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Research Article

Identification and Molecular Characterization of Genes Coding Pharmaceutically Important Enzymes from Halo-Thermo Tolerant *Bacillus*

Azam Safary[1,2], Rezvan Moniri[1,3]*, Maryam Hamzeh-Mivehroud[2,4], Siavoush Dastmalchi[2,4]*

[1] *Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran.*
[2] *Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*
[3] *Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran.*
[4] *School of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

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Abstract
Purpose: Robust pharmaceutical and industrial enzymes from extremophile microorganisms are main source of enzymes with tremendous stability under harsh conditions which make them potential tools for commercial and biotechnological applications.
Methods: The genome of a Gram-positive halo-thermotolerant *Bacillus* sp. SL1, new isolate from Saline Lake, was investigated for the presence of genes coding for potentially pharmaceutical enzymes. We determined gene sequences for the enzymes laccase (CotA), l-asparaginase (ansA3, ansA1), glutamate-specific endopeptidase (blaSE), l-arabinose isomerase (araA2), endo-1,4-β mannosidase (gmuG), glutaminase (glsA), pectate lyase (pelA), cellulase (bglC1), aldehyde dehydrogenase (ycbD) and allantoinases (pucH) in the genome of *Bacillus* sp. SL1.
Results: Based on the DNA sequence alignment results, six of the studied enzymes of *Bacillus* sp. SL-1 showed 100% similarity at the nucleotide level to the same genes of *B. licheniformis* 14580 demonstrating extensive organizational relationship between these two strains. Despite high similarities between the *B. licheniformis* and *Bacillus* sp. SL-1 genomes, there are minor differences in the sequences of some enzyme. Approximately 30% of the enzyme sequences revealed more than 99% identity with some variations in nucleotides leading to amino acid substitution in protein sequences.
Conclusion: Molecular characterization of this new isolate provides useful information regarding evolutionary relationship between *B. subtilis* and *B. licheniformis* species. Since, the most industrial processes are often performed in harsh conditions, enzymes from such halo-thermotolerant bacteria may provide economically and industrially appealing biocatalysts to be used under specific physicochemical situations in medical, pharmaceutical, chemical and other industries.

Introduction

Production of raw materials using enzymes is a rapidly expanding technology especially in the pharmaceutical and biotechnology industries. It is an excellent tool for replacing harmful traditional chemical synthesis by environmentally-friendly bio-based processes.[1-3] The chemical synthesis of compounds and pharmaceuticals has several disadvantages such as low catalytic efficiency, lack of chemo-, regio- and enantio-selectivity, and needs for specific conditions in terms of temperature, pH and pressure, just to mention a few. Also, the use of organic solvents leads to environmental issues brought about by organic waste and pollutants. The ability of enzymes to catalyze chemical reaction with high speed and specificity under mild reaction conditions has made them appropriate alternatives to conventional chemical reactions in industry[4] and progressively useful in preparation of diverse products[2,5] such as chiral medicines, biosensors, biofuels, detergents and textiles, pulp and paper, leather and animal feed.

The use of the therapeutic enzymes dates back around 40 years ago. The great affinity and specificity of such enzymes for binding and acting on their targets, distinguish them from all other types of drugs. Moreover enzymes can act as biocatalysts to convert several molecules to the desired products.[6] These distinct characteristics have resulted in the development of many therapeutic enzymes for a variety of diseases.[7] Enzyme-activation prodrug therapy is one of the promising area to overcome the limitations of

***Corresponding authors:** Rezvan Moniri, Siavoush Dastmalchi, Tel: +98 31 55540021, Email: moniri@kaums.ac.ir, moniri_re@yahoo.com Tel: +98 41 33364038, Fax: +98 41 33379420, Email: dastmalchi.s@tbzmed.ac.ir, siavoush11@yahoo.com

chemotherapeutic agents such as insufficient drug concentrations in tumors, systemic toxicity, lack of selectivity for tumor cells and drug-resistance problems in tumor cells.[8,9] The enzyme-prodrug cancer therapy is achieved by delivering the drug-activating enzyme gene or functional protein from nonhuman or human origin in to the tumor tissues accompanied with the simultaneous administration of a prodrug.[10,11] Pro-drugs are nontoxic chemically modified versions of the pharmacologically active agents that can be converted to the active and cytotoxic anticancer drugs with high local concentrations in tumors.[12]

The synthesis of biologically active compounds through enzymatic transformation can provide an efficient way of achieving novel antimicrobial agents required to overcome the global problem of antibiotic resistance development.[13]

Currently, out of almost 4000 enzymes, about 200 are used commercially. The majority of the industrial enzymes are of microbial origin[14] and prepared as recombinant proteins mostly in bacteria and/or yeast as the host expression system.[15-17] Microbial enzymes are preferred over plant or animal sources due to their economic production costs and simplicity of the modification and optimization process. They are relatively more stable than corresponding enzymes derived from plants or animals and they provide a greater diversity of catalytic activities.[18]

The special features of microbial enzymes which make them potential source for commercial and industrial applications include thermotolerance, thermophilic nature, and stability over broad range of temperature and pH, and other harsh reaction conditions.[1] Nature is the main source of enzyme producing microorganisms among them the great diversity of extremophiles supply the valuable enzymes with robust properties.[19] Extremophile microorganisms produce enzymes with great stability under harsh conditions which are regarded as incompatible environmental properties for biological systems.[15]

The halo-thermotolerant *Bacillus* sp. SL-1 is a Gram-positive, spore-forming bacterium that was isolated from Aran-Bidgol Saline Lake in central region of Iran as introduced in previous study.[20] Enzymes from halophiles tolerate such harsh environments by acquiring a large number of negatively-charged residues in their surfaces, which leads to their very low propensity for aggregation. Such a property has been taken as an advantage for these enzymes allowing them to function in non-aqueous media.[21] Phylogenetic analysis based on 16S rDNA gene sequence comparisons revealed that the isolate *Bacillus* sp. SL-1 was closely related to *Bacillus licheniformis* with 97% similarity. The *B. licheniformis* is an important producer of exoenzymes and has been used for decades in large-scale manufacturing the industrial and pharmaceutical enzymes such as different proteases, α-amylase, penicillinase, pentosanase, β-mannanase and several pectinolytic enzymes.[22,23] Based on complete genome sequence of B. *licheniformis*, many new genes for enzymes with potential biotechnological and pharmaceutical applications were found.[23] With the recent advances in biotechnology, various enzymes have been identified and designed or purposely engineered to produce more (and most likely new) chemicals and materials from cheaper (and renewable) resources, which will consequently contribute to establishing a bio-based economy.[15]

The halo-thermotolerant *Bacillus* sp. SL-1 as a new and locally isolated extremophile can be regarded as a resource for many useful pharmaceutical enzymes on commercial scales. In this study, based on the similarity observed for *Bacillus* sp. SL-1 and *B. licheniformis* (ATCC 14580), the two strains were considered closely related and hence the coding sequence for ten pharmaceutically and industrially important enzymes including laccase, l-asparaginase, glutamate-specific endopeptidase, L-arabinose isomerase, endo-1,4-β mannosidase, glutaminase, pectate lyase, cellulose, aldehyde dehydrogenase and allantoinases were inspected in the *Bacillus* sp. SL-1. The gene for these enzymes were identified, amplified, sequenced and compared with corresponding gene from the genome of the other *Bacillus* in database leading to the first analysis of enzymatic profile from *Bacillus* sp. SL-1. The results were indicative of opportunity for this organism as an industrial strain.

Materials and Methods

Materials

All reagents were of analytical grade. Tryptone and NaCl were purchased from Scharlau (Barcelona, Spain). Yeast extract, agar and glycerol were from Applichem (Darmstadt, Germany). DNA extraction kit was received from Qiagen (Germany). DNA ladders and Pfu PCR PreMix master mix were obtained from Fermentas (Russia) and Bioneer (South Korea), respectively. Safe stain was purchased from Thermo Scientific (USA). Primers used in this work were supplied from Bioron (Germany) ordered via FAZA Biotech (Tehran, Iran).

Strains and culture media

Halo-thermotolerant *Bacillus* sp. SL-1 was isolated from Aran-Bidgol Saline Lake in central region of Iran as introduced in previous study.[20] This isolate deposited in Iranian Biological Resources Center (approved by World Federation for Culture Collections: WDCM950) for availability to scientific community (IBRC-M **11052**). *Bacillus* sp. SL-1 was cultivated overnight in enriched Luria–Bertani (LB) medium at 35 °C and 180 rpm.

Primer Design

The design of primers for laccase (CotA), l-asparaginase (ansA1, ansA3), glutamate-specific endopeptidase (blaSE), l-arabinose isomerase (araA2), endo-1,4-β mannosidase (gmuG), glutaminase (glsA),

pectate lyase (pelA), cellulase (bglC1), aldehyde dehydrogenase (ycbD) and allantoinases (pucH) enzymes from *Bacillus* sp. SL-1 were based on the complete genome sequence of *B. licheniformis* (GenBank accession no. AE017333.1) and other closely related *Bacillus*, which permits the prediction of coding sequence for the ten selected enzymes according to the conserved regions in their sequences. Table 1 shows the list of designed primers for the enzymes.

Table 1. The primer pairs for amplification of enzyme genes from *Bacillus* sp.SL-1 genome. The primers designed according to the complete genome of *B. licheniformis* ATCC14580

Locus-tag	Genes	Primers (5′-3′)	PCR product size (bp)
BLi00679	CotA	TGGACATATGAAACTTGAAAAATTCGTTGACCGGC TGAACTCGAGTTGATGACGAACATCTGTCACTTC	1450
BLi04140	ansA3	GTAGCATATGAAAAAGTTACTGCTGTTGACCACC CTTGCGGCCGC TATGATGATATCGTCTGCAATCGG	980
BLi02433	ansA1	TCTTCATATGAATAAAAAAGTAGCTCTCATTACAACG AACAGTCGACCTAATAGCAGAATTTGTCTTTTATGCCTT	969
BLi00340	blaSE	ATATCATATGGTTAGTAAAAAGAGTGTTAAACGAGG GCCCTCGAGTTGTGCGCTGTTTTTCCAGTTGG	945
BLi03028	araA2	ATGTTAACAACAGGGAAAAAAGAATT TTACTTAATCACTACATATTCCAAGTC	1425
BLi00735	gmuG	CACACACCGTTTCTCCGGTG ACAGGCGTCAAAGAATCGCC	1003
BLi00274	glsA	ATGAATGAAGTATTGGAAGAACGCTATGAC TACTGAGATCCCATTCACGGGCTATATG	984
BLi04129	pelA	AGGGGTTGTCGGGTCATTGA TGGATTGATTTTGCCGACTCC	980
BLi00387	bglC1	GGGTGTCCCTTAACGAGCAA GCTCTTCCCCGTTTGTCTGA	945
BLi00285	ycbD	ATGTCTGTTGCAGCTGAAAGTAAAACG TTACGGTTTGACAAATACGGTTTTCAC	1460
BLi01126	pucH	ATCAAGTGCCAGCAACGGTA CCCTACTGCAATACTGCCTT	860

Polymerase Chain Reaction (PCR)

Genomic DNA from *Bacillus* sp. SL-1 was extracted by QIAamp DNA kit (Qiagen, Germany) according to the supplier's instruction. The amplification reaction mixtures were prepared to the final volume of 25 µl containing the 12 µl Pfu PCR PreMix master mix, 0.5 µl of forward and reverse primers (10 pmol/ µl), 2 µl DNA template (150 ng) and 10 µl PCR grade water. The PCR protocol adjusted for the amplification of the genes for all enzymes was performed using the following program: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 90 s, and extension at 72 °C for 2 min with final extension at 72 °C for 10 min. PCR products were analyzed on 1% agarose gel stained with safe stain and visualized under ultraviolet transillumination (Syngene InGenius, USA).

Sequence analysis

For further information about the sequence of the amplified genes the PCR products were sent out for sequencing at Sequetech, USA. Database search for the homologous sequences was performed using BLAST program from National Center for Biotechnology Information. Sequence alignments of the gene sequences were performed using CLUSTALW.[24]

Enzyme sequence accession numbers

The complete nucleotide sequences for the identified enzymes from *Bacillus* sp. SL-1 have been submitted to GenBank with following accession numbers: laccase (KU711667), l-asparaginase (KX129701, KX681674), glutamate-specific endopeptidase (KX129706), l-arabinose isomerase (KX129703), endo-1,4-β mannosidase (KX129704), glutaminase (KX129699), pectate lyase (KX129700), cellulose (KX129698), aldehyde dehydrogenase (KX129705) and allantoinase (KX129702).

Results and Discussion

Identification of putative Bacillus sp. SL-1 enzymes

According to the higher similarity (97%) observed between the16S rDNA gene sequences for *Bacillus sp*. SL-1 and *B. licheniformis* (ATCC 14580), the complete genome sequence from later was used as a template for designing appropriate primers for amplifying the corresponding enzyme genes from the genome of the SL-1 strain. The *B. licheniformis* belongs to the *B.*

subtilis group (group II) of the genus *Bacillus* together with other well-known species whose complete genome sequence has been determined.[23] The genome of *B. licheniformis* ATCC 14580 is in the form of a circular chromosome of 4,222,336 base-pairs (bp), which was predicted to be consist of 4,208 protein-coding sequences (CDSs). Based on a broad investigation on *B. licheniformis* genome, at least 82 of the 4,208 genes are likely to encode secreted proteins and enzymes. In addition, there are 27 predicted extracellular proteins encoded by the *B. licheniformis* (ATCC 14580) genome that are not found in *B. subtilis* 168. Due to the saprophytic lifestyle, the *B. licheniformis* encodes several secreted enzymes that hydrolyze polysaccharides, proteins, lipids and other nutrients.[22] Because of the biotechnological importance of this group of organisms we characterized the genes for ten industrial enzymes from the isolated halo-thermo tolerant *Bacillus* SL-1 and present the first analysis of data derived from the annotated sequences.

In this study selected enzyme genes were amplified from total genomic DNA of type strain SL-1 by using specific primer pairs and then the PCR products were visualized by gel electrophoresis on 1% agarose gel stained with safe stain. The gel image analysis indicated bands with sizes in the range of 860 to 1460 bp (Figure 1) that confirmed the presence of four industrially important classes of enzyme genes (EC1, EC3, EC4 and EC5) in *Bacillus* SL-1 genome. According to the Enzyme Commission the enzymes are divided into 6 class containing oxidoreductase (EC 1), transferase (EC 2), hydrolase (EC 3), lyase (EC 4), isomerase (EC 5) and ligase (EC 6).[25] The classification of the studied enzymes of *Bacillus* sp. SL-1 was shown in Table 2.

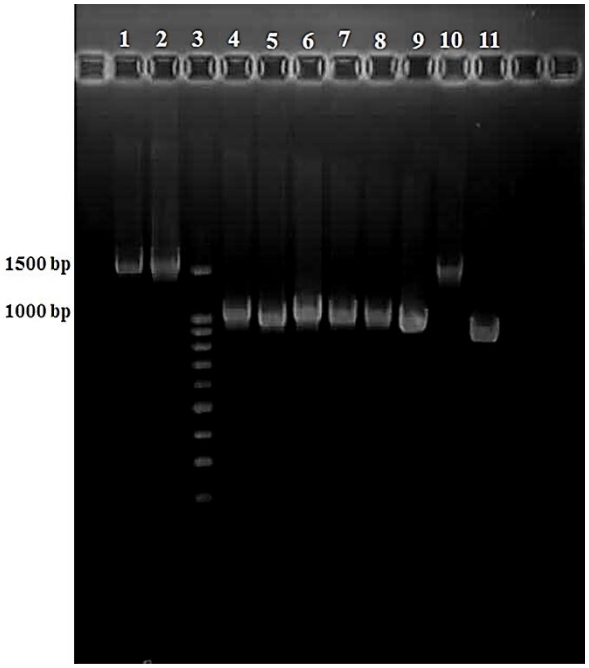

Figure 1. Gel electrophoresis results of enzyme genes from *Bacillus* sp. SL-1. Lane 1, l-arabinose isomerase (~1450 bp); lane 2, laccase (~1500 bp); lane 3, DNA Ladder; lane 4, glutamate-speicific endopeptidase (~950 bp); lane 5, l-asparaginase (~980 bp); lane 6, endo-1,4-β mannosidase (~1000 bp); lane 7, glutaminase (~990 bp); lane 8, pectate lyase (~980 bp); lane 9, cellulose (~950 bp); lane 10, aldehyde dehydrogenase (~1500 bp); lane 11, allantoinase (~860 bp).

Table 2. Industrial enzyme classes of *Bacillus* sp. SL-1 and types of reactions

Enzyme commission number (EC)	Class of enzymes	Industrial enzymes of *Bacillus* sp. SL-1
EC1	Oxidoreductases	Laccase Aldehyde dehydrogenase
EC3	Hydrolases	Cellulase Allantoinases Glutaminase L-asparaginase Endo-1,4-β mannosidase Glutamate-specific endopeptidase
EC4	Lyases	Pectate lyase
EC5	Isomerases	L-arabinose isomerase

Sequence analysis of Bacillus sp. SL-1 enzymes

Search for the homologs of the selected enzymes was performed by BLAST algorithm using the DNA sequences of the query enzymes against the nucleotide collection database. The most similar homologs for the studied enzymes were from *B. licheniformis* (ATCC 14580) with similarities ranging from 99 to 100% shown in Table 3. The table also contains the comparisons between the genes for the selected enzymes and the corresponding genes from *B. subtilis*, which is another closely related strain, albeit with less similarities (51-75%) in the case of these enzymes. Although the SL-1 isolate is considered closely related to the *B. licheniformis*, based on sequence similarities for the target enzymes, however, they are distinctive strains according to the 16S rDNA sequencing and some physicochemical properties described previously.[20] The comparative sequence analyses for the ten selected enzymes between *Bacillus* sp. SL-1 and *B. licheniformis* ATCC14580 were performed at the protein level by CLUSTAL-W pairwise alignment using the translated protein sequences (Table 4). More details regarding individual enzymes investigated in this work presented below.

Oxidoreductases (EC 1)

Oxidoreductase enzymes catalyze oxidation-reduction reactions where electrons are transferred. These electrons are usually in the form of hydride ions or hydrogen atoms. When a substrate is oxidized it acts as the hydrogen donor in the reaction and therefore the most common name used for the enzymes catalyzing this reaction is dehydrogenase. An oxidase is referred to when the oxygen atom is the electron acceptor.[26]

Table 3. Comparison of the corresponding gene from the genome of *Bacillus* sp. SL-1 with *B. licheniformis* ATCC14580 and *B. subtilis168*

Genes	Alignment (Identity %)		GenBank Accession numbers
	B. licheniformis 14580	*B. subtilis* 168	
CotA	99.94	64.65	KU711667
bglC1	100.0	76.43	KX129698
glsA	99.70	72.36	KX129699
pelA	100.0	51.12	KX129700
pucH	99.66	51.05	KX129702
ansA3	99.90	72.04	KX129701
ansA1	99.48	46.35	KX681674
araA2	100.0	57.52	KX129703
gmuG	100.0	72.48	KX129704
ycbD	100.0	74.23	KX129705
blaSE	100.0	56.80	KX129706

Table 4. Pairwise alignment of *Bacillus* sp. SL-1 enzymes with *B. licheniformis* (ATCC14580) complete genome

Enzymes	Protein sequence alignment		
	Nucleotide modification	*Amino acid modification*	*Identity (%)*
Laccase (CotA)	A948C	K316 to N	99.94
Cellulase (bglC1)	None	None	100.0
Glutaminase (glsA)	C345T	None	99.44
	A435G	I145 to M	
	A649T	I217 to F	
Pectate lyase (pelA)	None	None	100.0
Allantoinase (pucH)	G397A	D133 to N	99.36
	C850A	H284 to N	
	C896T	S299 to L	
L-asparaginase (ansA3)	T522A	None	100.0
L-asparaginase (ansA1)	A177T	None	99.07
	C184G	Q62 to E	
	G229A	D77 to N	
	C462T	None	
	A946T	I316 to L	
L-arabinose isomerase (araA2)	None	None	100.0
Endo-1,4-β mannosidase (gmuG)	None	None	100.0
Aldehyde dehydrogenase (ycbD)	None	None	100.0
Glutamate-specific endopeptidase (blaSE)	None	None	100.0

Laccases (EC 1.10.3.2), belonging to the superfamily of multicopper oxidases, catalyze the reduction of oxygen molecule into water molecule via transferring the electrons from substrates. Due to high capacity for the oxidation of wide range of phenols and polyphenols to highly reactive radicals, laccases have potential for applications in biotechnology, especially in the synthesis of new biologically active compounds and biomaterials.[3,27] These radicals can undergo coupling reactions with various types of compounds, which can lead to the formation of products with new structures and properties.[3] For example, laccase-catalyzed amination of dihydroxy aromatics is a new and promising method to synthesize novel antibiotics via enzymatic transformation.[13] These laccase mediated reactions are low-cost reactions which are conducted under mild reaction conditions, in aqueous solvent systems, normal pressure, and room temperature.[28,29] Up to now, novel cephalosporins, penicillins, and carbacephems were synthesized by amination of amino-

β-lactam structures using laccases.[13,28,30] Other examples of the potential application of laccases for organic synthesis include the oxidative coupling of katarantine and vindoline to produce vinblastine.[31] Vinblastine is an important anti-cancer agent, extensively used in treatment of leukemia.[31]

The CotA genes from *Bacillus* sp. SL-1 and *B. licheniformis* ATCC14580 are almost identical (99.94%), except for a single A948C nucleotide difference, which has led to K316N substitution as indicated in Table 3. Multiple sequence alignment of laccase from *Bacillus* sp. SL-1 with other laccase enzymes from different *Bacillus* strains indicated four conserved segments containing histidine-rich copper-binding sites which are characteristic for bacterial laccases. Moreover, the $CotA_{(SL-1)}$ gene from *Bacillus* sp. SL-1 shows 64.65% identity with CotA from *B. subtilis.* The most important advantages of using bacterial laccases are their higher activity as well as stability at various pH and temperature compared to the fungal laccase. Moreover, the expression level of laccase is anticipated to be higher in bacteria than other microorganisms, which may provide added economic values for its use. Besides, the industrial processes are often conducted in harsh conditions such as extreme pH, temperature, or ionic strength and therefore such robust enzymes supply economically appealing materials.[32,33] The isolation and comprehensively characterization of pure laccase from *Bacillus* sp. SL-1 revealed its high production yield and stability.[27]

Aldehyde dehydrogenase (ALDH; aldehyde: $NAD(P)^+$ oxidoreductase, EC 1.2.1.5) constitute a group of enzymes that catalyze the conversion of aldehydes to the corresponding acids mediated by an $NAD(P)^+$-dependent virtually irreversible reaction making it potentially useful in an industrial settings. The ALDH is very unstable because of the spontaneous oxidation,[34] therefore, there is considerable interest in production of stable ALDH, which can be used more efficiently in the pharmaceutical and fine chemicals industries for the production of aldehydes, ketones, and chiral alcohols. The production of chiral compounds is particularly desired because this is an increasingly important step in the synthesis of chirally pure pharmaceutical agents.[35] On the other hand, the ALDH family is the most important detoxifying enzyme due to its role in the removal of the accumulated aldehyde metabolits.[36] Many human diseases are associated with lack of ALDH enzymes and the increased level of aldehydes in the body contributes to the pathology of a variety of metabolic disorders. In this investigation, sequencing analysis of $ycbD_{(SL-1)}$ showed that this gene was composed of 1467 bp, corresponding to 488 amino acid residues with a molecular mass of 52,912 Da. The pairwise alignment of aldehyde dehydrogenase $_{(SL-1)}$ gene with DNA sequences of homologous enzymes from *B. licheniformis* (ATCC 14580) and *B. subtilis* 168 showed 100.0% and 74.23% identities, respectively.

Hydrolases (EC3)

Hydrolases catalyze hydrolysis using cleavage of substrates by water molecule. In biological systems, the reactions contain the cleavage of peptide bonds in proteins, glycosidic bonds in carbohydrates, and ester bonds in lipids. Generally, larger molecules are broken down to smaller fragments by hydrolases.[25]

The therapeutic enzyme L-asparaginase (EC 3.5.1.1; L-asparagine amidohydrolase) is an important antineoplastic agent primarily applied for management of acute lymphoblastic leukaemia (ALL). Eighty percentage of ALL type of leukemia affect children with only 20% of cases shown in adults.[37] L-asparaginase catalyzes the conversion of l-asparagine to l-aspartic acid and ammonia. The antileukemic activity of L-asparaginase is due to depletion of the circulating L-asparagine concentration in the extracellular fluid and hence reduction of its availability for the tumor cells which lack asparagine synthetase required for L-asparagine intracellular synthesis. This leads to the inhibition of protein synthesis in tumor cells and induction of apoptosis. However, the normal cells are not affected significantly due to their intact system for asparagine biosynthesis.[38] Currently, in the United States, three asparaginase formulations are widely used against ALL: native *E. coli* asparaginase, its pegylated form, and the product from cultures of *Erwinia chrysanthemi.*[39] Despite significant advancement in production of therapeutical forms of L-asparaginase, development of anti-asparaginase antibody in the patients is responsible for its major toxicity and resistance in asparaginase therapy and also reduces the therapeutic efficacy in some cancer cases. Considering that the patients do not develop cross reactivity, when a patient shows hypersensitivity to one type of L-asparaginase, it can be replaced with the enzymes obtained from different bacterial sources.[37] Pairwise alignment of ansA3 $_{(SL-1)}$ and ansA3 gene from *B. licheniformis* (strain ATCC 14580) showed 99.90% identity with just a single T522A silent substitution (Table 4). The sequence alignment of genes for L-asparaginase from *Bacillus* sp. SL-1 and that of *B. subtilis 168* indicated 72.04% identity (Table 3). Recently L-asparaginase from *B. licheniformis* with low glutaminase activity has been considered as a key therapeutic agent in the treatment of ALL.[40] Preliminary activity assay on recombinant L-asparaginase corresponding to ansA3 gene from *Bacillus* sp. SL-1in our lab showed that it is not functional. However, the gene for second homologous enzyme with asparaginase activity called ansA1 was also isolated from *Bacillus* sp. SL-1 with 99.07% similarity to ansA1 from *B. licheniformis* ATCC14580 (Table 4) and the corresponding recombinant protein was produced in high purity showing excellent enzymatic activity (unpublished data). Our findings are in contrast to the results reported by Sudhir et al where they showed that the enzyme encoded by ansA3 from

B. licheniformis MTCC 429 is active, while the enzyme from ansA1is highly unstable.[41]

Allantoinases (pucH) are members of amidohydrolase superfamily, which are involved in purine metabolism and also catalyze the hydrolysis of a broad range of substrates containing amide or ester functional groups at carbon and phosphorus centers.[42] Despite their importance in the purine catabolic pathway, sequences of microbial allantoinases with proven activity are scarce and only the enzymes from *Escherichia coli* has been studied in detail in this regard.[43] It has been reported that allantoinase from *B. licheniformis* presents an inverted enantioselectivity towards allantoin (R-enantioselective) that is not observed for other allantoinases, which makes it an interesting candidate for biotechnological applications.[42] The $pucH_{(SL-1)}$ sequence analysis from *Bacillus* sp. SL-1 showed 99.66 % similarity to *B. licheniformis* allantoinase gene with three nucleotide substitution (G397A, C850A and C896T) that led to three amino acid modifications in the corresponding protein sequence (Table 4).

Glutaminase (EC 3.5.1.2) is an oncolytic enzyme that catalyzes the deamination of L-glutamine to L-glutamic acid and ammonia with high specificity. L-glutaminase such as L-asparaginase is very significant anticancer enzyme in the treatment of acute lymphoblastic leukemia and other kinds of cancer through the L-glutamine amino acid depletion in cancerous cells.[44,45] These enzymes (i.e. L-glutaminase and L-asparaginase) are commonly used of therapeutic agents accounting for about 40% of the total worldwide enzyme sales.[45] Moreover, microbial glutaminases are enzymes with emerging potential in food industries.[44] One of the potential application of recombinant *B. licheniformis* glutaminase is the bioconversion of glutamine to flavor-enhancing glutamic acid in fermented food products.[46] There have been only a few reports on the characterization of recombinant glutaminases. The $glsA_{(SL-1)}$ from *Bacillus* sp. SL-1 with 99.70% similarity to glutaminase gene from *B. licheniformis* consists of 984 bp corresponding to 327 residues with A435G and A649T nucleotide substitutions leading to I145M and I217F residue changes in the protein sequence, respectively (Table 4).

A glutamate-specific endopeptidase (GSE) (EC 3.4.21.19) has the ability to cleave peptide bonds preceded by Glu and/or Asp residues. Its activity to Asp containing substrates contributes only 0.3% of that towards Glu substrates, demonstrating its high specificity for peptide bonds formed by α-carboxyl groups of Glu amino acids[47] The GSE from *B. licheniformis* has been used to hydrolyze α-lactalbumin, and the hydrolysate formed nanotubes due to the specificity of GSE-BL. It has been suggested that these nanotubes can be used as drug carriers and viscosifying agents with the advantages that they are biocompatible and of low toxicity.[48] Also, the biochemical properties of this type of enzyme are useful for its usage in protein structure analysis, solid phase peptide synthesis, and biochemistry industry.[49] Sequencing results of $blaSE_{(SL-1)}$ showed 100.0% and 56.80% identity with *B. licheniformis* and *B. subtilis*, respectively.

Cellulose is the major component of plant biomass, which originally comes from solar energy through the process known as photosynthesis and is the most abundant renewable energy on Earth.[50] Microorganisms produce multiple enzyme components to degrade cellulose, known as the cellulase (EC 3.2.1.4) system. The main application of these enzymes are in food and detergent industries.[51] Microbial cellulase applications have been widely studied in pharmaceutical industries, mainly because of its huge economic potential in the conversion of plant biomass into ethanol and other chemicals. Recently, improved novel tubular cellulose (TC), a porous cellulosic material has been produced by enzymatic treatment with cellolase in order to prepare nanotubes, which have gained broad attention as major nanomedicine tools in drug targeting and delivery systems.[52]

Also, mannan polysaccharide is one of the main polymers in hemicellulose, a major component of lignocellulose. The mannan endo-1,4-β-mannosidase (EC 3.2.1.78), commonly named β-mannanase, is an enzyme that can catalyze random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannans, glucomannans and galactomannans.[53] This enzyme has several applications in many industries including food, feed, pharmaceutical, pulp/paper industries, and gas well stimulation and pretreatment of lignocellulosic biomass for the production of second generation biofuel.[54] The application of mannan endo-1,4-β-mannosidase for the production of prebiotic mannan-oligosaccharides from byproducts of cheap agricultural sources has found increased interests.[55] Additionally, the β-mannanase and mannosidase secreted from the microflora in colon environment can degrade the hydrogel-based therapeutics and release the drug molecule from a galactomannan-based hydrogel.[56,57] The sequence analysis of $bglC1_{(SL-1)}$ and $gmuG_{(SL-1)}$ genes of *Bacillus* sp. SL-1 showed 100.0% homology with both of these genes from *B. licheniformis* as well as 76.43% and 72.48% identities with those of *B. subtilis* 168, respectively.

Lyases (EC4)

Lyases add some groups to double bonds or form double bonds through the elimination of groups. Thus bonds are cleaved by a principle different from hydrolysis. These are often referred to as synthase enzymes which differ from other enzymes in that one substrate is required in the forward direction, whereas two substrates are needed for the backward reaction.[26]

Pectin is the most complex polysaccharides widely found in plant cell wall, consist of a backbone of D-galacturonic acid residues, which are partially methylesterified. Pectate lyase (EC 4.2.2.2) (pelA) cleaves the α-1,4 glycosidic bond of polygalacturonic acid (PGA) via a β- elimination reaction and generates

a unsaturated bond at the non-reducing end of the newly formed oligogalacturonide.[58] These enzymes are of great commercial value for various industrial applications such as improving juice yields and clarity in fruit juice industry.[59] Genes encoding microbial pectate lyase have been identified from many microorganisms, including different *Bacillus* strains, and the corresponding enzymes form a superfamily based on their amino acid sequence similarity.[60] The sequence alignment of pelA from *Bacillus* sp. SL-1 revealed 100.0% and 51.12% homology with DNA sequences of *B. licheniformis* (ATCC 14580) and *B. subtilis* 168 pectate lyase coding genes, respectively.

Isomerases (EC5)

Isomerases mediate the transferring of groups from one position to another one in the same molecule. On the other hands, these enzymes change the structure of a substrate by rearranging its atoms.[25]

L-arabinose isomerase (EC 5.3.1.4) catalyzes the reversible isomerization of L-arabinose to L-ribulose involved in either the pentose phosphate or the phosphoketolase pathway in carbohydrate metabolism.[61] Isomerase enzymes play a crucial role in the synthesis of uncommon sugars, simply termed rare sugars. Due to their scarcity in the nature and uneconomical method of production, rare sugars are available only in limited amounts and at a high cost.[62] D-Tagatose, a natural rare monosaccharide, is an isomer of D-galactose which can be manufactured by the chemical or enzymatic isomerization of D-galactose.[63,64] Among the biocatalysts, L-arabinose isomerase has been mostly applied for D-tagatose production because of the industrial feasibility for the use of D-galactose as a substrate.[64] D-Tagatose has attracted a great attention in recent years for its low caloric diet and can be used as sweetener in several foods, beverages, and dietary supplements. There are numerous reports indicates the useful medical properties of this sugar such as prebiotic, antioxidant and tooth-friendly as well as reduction of symptoms associated with type 2 diabetes, anemia, hemophilia and hyperglycemia.[63,65]

L-arabinose isomerase from various bacteria have been identified and studied from a number of microbial sources, but little information is available about this enzyme from *B. licheniformis*, and L- arabinose isomerase specific towards only L-arabinose.[61] DNA sequence analysis of araA2 $_{(SL-1)}$ revealed an open reading frame of 1425 bp, capable of encoding a polypeptide of 474 amino acid residues with a calculated isoelectric point of pH 4.8 and a molecular mass of 53,500 Da. Based on analysis of the genome sequence, araA2 gene from *Bacillus* sp. SL-1 showed 100.0% and 57.52% identity with l- arabinose isomerase gene from *B. licheniformis* (ATCC 14580) and *B. subtilis* 168, respectively.

Conclusion

In the present study, halo-thermo tolerance *Bacillus* sp. SL1 isolate was evaluated for molecular characterization of its potential important pharmaceutical and industrial enzymes. Based on the sequence alignment results, 6 out of 10 studied enzymes from *Bacillus* sp. SL-1 showed 100.0% similarity at the nucleotide level to the corresponding genes of these enzymes in *B. licheniformis* (ATCC 14580) and demonstrated extensive organizational relationship between two strains. In the case of three studied enzymes (laccase, glutaminase, and allantoinase), their gene sequences showed more than 99% identity with *B. licheniformis* and the modifications in nucleotides translates to amino acid substitution in protein sequences. Asparaginase from *Bacillus* sp. SL1 was the only enzyme among others with just a single nucleotide silent substitution. Molecular characterizations of industrial enzyme sequences of newly isolated *Bacillus* sp. SL-1 provides useful information for comparative and evolutionary studies of different species within the industrial microorganisms including *B. subtilis*, *B. licheniformis* group. In the meantime, these studies may offer new information regarding the evolution and application of these closely related species. Since, the most industrial processes are often performed in harsh conditions like extreme pH, temperature, or ionic strength, therefore such robust enzymes provide economically appealing materials to be used under specific physicochemical situations.[27,66] Thus, halo-thermo tolerance *Bacillus* sp. SL-1 could represent an excellent source of enzymes that can function at extreme conditions working as biocatalysts in medical, pharmaceutical, chemical and other industries.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors declare no conflict of interests.

References

1. Nigam PS. Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules* 2013;3(3):597-611. doi: 10.3390/biom3030597
2. Li S, Yang X, Yang S, Zhu M, Wang X. Technology prospecting on enzymes: Application, marketing and

engineering. *Comput Struct Biotechnol J* 2012;2:e201209017. doi: 10.5936/csbj.201209017
3. Polak J, Jarosz-Wilkolazka A, Szalapata K, Graz M, Osinska-Jaroszuk M. Laccase-mediated synthesis of a phenoxazine compound with antioxidative and dyeing properties--the optimisation process. *N Biotechnol* 2016;33(2):255-62. doi: 10.1016/j.nbt.2015.09.004
4. Johnson EA. Biotechnology of non-*saccharomyces* yeasts-the *ascomycetes*. *Appl Microbiol Biotechnol* 2013;97(2):503-17. doi: 10.1007/s00253-012-4497-y
5. Sanchez S, Demain AL. Enzymes and bioconversions of industrial, pharmaceutical, and biotechnological significance. *Org Process Res Dev* 2010;15(1):224-30. doi: 10.1021/op100302x
6. Vellard M. The enzyme as drug: Application of enzymes as pharmaceuticals. *Curr Opin Biotech* 2003;14(4):444-50. doi: 10.1016/S0958-1669(03)00092-2
7. Mane P, Tale V. Overview of microbial therapeutic enzymes. *Int J Curr Microbiol App Sci* 2015;4(4):17-26.
8. Xu G, McLeod HL. Strategies for enzyme/prodrug cancer therapy. *Clin Cancer Res* 2001;7(11):3314-24.
9. Silverman RB, Holladay MW. *The organic chemistry of drug design and drug action*: 3rd ed. USA: Academic press; 2014.
10. Ferguson MJ, Ahmed FY, Cassidy J. The role of pro-drug therapy in the treatment of cancer. *Drug Resist Updat* 2001;4(4):225-32. doi: 10.1054/drup.2001.0207
11. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, et al. Prodrugs: Design and clinical applications. *Nat Rev Drug Discov* 2008;7(3):255-70. doi: 10.1038/nrd2468
12. Wu KM. A new classification of prodrugs: Regulatory perspectives. *Pharmaceuticals* 2009;2(3):77-81. doi: 10.3390/ph2030077
13. Mikolasch A, Hildebrandt O, Schluter R, Hammer E, Witt S, Lindequist U. Targeted synthesis of novel beta-lactam antibiotics by laccase-catalyzed reaction of aromatic substrates selected by pre-testing for their antimicrobial and cytotoxic activity. *Appl Microbiol Biotechnol* 2016;100(11):4885-99. doi: 10.1007/s00253-016-7288-z
14. Sharma R, Chisti Y, Banerjee UC. Production, purification, characterization, and applications of lipases. *Biotechnol Adv* 2001;19(8):627-62.
15. Adrio JL, Demain AL. Microbial enzymes: Tools for biotechnological processes. *Biomolecules* 2014;4(1):117-39. doi: 10.3390/biom4010117
16. Kirk O, Borchert TV, Fuglsang CC. Industrial enzyme applications. *Curr Opin Biotechnol* 2002;13(4):345-51.
17. Ran N, Zhao L, Chen Z, Tao J. Recent applications of biocatalysis in developing green chemistry for chemical synthesis at the industrial scale. *Green Chem* 2008;10(4):361-72. doi: 10.1039/B716045C
18. Adrio JL, Demain AL. Microbial Cells and Enzymes A Century of Progress. In: Barredo JL, editor. *Microbial enzymes and biotransformations*. USA: Humana Press; 2005.
19. Sarmiento F, Peralta R, Blamey JM. Cold and hot extremozymes: Industrial relevance and current trends. *Front Bioeng Biotechnol* 2015;3:148. doi: 10.3389/fbioe.2015.00148
20. Safary A, Moniri R, Mirhashemi SM, Nikzad H, Khiavi MA. Phylogenetic and biochemical characterization of a new halo-thermotolerant, biofilm-forming bacillus from saline lake of iran. *Pol J Microbiol* 2013;62(4):419-25.
21. Klibanov AM. Improving enzymes by using them in organic solvents. *Nature* 2001;409(6817):241-6. doi: 10.1038/35051719
22. Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, Zaretsky EJ, Tang M, et al. Complete genome sequence of the industrial bacterium bacillus licheniformis and comparisons with closely related bacillus species. *Genome Biol* 2004;5(10):R77. doi: 10.1186/gb-2004-5-10-r77
23. Veith B, Herzberg C, Steckel S, Feesche J, Maurer KH, Ehrenreich P, et al. The complete genome sequence of bacillus licheniformis dsm13, an organism with great industrial potential. *J Mol Microbiol Biotechnol* 2004;7(4):204-11. doi: 10.1159/000079829
24. Thompson JD, Higgins DG, Gibson TJ. Clustal w: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22(22):4673-80.
25. Gurung N, Ray S, Bose S, Rai V. A broader view: Microbial enzymes and their relevance in industries, medicine, and beyond. *Biomed Res Int* 2013;2013:329121. doi: 10.1155/2013/329121
26. Vasudevan DM, Sreekumari S, Vaidyanathan K. Textbook of biochemistry for medical students. 6th ed. New Delhi, India: Jaypee Brothers Medical Publishers (P) Ltd:2011
27. Safary A, Moniri R, Hamzeh-Mivehroud M, Dastmalchi S. A strategy for soluble overexpression and biochemical characterization of halo-thermotolerant bacillus laccase in modified e. Coli. *J Biotechnol* 2016;227:56-63. doi: 10.1016/j.jbiotec.2016.04.006
28. Mikolasch A, Niedermeyer TH, Lalk M, Witt S, Seefeldt S, Hammer E, et al. Novel penicillins synthesized by biotransformation using laccase from trametes spec. *Chem Pharm Bull (Tokyo)* 2006;54(5):632-8.
29. Mikolasch A, Niedermeyer TH, Lalk M, Witt S, Seefeldt S, Hammer E, et al. Novel cephalosporins synthesized by amination of 2,5-dihydroxybenzoic acid derivatives using fungal laccases ii. *Chem Pharm Bull (Tokyo)* 2007;55(3):412-6.
30. Mikolasch A, Wurster M, Lalk M, Witt S, Seefeldt S, Hammer E, et al. Novel beta-lactam antibiotics

synthesized by amination of catechols using fungal laccase. *Chem Pharm Bull (Tokyo)* 2008;56(7):902-7.
31. Kunamneni A, Camarero S, Garcia-Burgos C, Plou FJ, Ballesteros A, Alcalde M. Engineering and applications of fungal laccases for organic synthesis. *Microb Cell Fact* 2008;7:32. doi: 10.1186/1475-2859-7-32
32. Santhanam N, Vivanco JM, Decker SR, Reardon KF. Expression of industrially relevant laccases: Prokaryotic style. *Trends Biotechnol* 2011;29(10):480-9. doi: 10.1016/j.tibtech.2011.04.005
33. Brander S, Mikkelsen JD, Kepp KP. Characterization of an alkali- and halide-resistant laccase expressed in e. Coli: Cota from bacillus clausii. *PLoS One* 2014;9(6):e99402. doi: 10.1371/journal.pone.0099402
34. Lo HF, Chen YJ. Gene cloning and biochemical characterization of a nad(p)+ -dependent aldehyde dehydrogenase from bacillus licheniformis. *Mol Biotechnol* 2010;46(2):157-67. doi: 10.1007/s12033-010-9290-5
35. Machielsen R, Uria AR, Kengen SW, van der Oost J. Production and characterization of a thermostable alcohol dehydrogenase that belongs to the aldo-keto reductase superfamily. *Appl Environ Microbiol* 2006;72(1):233-8. doi: 10.1128/AEM.72.1.233-238.2006
36. Chen CH, Ferreira JC, Gross ER, Mochly-Rosen D. Targeting aldehyde dehydrogenase 2: New therapeutic opportunities. *Physiol Rev* 2014;94(1):1-34. doi: 10.1152/physrev.00017.2013
37. Zuo S, Xue D, Zhang T, Jiang B, Mu W. Biochemical characterization of an extremely thermostable l-asparaginase from *thermococcus gammatolerans* EJ3. *J Mol Catal B Enzym* 2014;109:122-9. doi: 10.1016/j.molcatb.2014.08.021
38. Kotzia GA, Labrou NE. L-asparaginase from erwinia chrysanthemi 3937: Cloning, expression and characterization. *J Biotechnol* 2007;127(4):657-69. doi: 10.1016/j.jbiotec.2006.07.037
39. Jia M, Xu M, He B, Rao Z. Cloning, expression, and characterization of l-asparaginase from a newly isolated bacillus subtilis b11-06. *J Agric Food Chem* 2013;61(39):9428-34. doi: 10.1021/jf402636w
40. Mahajan RV, Kumar V, Rajendran V, Saran S, Ghosh PC, Saxena RK. Purification and characterization of a novel and robust l-asparaginase having low-glutaminase activity from bacillus licheniformis: In vitro evaluation of anti-cancerous properties. *PLoS One* 2014;9(6):e99037. doi: 10.1371/journal.pone.0099037
41. Sudhir AP, Dave BR, Prajapati AS, Panchal K, Patel D, Subramanian RB. Characterization of a recombinant glutaminase-free l-asparaginase (ansa3) enzyme with high catalytic activity from bacillus licheniformis. *Appl Biochem Biotechnol* 2014;174(7):2504-15. doi: 10.1007/s12010-014-1200-z
42. Conejero-Muriel M, Martinez-Gomez AI, Martinez-Rodriguez S, Gavira JA. Cloning, expression, purification, crystallization and preliminary x-ray characterization of allantoinase from bacillus licheniformis atcc 14580. *Acta Crystallogr F Struct Biol Commun* 2014;70(Pt 11):1513-6. doi: 10.1107/S2053230X14021608
43. Martinez-Gomez AI, Soriano-Maldonado P, Andujar-Sanchez M, Clemente-Jimenez JM, Rodriguez-Vico F, Neira JL, et al. Biochemical and mutational studies of allantoinase from bacillus licheniformis cect 20t. *Biochimie* 2014;99:178-88. doi: 10.1016/j.biochi.2013.12.002
44. Nandakumar R, Yoshimune K, Wakayama M, Moriguchi M. Microbial glutaminase: Biochemistry, molecular approaches and applications in the food industry. *J Mol Catal B Enzym* 2003;23(2):87-100. doi: 10.1016/S1381-1177(03)00075-4
45. El-Ghonemy DH. Microbial amidases and their industrial applications: A review. *J Med Microbiol Diagn* 2015;4(1):1.
46. Sinsuwan S, Yongsawatdigul J, Chumseng S, Yamabhai M. Efficient expression and purification of recombinant glutaminase from bacillus licheniformis (glsa) in escherichia coli. *Protein Expr Purif* 2012;83(1):52-8. doi: 10.1016/j.pep.2012.03.001
47. Wehofsky N, Wissmann J, Alisch M, Bordusa F. Engineering of substrate mimetics as novel-type substrates for glutamic acid-specific endopeptidases: Design, synthesis, and application. *Biochim Biophys Acta* 2000;1479(1-2):114-22.
48. Ye W, Wang H, Ma Y, Luo X, Zhang W, Wang J, et al. Characterization of the glutamate-specific endopeptidase from bacillus licheniformis expressed in escherichia coli. *J Biotechnol* 2013;168(1):40-5. doi: 10.1016/j.jbiotec.2013.08.009
49. Ye W, Liu J, Wang H, Wang J, Wang X. Cloning, expression, purification, and characterization of a glutamate-specific endopeptidase from bacillus licheniformis. *Protein Expr Purif* 2012;82(1):138-43. doi: 10.1016/j.pep.2011.12.001
50. Liu Y, Zhang J, Liu Q, Zhang C, Ma Q. Molecular cloning of novel cellulase genes cel9a and cel12a from bacillus licheniformis gxn151 and synergism of their encoded polypeptides. *Curr Microbiol* 2004;49(4):234-8. doi: 10.1007/s00284-004-4291-x
51. Maeda RN, Barcelos CA, Santa Anna LM, Pereira N, Jr. Cellulase production by penicillium funiculosum and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. *J Biotechnol* 2013;163(1):38-44. doi: 10.1016/j.jbiotec.2012.10.014
52. Koutinas A, Papafotopoulou-Patrinou E, Gialleli AI, Petsi T, Bekatorou A, Kanellaki M. Production of nanotubes in delignified porous cellulosic materials after hydrolysis with cellulase. *Bioresour Technol* 2016;213:169-71. doi: 10.1016/j.biortech.2016.03.065

53. Songsiriritthigul C, Buranabanyat B, Haltrich D, Yamabhai M. Efficient recombinant expression and secretion of a thermostable gh26 mannan endo-1,4-beta-mannosidase from bacillus licheniformis in escherichia coli. *Microb Cell Fact* 2010;9:20. doi: 10.1186/1475-2859-9-20
54. Gubitz G, Sachslehner A, Haltrich D. Microbial mannanases: Substrates, production, and applications. In: Himmel ME, Baker JO, Saddler JN, editors. *Glycosyl Hydrolases for Biomass Conversion*. Washington: American Chemical Society; 2001.
55. Biggs P, Parsons CM. The effects of several oligosaccharides on true amino acid digestibility and true metabolizable energy in cecectomized and conventional roosters. *Poult Sci* 2007;86(6):1161-5.
56. Burke MD, Park JO, Srinivasarao M, Khan SA. A novel enzymatic technique for limiting drug mobility in a hydrogel matrix. *J Control Release* 2005;104(1):141-53. doi: 10.1016/j.jconrel.2005.01.017
57. Dionisio M, Grenha A. Locust bean gum: Exploring its potential for biopharmaceutical applications. *J Pharm Bioallied Sci* 2012;4(3):175-85. doi: 10.4103/0975-7406.99013
58. Vierhuis E, Korver M, Schols HA, Voragen AG. Structural characteristics of pectic polysaccharides from olive fruit (olea europaea cv moraiolo) in relation to processing for oil extraction. *Carbohyd Polym* 2003;51(2):135-48. doi: 10.1016/S0144-8617(02)00158-3
59. Berensmeier S, Singh SA, Meens J, Buchholz K. Cloning of the pela gene from bacillus licheniformis 14a and biochemical characterization of recombinant, thermostable, high-alkaline pectate lyase. *Appl Microbiol Biotechnol* 2004;64(4):560-7. doi: 10.1007/s00253-003-1446-9
60. Hatada Y, Kobayashi T, Ito S. Enzymatic properties of the highly thermophilic and alkaline pectate lyase pel-4b from alkaliphilic bacillus sp. Strain p-4-n and the entire nucleotide and amino acid sequences. *Extremophiles* 2001;5(2):127-33.
61. Prabhu P, Tiwari MK, Jeya M, Gunasekaran P, Kim IW, Lee JK. Cloning and characterization of a novel l-arabinose isomerase from bacillus licheniformis. *Appl Microbiol Biotechnol* 2008;81(2):283-90. doi: 10.1007/s00253-008-1652-6
62. Izumori K. Bioproduction strategies for rare hexose sugars. *Naturwissenschaften* 2002;89(3):120-4.
63. Wanarska M, Kur J. A method for the production of d-tagatose using a recombinant pichia pastoris strain secreting beta-d-galactosidase from arthrobacter chlorophenolicus and a recombinant l-arabinose isomerase from arthrobacter sp. 22c. *Microb Cell Fact* 2012;11:113. doi: 10.1186/1475-2859-11-113
64. Oh DK. Tagatose: Properties, applications, and biotechnological processes. *Appl Microbiol Biotechnol* 2007;76(1):1-8. doi: 10.1007/s00253-007-0981-1
65. Jung ES, Kim HJ, Oh DK. Tagatose production by immobilized recombinant escherichia coli cells containing geobacillus stearothermophilus l-arabinose isomerase mutant in a packed-bed bioreactor. *Biotechnol Prog* 2005;21(4):1335-40. doi: 10.1021/bp050078p
66. Gomes J, Steiner W. The biocatalytic potential of extremophiles and extremozymes. *Food Technol Biotechnol* 2004;42(4):223-35.