In silico* analysis of signal peptides for secretory production of α -amylase in *Bacillus subtilis

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Abstract. α -Amylases are important commercial enzymes and have a broad application in industrial processes and medicine. Gram-positive bacteria such as *Bacillus subtilis* are possible host organisms for α -amylases secretory production. Secretion of α -amylases to the culture medium versus intracellular production has several advantages such as prevention of inclusion bodies accumulation, higher product stability and solubility. Signal peptides are considered as one of the most essential elements for successful secretory synthesis of the recombinant proteins. Therefore, by the selection of an efficient signal peptide, secretion of the recombinant protein can be enhanced. The goal of this investigation was the *in silico* evaluation of several peptides to find the most suitable leader peptides for secretory production of α -amylase in *B. subtilis*. In present work, 30 signal peptides were selected, and numerous online servers such as SignalP, ProtParam, SOLpro, PRED-TAT and ProtComp was used for investigation of suitable signal peptides. According to *in silico* predictions all other signal peptides connected to α -amylase were stable and soluble except PPBD_BACSU. PPBD_BACSU because of having D-score below cut-off could not be recognized as a suitable signal peptide for α -amylase. Computational analysis identified QOX2_BACSU may direct protein into transmembrane location and was ignored. All 28 remained were predicted as secretory signal peptides which can excrete protein out of the bacteria. The signal peptides recommended by the present study are valuable for rational designing of secretory soluble α -amylase. Although, such information can be useful for future experimental production of these mentioned secretory proteins.

Keywords: α -Amylase, *in silico*, recombinant protein, *Bacillus subtilis*, signal peptide

INTRODUCTION

Recombinant proteins, including commercial recombinant enzymes and biopharmaceutical proteins, have a multibillion-dollar sale per year (Freudl, 2018). α -Amylases are important enzymes in market that comprise 30% of the world's enzymes market (Mobini-Dehkordi and Javan, 2012). In medicine, these enzymes are used to treat pancreatic insufficiency. Patients with cystic fibrosis can also be treated with enzyme-

replacement therapies such as α -amylase to prevent malnutrition because in cystic fibrosis disease the secretion of α -amylase is obstructed (Li and Somerset, 2014, Azzopardi *et al.*, 2016). These enzymes have a broad application in industrial processes, like the textile industry, paper, food, detergent and pharmaceutical field (Nascimento, 2013). The utilization of these biocatalysts in food industries improves the quality of

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products while reducing the use of different chemical materials, thereby helping the consumers' health (Mehta and Satyanarayana, 2016). Additionally, for reduction of processing time, cost-effectiveness, low energy consumption, nontoxicity and economic efficiency, the application of α -amylases in various industries is increasing (Singh *et al.*, 2016).

α -Amylases grouped into the GH-13 family of glycosyl hydrolases are extracellular enzymes which randomly break the α (1-4) connection between two contiguous glucose units in the linear amylose chain and finally produce glucose, maltose, and maltotriose units. α -Amylases can be synthesized by various living sources, including microorganisms, plants, and animals. However, bacterial and fungal enzymes have more commercial and industrial application due to their high efficiency and resistance to pH and temperature (Nascimento, 2013). However, it seems that bacterial sources are a better choice due to their stability at higher temperatures (Kindle, 1983). Among bacillus species, *Bacillus licheniformis* is known to be a good producer of thermostable α -amylases (Schallmey *et al.*, 2004).

Many different expression systems exist for the recombinant production of α -amylase protein including different pro- and eukaryotic expression systems. Even though, for using as expression hosts, bacteria are more interesting (Freudl, 2018).

Escherichia coli (*E. coli*) is one of the most broadly applied hosts for the recombinant proteins synthesis but it cannot excrete proteins out of the cell. So, overexpression of proteins in it usually results in inclusion body formation or protein aggregation in the cytoplasm, which requires sophisticated refolding mechanisms to achieve biologically active proteins and consequently result in decreasing of active proteins yields (Cui *et al.*, 2017, Wang *et al.*, 2016). Therefore, there is some benefits for recombinant proteins secretion into extracellular medium, such as prevention of inclusion bodies accumulation, reduction or even alleviation of toxic effect that applied by target proteins, correct construction of disulfide bonds that are necessary for the final conformations, and simplification of product recovery (Freudl, 2018, Anné *et al.*, 2016).

Gram-positive bacteria are considered as potential hosts for recombinant protein secretion. This type of bacteria contains only one lipid

bilayer with a broad peptidoglycan cell wall and export target protein directly into the culture medium that is an ideal condition for correct folding of recombinant proteins (Green and Meccas, 2016).

Bacillus subtilis is a gram-positive, safe bacterial strain that has a generally recognized as safe (GRAS) condition with an innate capability to excrete proteins into the medium (Zobel *et al.*, 2015). The most significant normal secretion pathways in *B. subtilis* are twin-arginine translocation (Tat) pathway, Sec pathway and ATP-binding cassette (ABC) pathway (Tjalsma *et al.*, 2004, Anné *et al.*, 2016). Signal peptides mark the proteins to follow the appropriate secretion pathway (Owji *et al.*, 2018). Three separate domains of H, N, and C are observed in signal peptides. The C domain containing the cleavage site for the signal peptidase while the h and n-regions are necessary for efficient protein transferring (Freudl, 2018, Tjalsma *et al.*, 2004).

Since the secretion of a given recombinant protein can be enhanced by the selection of an efficient signal peptide, acquiring the most appropriate one for a given target protein is a critical step of an efficient secretory production procedure (Guan *et al.*, 2016).

Despite different studies and deep considerations, there is no assurance in selecting a suitable signal sequence and confirm efficient secretion of the recombinant protein experimentally, and it needs a trial-and-error approach. One alternative strategy is employing the bioinformatics tools to perform *In silico* studies (Taheri-Anganeh *et al.*, 2019). For example, numerous online servers such as SignalP, ProtParam, SOLpro, PRED-TAT and ProtComp are used for prediction of the best signal peptides (Mousavi *et al.*, 2017, Negahdaripour *et al.*, 2017). Thus, in our work, an *in-silico* approach is chosen to analyze several signal peptides for acquiring the most suitable candidates for secretory synthesis of α -amylase in *B. subtilis*.

MATERIALS AND METHODS

Dataset collection. In the first step of the research, the primary structure of *Bacillus licheniformis* α -amylase was retrieved from the

UniProt server at <http://www.uniprot.org/>. In the second step, the amino acid sequences of the prokaryotic signal peptides were gained from the Signal Peptide Website at <http://www.signalpeptide.de/> and validated by UniProt.

The prediction of cleavage site location of the signal peptides. There are several online servers for signal peptides prediction and determination of their cleavage site location. SignalP server at <http://www.cbs.dtu.dk/services/SignalP/>, was used to evaluate the presence of signal peptides and their cleavage sites locations by combining several artificial neural networks (Petersen *et al.*, 2011).

Signal peptides physicochemical aspects prediction. ProtParam, as a part of ExPASy, is online at <https://web.expasy.org/protparam/>, was used for the computation of various physicochemical properties of the signal peptides, such as molecular weight (MW), isoelectric point (pI), aliphatic index, instability index, positively charge residues and grand average of hydrophobicity (GRAVY) (Gasteiger *et al.*, 2005).

Protein solubility prediction. SOLpro server at <http://scratch.proteomics.ics.uci.edu/>, was employed for solubility prediction. Using this server, the solubility of proteins can be analyzed, based on their amino acid sequences. SOLpro employs two-stage SVM structure based on primary sequences of proteins (Magnan *et al.*, 2009).

Evaluation of signal peptides secretion pathway and sub-cellular localization. *In silico* prediction of secretion pathways of signal peptide connected fusions was implemented by “PRED-TAT” online server (<http://www.compgen.org/tools/PRED-TAT>). This server defines Sec or Tat pathway for secretion with a score in the 0-1 range. “ProtCompB” online server (<http://www.softberry.com>) was employed to evaluate sub-cellular location of fusion proteins (Bagos *et al.*, 2010, Mohammadi *et al.*, 2018).

RESULTS AND DISCUSSION

Retrieval of signal peptides and sequence definition. According to the Signal Peptide Website, 30 signal peptides including the natural *Bacillus licheniformis* α -amylase and 29 other prokaryotic signal peptides were selected. 21 signal peptides were from gram-positive Bacilli, and nine signal peptides were selected from *E. coli*, which are listed in Table 1.

The prediction of cleavage site location. The presence of signal peptides and their cleavage site locations prediction are shown in Table 2. D-score >0.57 for a sequence means that it is determined as a signal peptide. Based on these results, D-score of the PPBD_BACSU was under the cut-off (0.57) and was neglected for next steps of study.

Prediction of physicochemical aspects of signal peptides. Physicochemical properties of the signal peptides are shown in Table 3. All signal peptides were in the range of 19 and 41 amino acids in length. All the selected signal peptides had net positive charges of 1 to 6 based on ProtParam results. The highest GRAVY values belonged to QOX2_BACSU, CWBA_BACSU, and OMPC_ECOLI and GUB_BACAM respectively (2.242, 1.596, 1.552, and 1.508). The results showed that QOX2_BACSU, OMPC_ECOLI, CWBA_BACSU, and MPF_ECOLI, respectively (198.46, 171.90, 160, and 150.91) had the highest aliphatic index. Instability index represents that how much a signal peptide alone and fused to α -amylase protein can be stable. Based on our results, α -amylase fused with all signal peptides were predicted to be stable.

In silico evaluation of protein solubility. The solubility of the α -amylase in fusion with different signal peptides is shown in Table 3. Based on the results, α -amylase in the fused form with all signal peptides was soluble.

Table 1. Qualitative analyzes of protein spots detected of untreated, 5-Aza and TSA-treated HL-60.

	Uniprot Accession No.	Signal Peptide	Protein Name	Source	Amino Acid Sequence
1	P06278	AMY_BAC LI	Alpha amylase	<i>Bacillus licheniformis</i>	MKQQKRLYARLLTLFALIF <u>LI</u> PHSAAAA
2	P00808	BLAC_BA CLI	Beta-lactamase	<i>Bacillus licheniformis</i>	MKLWFSTLKLKKA AAVLLF <u>SCVALAG</u>
3	P16397	SUBF_BAC SU	Bacillopeptidase F	<i>Bacillus subtilis</i>	MRKKTKNRL ISSVLSTVVISS <u>LLFPGAAGA</u>
4	Q02113	CWBA_BA CSU	Amidase enhancer	<i>Bacillus subtilis</i>	MKSCCKQLIVCSLAA ILLIPS <u>VSFA</u>
5	P42111	YXAL_BA CSU	Uncharacterized protein yxaL	<i>Bacillus subtilis</i>	MVKSFRMKALIAGA AAVAAA <u>VSAGAVSDVPAAKVLQPTA</u> <u>AYA</u>
6	O31737	YLQB_BA CSU	Uncharacterized protein ylbB	<i>Bacillus subtilis</i>	MKKIGLLFMLCLA ALFTIGF <u>PAQQADA</u>
7	P00691	AMY_BAC SU	Alpha-amylase	<i>Bacillus subtilis</i>	MFAKRFKTSLPLF AGFLLL <u>FHLVLAG</u>
8	P39116	PEL_BACS U	Pectate lyase	<i>Bacillus subtilis</i>	MKKVMLATALFLGL TPAGA <u>NA</u>
9	O07532	LYTF_BA CSU	Endopeptidase lytF	<i>Bacillus subtilis</i>	MKKKLAAGLTASA IVGTTLY <u>VTPAEA</u>
10	P42251	PPBD_BA CSU	Alkaline phosphatase D	<i>Bacillus subtilis</i>	MAYDSRFDEWVQKL KEESF QNNTFDRRKFIQGAGKIAG <u>LSLGLTIAQSVGA</u> FEVNA
11	P34957	QOX2_BA CSU	Quinol oxidase subunit 2	<i>Bacillus subtilis</i>	MVIFLFRALKPLL VLALLTV <u>VFVLGG</u>
12	P04957	GUB_BAC SU	Beta-glucanase	<i>Bacillus subtilis</i>	MPYLKRVL LLLVLTGLFMSLF <u>AVTATASA</u>
13	P00648	RNBR_BA CAM	Ribonuclease	<i>Bacillus amyloliquefaciens</i>	MMKMEGIALKKRL SWISVC LLVLVSAAGMLFSTA
14	P21130	SACB_BA CAM	Levansucrase	<i>Bacillus amyloliquefaciens</i>	MNIKKIVKQATVLT FTTALL <u>AGGATQAEA</u>
15	P00692	AMY_BAC AM	Alpha-amylase	<i>Bacillus amyloliquefaciens</i>	MIQKRKR TVSFRLVLMCTLL <u>FVSLPITK</u> TSA
16	P07980	GUB_BAC AM	Beta-glucanase	<i>Bacillus amyloliquefaciens</i>	MKRVL LLLVLTGLFMSLCGITS <u>SVSA</u>
17	Q05523	DACA_BA CST	D-alanyl-D- alanine carboxypeptidase dacA	<i>Bacillus stearothermophilus</i>	MRRRKQNW LFWLLSICLCL <u>TFGP</u> EQQT VKA
18	P31797	CDGT_BA CST	Cyclomaltodextri n glucanotransfera se	<i>Bacillus stearothermophilus</i>	MRRWLSLVLSMS SVFSAIFIV SDT QKV TVEA
19	P06874	THER_BA CST	Thermolysin	<i>Bacillus stearothermophilus</i>	MNKRA MLGAIGLAFGLLAA <u>PIGASA</u>
20	P40943	XYN1_BA CST	Endo-1,4-beta- xylanase	<i>Bacillus stearothermophilus</i>	MRNVVRKPL TIGLALTL LLP <u>MGMTATSA</u>
21	P06279	AMY_BAC ST	Alpha-amylase	<i>Bacillus stearothermophilus</i>	MLTFHRIIRK GWMFLLAFL <u>LTALLFCPTGQPAKA</u>
22	P02932	PHOE_EC OLI	Outer membrane pore protein E	<i>Escherichia coli (strain K12)</i>	MKKSTLALVVM GIVASASVQ <u>A</u>
23	P00634	PPB_ECO LI	Alkaline phosphatase	<i>Escherichia coli (strain K12)</i>	MKQST IALLPLLE FTP VTK <u>A</u>

24	P0A910	OMPA_EC OLI	Outer membrane protein A	<i>Escherichia coli</i> (strain K12)	MKKT AIAIAVALAGE FATVA QA
25	P02931	OMPF_EC OLI	Outer membrane protein F	<i>Escherichia coli</i> (strain K12)	MMKR NILAVIVPALL VAGT ANA
26	P09169	OMPT_EC OLI	Protease 7	<i>Escherichia coli</i> (strain K12)	MRAK LLGIVLTTP IAISSFA
27	P06996	OMPC_EC OLI	Outer membrane protein C	<i>Escherichia coli</i> (strain K12)	MKVK VLSLLVPALL VAGAA NA
28	P02943	LAMB_EC OLI	Maltoporin	<i>Escherichia coli</i> (strain K12)	MMIT LRKLPLAVAVAAG YVM SAQAMA
29	P0AEX9	MALE_EC OLI	Maltose-binding periplasmic protein	<i>Escherichia coli</i> (strain K12)	MKIKT GARILALSALT TMME SASALA
30	P0AEG4	DSBA_EC OLI	Thiol:disulfide interchange protein dsbA	<i>Escherichia coli</i> (strain K12)	MKKI WLALAGLVLA FSASA

The amino acids in the n-region are shown in boldface and the underlined amino acids represent the c-region.

Table 2. *In silico* analysis of the signal peptides sequences by SignalP 4.1 server.

Protein Name	n-region	h-region	c-region	Cleavage Site	C-score	Y-score	S-score	S-mean	D-score
AMY_BACLI	1-10(10)	11-22(12)	24-30(7)	AAA	0.702	0.818	0.996	0.945	0.888
BLAC_BACLI	1-12(12)	13-22(10)	23-26(4)	ALA	0.139	0.359	0.968	0.926	0.671
SUBF_BACSU	1-9(9)	10-14(15)	15-20(6)	AGA	0.769	0.849	0.973	0.942	0.900
CWBA_BACSU	1-5(5)	6-20(15)	20-24(5)	SFA	0.825	0.890	0.985	0.941	0.918
YXAL_BACSU	1-8(8)	9-34(26)	35-41(7)	AYA	0.690	0.528	0.964	0.723	0.635
YLQB_BACSU	1-3(3)	4-18(15)	19-27(9)	ADA	0.668	0.763	0.985	0.889	0.832
AMY_BACSU	1-5(5)	6-22(17)	23-27(5)	AGA	0.208	0.439	0.970	0.924	0.706
PEL_BACSU	1-3(3)	4-14(11)	15-21(7)	ANA	0.613	0.735	0.961	0.880	0.814
LYTF_BACSU	1-4(4)	5-21(17)	22-26(5)	AEA	0.724	0.774	0.905	0.828	0.803
PPBD_BACSU	*	*	*	*	0.397	0.441	0.706	0.273	0.348
QOX2_BACSU	1(1)	2-23(22)	24-26(3)	LGG	0.151	0.380	0.984	0.961	0.700
GUB_BACSU	1-3(3)	4-19(16)	20-25(6)	ASA	0.676	0.798	0.993	0.939	0.876
RNBR_BACAM	1-11(11)	12-32(21)	33-34(2)	STA	0.400	0.614	0.985	0.899	0.771
SACB_BACAM	1-5(5)	6-24(19)	25-29(5)	AFA	0.761	0.799	0.936	0.848	0.826
AMY_BACAM	1-6(6)	7-23(17)	24-28(5)	TSA	0.491	0.680	0.979	0.954	0.831
GUB_BACAM	1-3(3)	4-19(16)	20-25(6)	VSA	0.512	0.691	0.978	0.924	0.819
DACA_BACST	1-5(5)	6-23(18)	24-30(7)	VKA	0.699	0.744	0.906	0.814	0.783
CDGT_BACST	1-3(3)	4-22(19)	23-31(9)	VEA	0.686	0.664	0.930	0.759	0.716

THER_BACST	1-4(4)	5- 21(17)	22-25(4)	ASA	0.792	0.852	0.969	0.890	0.873
XYN1_BACST	1-3(3)	4- 23(20)	24-28(5)	TSA	0.683	0.795	0.986	0.927	0.867
AMY_BACST	1- 10(10)	11- 26(16)	27-33(7)	AKA	0.848	0.871	0.972	0.908	0.891
PHOE_ECOLI	1-4(4)	5- 15(11)	16-21(6)	VQA	0.169	0.390	0.960	0.881	0.660
PPB_ECOLI	1-4(4)	5- 16(12)	17-21(5)	TKA	0.426	0.618	0.944	0.874	0.759
OMPA_ECOLI	1-4(4)	5-13(9)	14-21(8)	AQA	0.760	0.816	0.944	0.859	0.840
OMPF_ECOLI	1-4(4)	5- 17(13)	18-22(5)	ANA	0.786	0.847	0.959	0.918	0.886
OMPT_ECOLI	1-4(4)	5-12(8)	13-20(8)	SFA	0.197	0.415	0.913	0.835	0.646
OMPC_ECOLI	1-4(4)	5- 16(12)	17-21(5)	ANA	0.542	0.708	0.982	0.929	0.829
LAMB_ECOLI	1-7(7)	8-16(9)	17-25(9)	AMA	0.269	0.491	0.956	0.911	0.722
MALE_ECOLI	1-5(5)	6- 20(15)	21-26(6)	ALA	0.300	0.529	0.979	0.924	0.747
DSBA_ECOLI	1-3(3)	4-15 (12)	16-19(4)	ASA	0.193	0.417	0.941	0.889	0.677

N region: positively charged N terminal of SP. H region: aliphatic region of SP. C region: C-terminal region of SP. The S and C-score recognize signal peptide positions and cleavage sites respectively. The combination of C-score and S-score is Y-score, and the average of the S-score is S-mean. D-score (discrimination score) is a simple average of the S-mean and Y-max score.

*: no signal peptide detected.

Table 3. Physicochemical properties of the signal peptides was determined by ProtParam server.

Signal Peptide	Amino Acid Length (bp)	MW (Da)	pI	Net Positive Charge	Aliphatic Index	GRAVY	Instability Index of SP	Instability Index of SP+ amyS	Solubility
AMY_BA CLI	29	3299.07	11.10	4	141.72	0.752	Unstable (50.51)	Stable (29.94)	soluble
BLAC_B ACLI	26	2810.54	10.04	4	131.54	1.192	Stable (15.30)	Stable (27.81)	soluble
SUBF_B ACSU	30	3145.80	12.02	5	117.00	0.497	Stable (20.75)	Stable (28.20)	soluble
CWBA_B ACSU	25	2649.35	8.89	2	160.00	1.596	Unstable (42.97)	Stable (29.37)	soluble
YXAL_B ACSU	41	3974.74	10.00	3	105.12	0.915	Stable (30.66)	Stable (28.82)	soluble
YLQB_B ACSU	27	2912.60	7.95	1	119.63	1.122	Stable (31.87)	Stable (28.83)	soluble
AMY_BA CSU	27	3051.82	11.17	3	137.41	1.352	Stable (23.27)	Stable (28.21)	soluble
PEL_BA CSU	21	2118.62	10.00	2	111.90	0.948	Stable (10.61)	Stable (27.91)	soluble
LYTF_B ACSU	26	2542.07	9.70	2	116.54	0.769	Stable (0.70)	Stable (27.23)	soluble
QOX2_B ACSU	26	2843.68	11.00	2	198.46	2.242	Stable (8.20)	Stable (27.45)	soluble
GUB_BA CSU	28	3027.76	9.99	2	142.86	1.443	Unstable (40.54)	Stable (29.31)	soluble
RNBR_B ACAM	34	3699.65	9.79	3	129.12	1.153	Stable (28.71)	Stable (28.66)	soluble

SACB_B ACAM	29	3008. 61	10.30	3	107.93	0.710	Stable (26.18)	Stable (28.52)	soluble
AMY_BA CAM	31	3582. 47	11.74	6	119.35	0.606	Unstable (47.44)	Stable (29.79)	soluble
GUB_BA CAM	25	2640. 30	9.50	2	148.00	1.508	Unstable (45.44)	Stable (29.49)	soluble
DACA_B ACST	30	3685. 47	10.93	5	91.00	0.117	Unstable (86.84)	Stable (32.06)	soluble
CDGT_B ACST	31	3562. 25	8.50	1	116.13	0.887	Unstable (46.86)	Stable (29.76)	soluble
THER_B ACST	25	2414. 95	11.00	2	121.60	1.100	Stable (33.02)	Stable (28.88)	soluble
XYN1_B ACST	28	2969. 70	12.01	3	128.93	0.814	Stable (11.82)	Stable (27.74)	soluble
AMY_BA CST	34	3906. 86	11.01	4	115.00	0.876	Stable (35.93)	Stable (29.14)	soluble
PHOE_E COLI	21	2104. 59	10.00	2	130.00	1.195	Stable (1.44)	Stable (27.53)	soluble
PPB_EC OLI	21	2256. 82	10.00	2	139.52	0.971	Unstable (56.02)	Stable (29.80)	soluble
OMPA_E COLI	21	2046. 50	10.00	2	121.43	1.295	Stable (9.52)	Stable (27.86)	soluble
OMPF_E COLI	22	2266. 83	11.00	2	150.91	1.259	Unstable (67.18)	Stable (30.34)	soluble
OMPT_E COLI	20	2102. 61	11.00	2	146.50	1.290	Stable (2.62)	Stable (27.63)	soluble
OMPC_E COLI	21	2078. 63	10.00	2	171.90	1.552	Stable (14.37)	Stable (28.07)	soluble
LAMB_E COLI	25	2545. 22	11.00	2	125.20	1.332	Unstable (42.97)	Stable (29.37)	soluble
MALE_E COLI	26	2698. 34	11.17	3	113.08	1.012	Stable (2.85)	Stable (27.34)	soluble
DSBA_E COLI	19	1990. 48	10.00	2	144.21	1.416	Stable (11.50)	Stable (28.01)	soluble

MW: molecular weight. pI: isoelectric point. GRAVY: grand average of hydrophobicity.

Secretion pathway and sub-cellular localization. Predicted secretion pathway for signal peptides connected α -amylase are shown in Table 4. All predicted pathways were Sec pathway except for QOX2_BACSU. This signal peptide was ignored for last step. In the last step, all remained signal peptides fusions were evaluated as secreted proteins.

DISCUSSION

Recombinant protein secretion to the extracellular space includes several important privileges in

comparison to intracellular expression approaches, including high stability and solubility of the product, enhanced biological activity and simplified downstream processing. Secretion of a specific protein into the extracellular space can be mediated via an expression system by addition a signal peptide to the N-terminal of a protein (Anné *et al.*, 2016, Song *et al.*, 2015). The signal peptide is considered as a momentous factor that has an essential effect on the secretion pathway selection and consequently, on the yield of the recombinant protein. So, secretion of the recombinant protein can be enhanced by the choice of an efficient signal peptide (Guan *et al.*, 2016).

Table 4. Secretion sorting and sub-cellular location of signal peptides

Signal peptides	Secretion Pathway		Sub-cellular localization			
	Type of SP	Reliability Score (%)	Cytoplasmic	Membrane	Secreted (extracellular)	Final prediction site
AMY_BACLI	Sec pathway	0.995	3.36	0.00	6.63	Secreted
BLAC_BACLI	Sec pathway	0.994	3.35	0.05	6.58	Secreted
SUBF_BACSU	Sec pathway	1.000	3.32	0.05	6.61	Secreted
CWBA_BACSU	Sec pathway	1.000	3.34	0.01	6.63	Secreted
YXAL_BACSU	Sec pathway	0.937	3.32	0.15	6.50	Secreted
YLQB_BACSU	Sec pathway	0.993	3.33	0.04	6.62	Secreted
AMY_BACSU	Sec pathway	0.990	3.36	0.03	6.59	Secreted
PEL_BACSU	Sec pathway	1.000	3.32	0.01	6.65	Secreted
LYTF_BACSU	Sec pathway	1.000	3.33	0.04	6.62	Secreted
QOX2_BACSU	TM segment	0.810	*	*	*	*
GUB_BACSU	Sec pathway	0.999	3.35	0.00	6.63	Secreted
RNBR_BACAM	Sec pathway	0.986	3.36	0.02	6.61	Secreted
SACB_BACAM	Sec pathway	1.000	3.29	0.52	6.10	Secreted
AMY_BACAM	Sec pathway	0.999	3.36	0.00	6.63	Secreted
GUB_BACAM	Sec pathway	1.000	3.32	0.00	6.67	Secreted
DACA_BACST	Sec pathway	1.000	3.32	0.00	6.66	Secreted
CDGT_BACST	Sec pathway	0.993	3.31	0.04	6.62	Secreted
THER_BACST	Sec pathway	1.000	3.32	0.00	6.67	Secreted
XYN1_BACST	Sec pathway	0.997	3.33	0.00	6.65	Secreted
AMY_BACST	Sec pathway	1.000	3.32	0.00	6.66	Secreted
PHOE_ECOLI	Sec pathway	1.000	3.32	0.12	6.54	Secreted
PPB_ECOLI	Sec pathway	1.000	3.32	0.15	6.50	Secreted
OMPA_ECOLI	Sec pathway	1.000	3.33	0.12	6.52	Secreted
OMPF_ECOLI	Sec pathway	1.000	3.32	0.07	6.59	Secreted
OMPT_ECOLI	Sec pathway	1.000	3.32	0.10	6.55	Secreted

OMPC_ECOLI	Sec pathway	0.999	3.32	0.14	6.51	Secreted
LAMB_ECOLI	Sec pathway	1.000	3.32	0.03	6.63	Secreted
MALE_ECOLI	Sec pathway	1.000	3.32	0.03	6.63	Secreted
DSBA_ECOLI	Sec pathway	1.000	3.32	0.11	6.54	Secreted

Today, several online tools are used for identification of signal peptides to reduce the cost and improving the quality of experimental investigation (Gasteiger *et al.*, 2005, Magnan *et al.*, 2009, Petersen *et al.*, 2011). For this purpose, in this study, 30 prokaryotic signal peptides were selected. These signal peptides have illustrated positive effects on secretion of some proteins in previous investigations (Watanabe *et al.*, 2009, Chen *et al.*, 2015, Degering *et al.*, 2010). Then numerous online servers such as SignalP, ProtParam, and SOLpro are used for prediction of the presence and cleavage site location, physico-chemical properties and protein solubility of signal peptides. These factors are important to improve the protein secretion levels.

In this study, for the prediction of cleavage site presence and its location on the signal peptide sequences SignalP server was applied. The graphical output from SignalP comprises three different scores, C, S and Y, The S and C-score recognize signal peptide positions and cleavage sites respectively. The combination of C-score and S-score is Y-score, and the average of the S-score is S-mean. D-score (discrimination score) is a simple average of the S-mean and Y-max score and when a value > 0.57 represent a secretory protein and value of < 0.57 is interpreted as a non-secretory protein. As shown in Table 2, D-scores of the PPBD_BACSU was under the cutoff (0.57), which indicated that the cleavage site of it cannot appropriately be recognized by peptidase enzyme. Amino acids with small neutral side chains like alanine in the -3 and the -1 position of c-region are needed to correct cleavage of the signal peptide. The important sites of the C-region are position 1 and 3 before the cleavage site that is recognized as the $(-3, -1)$ rule or AXA motif. This motif is universal in prokaryotes, eukaryotes organelles SPs. Table 2 shows that most of the signal peptides had a proper cleavage site. Ala amino acid is conserved at positions -3 and -1 .

Replacing of Ala at these positions with other residues, specifically at position -1 , disturbs the cleavage site (Owji *et al.*, 2018). Exceptionally, as PPBD_BACSU have a D-scores 0.348, it is not applicable for the secretion of the α -amylase protein.

To design a secreted protein properly, structural and physicochemical features of the signal peptides should be considered. Numerous bioinformatics servers are provided to anticipate and analyze the physicochemical properties of signal peptides. In the present study, for investigation of the structural and physicochemical features of different signal peptides, ProtParam online server was used. The results were included in molecular weight, amino acid length, net positive charge, instability and aliphatic indexes, pI and GRAVY (Gasteiger *et al.*, 2005).

Improving an applicable signal peptide is depending on the n region with positively charged amino acids. Positively charged amino acids interacts with the membrane phospholipids and it would be an advantage. Therefore, if one or more basic amino acids present in the signal peptide n-region, it may be more useful. Regarding these facts, when the net positive charge of the n region lowered to zero or get a negative value the rate of transportation across the membrane can significantly decrease. (Freudl, 2018, Owji *et al.*, 2018). The net positive charge for most of the studied signal peptides was $+2$, and AMY_BACAM had the highest net positive charge in this study.

The h-region's hydrophobicity is essential for transferring of the protein out of the membrane. So elevated hydrophobicity levels and increased h-region length can help to improve the protein secretion. On the other hand, a polar or charged amino acid residue can disturb the core hydrophobicity and reduces or stops

transportation across the membrane (Chen *et al.*, 1996).

To study the hydrophobicity of the adopted signal peptides, GRAVY and aliphatic index were measured. The increase in GRAVY and aliphatic index means high hydrophobicity. The grand average of hydrophobicity (GRAVY) protein is the sum of hydrophobicity values of all the amino acid residues and dividing by the number of residues in the sequence (Kyte and Doolittle, 1982). The aliphatic index is interpreted as the relative volume taken up by aliphatic side chains (valine, leucine, isoleucine, and alanine) (Ikai, 1980). According to the values of aliphatic index and GRAVY that are shown in Table 3, the hydrophobicity amounts of the signal peptides, QOX2_BACSU, OMPC_ECOLI and CWBA_BACSU were the highest levels among all 30 signal peptides that could interact with hydrophobic interior side of the membrane transporter proteins.

Protein stability that is the sum of all forces predict that a protein in the experimental stages remain in its correct conformation or it becomes misfolded. Generally, the protein stability is affected by hydrophobic interactions, hydrogen bonds, and conformational entropy and the fate of unstable proteins is usually to be degraded using ATP-dependent cytosolic systems in both bacterial and eukaryotic cells (Golden Zweig and Fleishman, 2018, Gur and Sauer, 2008). Based on ProtParam, instability index reflects the instability of the signal peptides linking to α -amylase protein. If instability index of a protein is to be lower than 40 (instability index <40), it will be stable, a value of > 40 can be an indication of unstable protein (Guruprasad *et al.*, 1990). The results showed α -amylase fused to all signal peptides were predicted to be stable.

In the experimental stages of a study, protein insolubility can produce some difficulties, so accurate analysis of the protein solubility is necessary. An insoluble protein has more exposed hydrophobic regions which can interact with each other and cause protein aggregation. A lot of experimental investigations have been done to get the soluble form of recombinant proteins, including chaperone co-expression, culture temperature regulation, efficient host strain and vector that require long time trial-and-error approaches (Rosano and Ceccarelli, 2014). Thus,

the *in-silico* approaches are essential for analyzing protein solubility. Among different available servers, SOLpro is a sequence-based solubility predictor tool that has an accuracy value of 74% (Magnan *et al.*, 2009). In the present study, SOLpro online server was used for analysis of overexpressed α -amylase solubility when linked to the various signal peptides in *B. subtilis*. According to Table 4, α -Amylase was predicted to be soluble in fusion with all the signal peptides.

Protein export pathways in *B. subtilis* are twin-arginine translocation (Tat) pathway, Sec pathway and ATP-binding cassette (ABC) pathway. However Sec and Tat pathways are used most commonly for recombinant protein secretion in *B. subtilis*. In the Sec pathway unfolded proteins and in the Tat pathway folded proteins across the cytoplasmic membrane. So, it seems secretion recombinant proteins folding in Tat pathway causes protein aggregation and making inclusion bodies. So, secretion of proteins in unfolded state in the sec pathway is more appropriate (van Dijk and Hecker, 2013, Rusch and Kendall, 2007, Natale *et al.*, 2008). Except QOX2_BACSU that may translocate into transmembrane segment, all signal peptides were predicted to direct α -amylase through Sec pathway. Ultimately, all remained signal peptides may be useful for the secretory production and can target protein into the culture medium with a reliability score more than 0.6 (Table 4).

CONCLUSION

Finally, GUB_BACAM and THER_BACST can be useful for the future practical investigations in α -amylase secretion. *In silico* investigations and analysis of signal peptides using online tools are acceptable, but these results need to be approved by experimental analysis.

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