasses etc. are the cheapest and abundantly available natural carbon sources that can be

used for production of serine alkaline proteases. Some other carbon sources reported for production of SAPs by microorganisms include glucose, sucrose, dextrose, starch, maltose, malt extract, lactose and fructose etc. The summary of carbon sources used by microorganisms for production of serine alkaline proteases is described in the Table 4.

Table 3. Some cultural parameters affecting the production of serine alkaline proteases.

Micro organism	**Medium pH**	**Incubation temperature (°C)**	**Incubation period**	**Inoculum**	**Agitation (rpm)**	**References**
Streptomyces sp. strain AH4	9	30	6 days	~10^7 spore/ml)	200	(Touioui *et al.*, 2015)
Virgibacillus natechei sp. nov., Strain FarDT	7.4	35	36h	-	200	(Mechri *et al.*, 2019)
Marine *Bacillus flexus* APCMST-RS2P	7	32	72 h	10%(v/v)	150	(Maruthiah *et al.*, 2014)
Aspergillus tamarii URM4634	7	30	72h	10^7 spores/ml	150	(Da Silva *et al.*, 2018)
Purpureocillium lilacinum LPS # 876	6	28	111h	2×10^7conidia/ml	200	(Cavello *et al.*, 2013)
Pediococcus acidilactici NCDC 252	8	37	24h	Overnight grown culture	50	(Bansal *et al.*, 2020)
Streptomyces koyangensis TN650	7.2	30	6 days	10^8spore/ml	190	(Elhoul *et al.*, 2015)
Neocosmospora sp. N1	-	30	96 h	10^7spore/ml	-	(Matkawala *et al.*, 2019)
Bacillus caseinilyticus	9	37	48 h	1%(v/v)	180	(Mothe and Sultanpuram, 2016)
Penicillium chrysogenium X5	5.6	30	108h	10^8spore/ml	150	(Benmrad *et al.*, 2018)
Bacillus pumilus SG2	9	37	48h	5% (v/v)of overnight culture	180	(Sangeetha and Arulpandi, 2019)
Geotrichum candidum QAUG C01	4	25	72h	5 ± 1x10^8 cells /ml	200	(Muhammad *et al.*,2019)
Melghiribacillus thermohalophilus Nari2AT	7.4	55	52h	-	160	(Mechri *et al.*, 2019)
S. agaradhaerens AK-R	10.5	35	32h	2%(v/v) of 18 h old culture	150	(Ibrahim *et al.*, 2019)

Table 4. List of different carbon sources used by microorganisms for the production of serine alkaline proteases.

Microorganism	Carbon source	Concentration (w/v)	References
Bacillus cereus strain S8	Molasses	1%	(Lakshami *et al.*,2018)
Streptomyces koyangensis TN650	Malt extract	1%	(Elhoul *et al.*, 2015)
Bacillus megaterium	Sucrose	2%	(Jeong *et al.*,2017)
Bacillus caseinilyticus	Fructose	1%	(Mothe and Sultanpuram, 2016)
Bacillus safensis strain RH12	Galactose	1%	(Rekik *et al.*,2019)
Streptomyces sp. GS-1	Wheat bran	5%	(Sarkar andSuthindhiran, 2020)
Bacillus sp. NPST-AK15	Fructose	2%	(Ibrahim *et al.*, 2015)
Chryseobacterium sp.	Starch	1%	(Mageswari *et al.*, 2017)
Salipaludibacillus agaradhaerens strain AK-R	Wheat bran	1.5%	(Ibrahim *et al.*, 2019)
Bacillus licheniformis NCIM-2042	Starch	3.08%	(Bhunia *et al.*, 2013)
Bacillus pumilus SG2	Glucose	1%	(Sangeetha and Arulpandi, 2019)
Bacillus subtilis AP-MSU 6	Glucose	1%	(Maruthiah *et al*., 2013)
Bacillus pumilus TMS55	Maltose	0.5%	(Ibrahim *et al.*, 2011)
Serratia sp.	Wheat flour	1%	(Li *et al.*, 2011)
Trametes cingulata strain CTM10101	Lentil flour, glucose	1%, 1%	(Benmrad *et al.*, 2016)
Halobacterium sp.	Lactose	1%	(Vijayaraghavan *et al*.,2012)
Bacillus subtilis	Glucose	0.5%	(Borkar *et al.*,2018)
Bacillus sp. DEM07	Citric acid, sucrose	0.5%, 0.5%	(Nazari and Mehrabi,2019)

Nitrogen sources

Nitrogen is a very essential component for the growth of microbes and subsequent production of enzymes. Both organic and inorganic nitrogen sources can be used in the fermentation media. The inorganic nitrogen sources are usually supplied as ammonium salts such as ammonium sulfate, diammonium hydrogen phosphate, ammonia and nitrates etc. However, the organic nitrogen sources include urea, amino acids and proteins. The agro industrial by-products such as soybean meal and urea etc. can be used as the cheap sources of nitrogen for production of serine alkaline proteases. Other nitrogen sources that have been reported for production of SAPs include casein, gelatin, yeast extract, beef extract, peptone, diammonium hydrogen phosphate, ammonium sulfate and ammonium chloride etc. The summary of nitrogen sources used by microorganisms for the production of SAPs is shown in the Table 5.

Metal ions

In addition to other growth components, microorganisms also require some metal ions for the production of enzymes. The examples of divalent metal ions that are added in fermentation media include Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Na^{2+} , Mg^{2+} and Mn^{2+} etc. (Bhunia *et al.*, 2012). The metal ions are used in the media in the form of salts such as sodium chloride, magnesium sulfate, potassium hydrogen phosphate, manganese chloride and nickel chloride etc. These metal ions act as cofactors and help in inducing the production of enzymes by microorganisms. Among all the ions, Na^{2+}, Mg^{+}, K^{+} and Ca^{2+} ions are reported to be used for production of serine alkaline proteases. Some microorganisms require a single metal ion and some require the combination of two or more than two metal ions for production of serine alkaline proteases as listed in the Table 6.

Table 5. List of different nitrogen sources used by microorganisms for production of serine alkaline proteases.

Micro organism	Nitrogen source	Concentration (w/v)	References
Bacillus safensis strain RH12	Yeast extract	0.4%	(Rekik *et al.*,2019)
Streptomyces koyangensis TN650	Casein, yeast extract	1.5% and 0.5%	(Elhoul *et al.*, 2015)
Bacillus licheniformis NCIM-2042	Soybean meal	8.16%	(Bhunia *et al.*, 2013)
Pediococcus acidilactici NCDC 252	Yeast extract	1%	(Bansal *et al.*, 2020)
Bacillus cereus strain S8	Potassium nitrate	0.75%	(Lakshmi *et al.*,2018)
Bacillus sp. NPST-AK15	Yeast extract	0.75%	(Ibrahim *et al.*, 2015)
Trametes cingulata strain CTM10101	Yeast extract	0.2%	(Benmrad *et al.*, 2016)
Bacillus subtilis	Peptone	0.75%	(Borkar *et al.*, 2018)
Bacillus pumilus TMS55	Beef extract, soybean meal	0.5%, 1%	(Ibrahim *et al.*, 2011)
Bacillus licheniformis BA17	Yeast extract	0.5%	(Öztürk *et al.*, 2009)
Bacillus flexus APCMST-RS2P	Yeast extract, peptone	1%, 1%	(Maruthiah *et al.*, 2014)
Chryseobacterium sp.	urea	0.5%	(Mageswari *et al.*, 2017)
Salipaludibacillus agaradhaerens strain AK-R	Gelatin and yeast extract	1% and 0.5%	(Ibrahim *et al.*, 2019)
Halobacterium sp.	Ammonium chloride	1%	(Vijayaraghavan *et al.*,2012)
Bacillus pumilus SG2	Yeast extract	0.3%	(Sangeetha and Arulpandi, 2019)
Bacillus sp. DEM07	Yeast extract	1%	(Nazari and Mehrabi,2019)

Table 6. List of metal ions added in fermentation media for production of serine alkaline proteases.

Micro organism	Metal salts	Concentration (w/v)	References
Geotrichum candidum QAUGC01	NaCl	0.5%	(Muhammad *et al.*, 2019)
Bacillus sp. DEM07	$CaCl_2.2H_2O$	0.01%	(Nazari and Mehrabi, 2019)
A. fumigatus	KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and KCl	0.1%,0.05%.0.05%	(Hernández-Martínez *et al.*, 2011)
Bacillus pumilus TMS55	MgSO4, K2HPO4 and NaCl	0.2%,0.5%,0.5%	(Ibrahim *et al.*, 2011)
Penicillium chrysogenium strain X5	$CaCl_2$, KH_2PO_4 and K_2HPO_4	0.2%,0.05%,0.05%	(Benmrad *et al.*, 2018)
Streptomyces koyangensis TN650	KH_2PO_4 and K_2HPO_4	0.1%,0.1%	(Elhoul *et al.*, 2015)
Bacillus atrophaeus NIJ	K_2HPO_4, KH_2PO_4 and $CaCl_2$	0.15%,0.15%,0.1%	(Rahem *et al.*, 2021)
Bacillus licheniformis BA17	K_2HPO_4, Na_2NO_3 and $MgSO_4{\cdot}7H_2O$	0.1%,1%,0.02%	(Öztürk *et al.*, 2009)
Streptomyces sp. strain AH4	$CaCl_2$,K_2HPO_4 and KH_2PO_4	0.5%,0.1%,0.1%	(Touioui *et al.*, 2015)
Bacillus pumilus SG2	$CaCl_2$, $MgCl_2$ and NaCl	0.04%,0.02%,0.5%	(Sangeetha and Arulpandi, 2019)
Bacillus safensis strain RH12	K_2HPO_4, KH_2PO_4 and $CaCl_2$	0.05%,0.05%,0.2%	(Rekik *et al.*, 2019)
Bacillus licheniformis NCIM-2042	K_2HPO_4,KH_2PO_4,$MgSO_4$ and NaCl	0.3%,0.1%,0.05%, 0.53%	(Bhunia *et al.*, 2013)

Applications of serine alkaline proteases

Serine alkaline proteases possess distinctive properties due to which they are commercially used for the hydrolysis of protein in various industries such as medical industry, leather industry, silk degumming, waste management, detergent industry, food and feed industry etc. as graphically illustrated in Figure 5.

Fig. 5. Graphical Illustration of applications of serine alkaline proteases.

Detergent industry

Alkaline proteases are commercially used in detergent industry because of their ability to remove various types of stains such as blood, egg, gravy, milk, curd etc. at alkaline pH conditions. They are used in formulation of household detergents, laundry detergents and cleaning detergents. The use of non-enzymatic processes can leave the stains on clothes due to use of chemicals such as bleach (Akbar and Sharma, 2017; Thakur *et al*., 2018; Salwan and Sharma, 2019). Trypsin and chymotrypsin were used for degrading protein stains for the first time in 1913. Currently, a variety of commercial detergents are available in the market that include Era plus® (Procter and Gamble), Dynamo® and opticlean etc. that contain different enzymes including proteases (Matkawala *et al*., 2021). Different researchers have characterized serine alkaline proteases from different microorganisms that can be used as additives in detergents such as *Bacillus* sp., *Penicillium chrysogenum* strain X5, marine *Engyodontium album* BTMFS10, *Bacillus mojavensis* A21, *A. pallidus* C10, halo-alkaliphilic bacterium sp. AH-6 and *Bacillus subtilis* PE-11 etc. (Adinarayana *et al.*, 2003; Dodia *et al*., 2008; Haddar *et al.*, 2009; Chellappan *et al.*, 2011; Kamran *et al.*, 2015; Yildirim *et al.*, 2017; Benmrad *et al.*, 2018).

Leather industry

Leather is an end product formed after processing of animal skin and hides. Proteases play an essential role in treating raw leather in tanneries.Serine alkaline proteases are most effective for use to treat leather. Alkaline proteases are used in soaking stage, a process in which blood, dung or dirt is removed from the animal hides. They solubilize albumin or globulin proteins and thus are important for removing dirt in the soaking stage. Traditionally, lime-sulfide process has been used for dehairing of animal skins (Anwar and Saleemuddin, 1998) but due to release of some toxic chemicals, this conventional method has many objections globally. The use of alkaline proteases has become more worldwide for the removal of hairs (Singh and Bajaj, 2017). The proteases with keratinase activity can degrade the keratin in hair and thus help in the easy removal of hair (Contesini *et al.,* 2017). They also increase the surface area and make the skin clean with easy uptake of dyes. Serine alkaline proteases that show dehairing ability have been reported to be produced by many microorganisms such as *Bacillus circulans* strain DZ100, *B. subtilis*, *Vibrio metschnikovii* NG155, *Bacillus circulans*, *Bacillus altitudinis* GVC11 and *Bacillus pumilus* BA06etc. (Mukhtar and Haq, 2008; Rao *et al.,* 2009; Kumar *et al.,* 2011; Zhao *et al.,* 2012; Benkiar *et al.,* 2013; George *et al.,* 2014).The conventional method of bating involves the use of pancreatic trypsin, but nowadays microbial proteases are more economical to use.

Food industry

Proteases produced by microorganisms have largely been used in dairy, baking and food processing industries for various purposes. Serine alkaline proteases are used for production of high nutrition protein hydrolysates by hydrolyzing proteins e.g. chymotrypsin can be utilized for production of protein hydrolysates such as casein, whey, soy-isolate and wheat gluten etc. The protein hydrolysates have wide applications in infant food formulations, processed food items, dietary food products, fortification of soft drinks and fruit juices and also as bioactive compounds in nutraceuticals (Contesini *et al.,* 2017). Alkaline proteases are also used in making bread, different types of cheese, for removing bitterness in the food and for making it sweeter (Banerjee and Ray, 2017). Alkaline proteases from *Streptococcus cremoris, Amycolata sp. and Amycolatopsis sp*. have been largely used for industrial production of cheese (Sundus *et al.,* 2016).

Medical industry

Microbial proteases have been widely used in medical industry. Many proteases have been approved by FDA for treating various clinical conditions. Immobilized subtilisins have been utilized for producing soft-gel based formulas, ointments, gauzes, materials used for bandaging and treatment of burns and wounds etc. (Chanalia *et al*., 2011). Sangeetha and Arulpandi have reported the anti-inflammatory activity of a serine protease produced from *Bacillus pumilus* SG2 (Sangeetha and Arulpandi, 2019).Various other serine alkaline proteases are also used for treating various diseases such as factor VIIa for treating hemophilia activated protein C for sepsis, asparaginase for lymphocytic leukemia, elastoterase for treating abscesses, wounds and burns (Varia *et al.,* 2019; Matkawala *et al.,* 2021).The alkaline proteases with fibrinolytic activity can be utilized in thrombolytic therapy and in the development of various anti-cancer drugs (Thakur and Kumar, 2018). Research is also going on determining new proteases with therapeutic applications.

Waste management

Another important application of serine alkaline proteases is their use in the management of wastes that are generated as a result of household activities and also from many food processing industries. These proteases solubilize the proteinaceous matter present in waste and thus help in reducing BOD of water. Serine alkaline proteases can also be used to hydrolyse the waste of animal feathers into soluble proteins and amino acids in cheap and milder conditions for the production of products that can be valuable to use as feed additives and also as fertilizers. The waste generated from leather and poultry industries contain a large amount of keratin. Several million tons of feather waste is generated annually by poultry industries and the degradation of such waste by using chemical methods of hydrolysis is not environmentally friendly and the best method is to use alkaline proteases (Varia *et al.,* 2019). The feathers are first treated with alkali such as NaOH for mechanical disintegration and then these are treated with enzymes which totally solubilize the feathers.

The marine industry also generates a lot of waste that includes chitin, proteins and calcium carbonate. This causes a lot of environmental pollution. This waste can be utilized as a carbon and nitrogen source for production of proteases and can decrease both the pollution and cost of production of proteases (Barzkar, 2020). Many researchers have reported different serine alkaline proteases that can be utilized for this process. For example, serine alkaline protease was produced from *Bacillus licheniformis* MP1 for deproteinization of shrimp waste (Jellouli *et al.,* 2011).

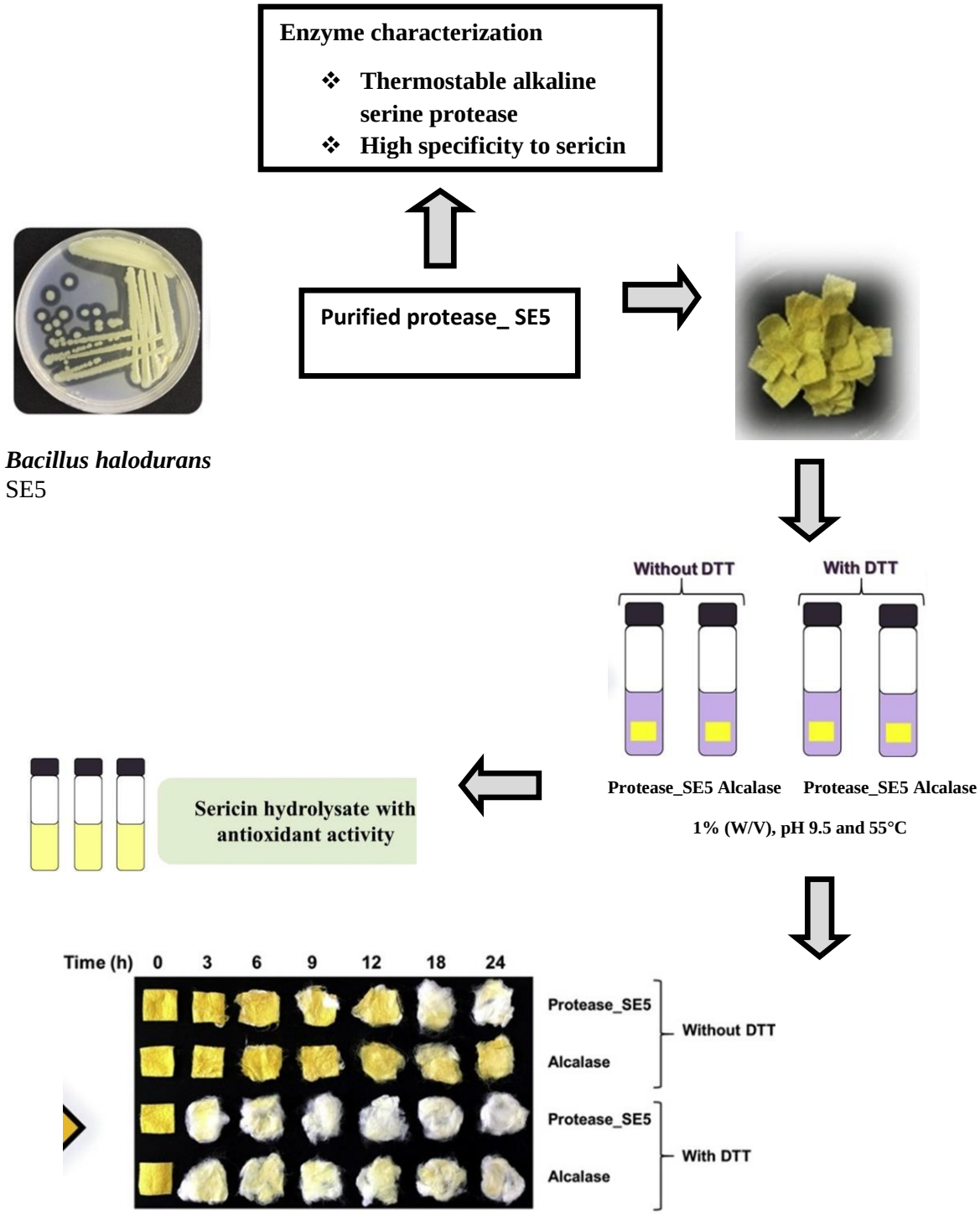

Fig. 6. Graphical representation of silk degumming by *Bacillus halodurans* SE5 (modified from Yakul *et al.*, 2019). Dithiothreitol (DTT).

Silk degumming

Silk consists of cocoon proteins and is produced by silk worms. The silk making process consists of six steps that include cooking, reeling, degumming, dyeing, weaving and finishing. Degumming of silk is a process in which sericin protein is entirely removed from the silk fibroin (Verma *et al.*, 2011). This process increases the smoothness of silk and also enhances its ability to uptake dye better as compared to raw or untreated silk (Suwannaphan *et al.*,

2017). The conventional method of silk degumming involves the use of alkaline soap solution or use of starch for shrink-proofing and twist setting of silk yarns. These methods have certain disadvantages that include requirement of high power, increased cost and environmental problems (Varia *et al.,* 2019). Therefore, serine alkaline proteases are a good choice because they are less expensive, environmentally friendly. They also do not cause damage to the fibres. They break the peptide bonds present in sericin protein. The sericin powder produced as a by-product in the silk degumming process can be utilized as a moisturizer in the cosmetics industry. Thermostable serine alkaline portease (Protease_SE5) purified from *Bacillus halodurans* has the ability to decompose sericin. Protease_SE5 along with commercial alacalase showed enhanced degumming ability in presence of Dithiothreitol (DTT) (Yakul *et al.,* 2019). The graphical representation of silk degumming by *Bacillus halodurans* SE5 is described in Figure 6.

Diagnostic applications

Proteases have also been utilized for diagnostic purposes e.g. proteases isolated form *A. oryzae* can be used for diagnosis of certain enzyme deficient syndrome (Sundus *et al.,* 2016). Fecal elastase can be used for determining exocrine activity of pancreas e.g. in cystic fibrosis. Mast cells also have the ability to releases serine proteases which is an important diagnostic marker for type 1 hypersensitivity reaction. Various serine proteases also act as coagulation factors and thus their level in the body can be utilized for diagnosis of hemorrhagic or thrombotic conditions.

Recovery of silver

Silver being a very noble metal is largely used in photographic industry. The best source of silver recovery is the photographic film that contains the black metallic silver spread in the gelatin layer. The traditional method of silver recovery involves the direct burning of films, then oxidizing metallic silver, and then using chemical solutions to remove the gelatin–silver layer (Kamal *et al.,* 2016). Although these methods are cost effective, but these are not environmentally friendly and can also pose some serious health hazards (Singh AND Bajaj, 2017). Therefore, enzyme based methods can be utilized, and among them alkaline proteases are very helpful (Sharma *et al.,* 2019).

The hydrolysis of gelatin by using proteases also helps in recycling of the film base made of polyester. A SAP produced by *Purpureocillium lilacinum* has been reported to hydrolyse the gelatin layer and the silver was recovered in 6 minutes (Cavello *et al.,* 2013). A recombinant SAP produced by *Bacillus lehensis* has been reported for removal of the gelatin layer in photographic film (Joshi and Satyanarayana, 2013). Alkaline proteases produced by *Aspergillus versicolor* has ability to recover silver from used X-ray films. The silver particles were recovered from dried slurry produced by enzymatic treatment of X-ray film as shown in Figure 7 (Choudhary AND Vishwavidylaya, 2013).

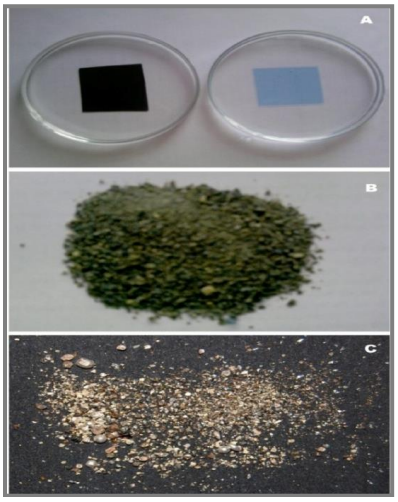

Fig. 7. Recovery of silver from used X-ray films by using crude alkaline protease produced by *Aspergillus versicolor*. (A) Used x-ray film and film after hydrolysis of gelatin-silver layer. (B) Dried slurry after enzymatic treatment of x-ray film. (C) Recovery of silver particles from slurry (Choudhary and Vishwavidylaya, 2013).

Conclusion

Serine alkaline proteases are one of the most important groups of proteases. Their increased industrial applications are due to their broad range of substrate specificity and stability and activity at high temperature and pH. Researchers are more urged to produce and characterize serine alkaline proteases from different microorganisms

by using both solid state fermentation and submerged fermentation to meet their increased global demand. These enzymes are beneficial to use as compared to chemical methods because they are environmentally friendly. More work is needed on these enzymes to get full insight of their other commercial applications.

Table 7. List of some commercially available serine alkaline proteases and their industrial applications (Razzaq *et al.*, 2019; Matkawala *et al*., 2021).

Commercial name	**Formulation**	**Manufacturing company**	**Industrial Applications**
Alcalase	liquid	Novo enzymes	Food processing
Subtilisin A	powder	Sigma Aldrich	Food and pharmaceutical
Savinase	Granulate	Novo enzymes	Food processing/detergent
Esperase	liquid	Novo enzymes	Food processing
SEBZyme AP200	-	Advanced enzymes	Leather
Opticlean	liquid	Solvay enzymes	Detergent
Addclean PRO L/PRO S	Powder/liquid	Advanced enzymes	Detergent
Bio-Sorb-ALKP	powder	Noor enzymes	Animal feed
SEBDigest F59 P	powder	Advanced enzymes	Food processing
Bio-Tan-ALKP	Powder/liquid	Noor enzymes	Detergent
COROLASE	Liquid	AB enzymes	detergent
Bio-pro-ALKP	powder	Noor enzymes	Baking
Multifect PR 6L	liquid	DuPont industrial biosciences	Food processing/silver recovery
Actinase E	powder	Sigma Aldrich	Pharmaceutical
Flavourzyme	Granulate	Novo enzymes	Food processing
PRAL800	powder	Sinobios(Shangai)Imp.and Exp. Co., Ltd	Detergent/leather/silk degumming

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