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Identification of a New *Bacillus B. amyloliquefaciens* Isolate with Enzymatic and Antifungal Potential

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Abstract

Bacillus has become the bacterium of the choice for its versatility and capacity to synthesize hydrolytic enzymes and release extracellular antifungal metabolites. In this work, out of 525 isolates, the *Bacillus* isolate SY123A was the best xylanase and amylase producer (391.38 U/g; pH 7.0 and 195.46 U/g; pH 6.0, respectively) through solid-state fermentation (SSF), which was associated with high antagonistic activities (up 100%) against four common and destructive soil- borne phytopathogenic fungi in nutrient agar (NA) medium. According to the colony morphology and 16S rRNA gene sequencing, the isolate was identified as *B. amyloliquefaciens.* The biotechnological potential of SY123A could suggest this isolate as very promising for agro-industry and the environmental aspect.

Keywords*: Bacillus amyloliquefaciens*, xylanase, α-amylase, antagonism, filamentous fungi

1.Introduction

Bacillus is an important genus as it is a source of a large number of biologically active metabolites, including antibiotics, enzymes, and probiotics. The Gram-positive strain *B. amyloliquefaciens* in particular has been an attractive species over many years and, is considered as an industrial important and a root-colonizing biocontrol bacterium, since it has a capacity to produce various important industrial enzymes [6], and bacteriocins such as subtilin and barnase which have antibacterial effects against plant diseases caused by soil-borne microorganisms [20].

B. amyloliquefaciens is broadly used industrially to produce different hydrolytic thermostable enzymes [9, 10] such as xylanase (EC 3. 2. 1. 8) which is one of an important industrial enzyme that hydrolyzes xylan by breaking the hemicelluloses of the plant cell wall. Recently, it has markedly increased due its wide variety of food applications such bread making, the production of corn starch, clarification of fruit juice and wine; animal feeds, and alcoholic fermentation [8].

Another industrially *important enzyme* produced by *B. amyloliquefaciens* is alpha-amylase (EC 3.2.1.1, a-1, 4-glucan-4-glucanohydrolase), which catalyses the endocleavage of the a-1, 4-glycoside linkages and the release of short oligosaccharides and a-limit dextrin. This enzyme has been used commercially for sugar syrups production from starch, and for starch liquefaction and paper, food, pharmaceutical and sugar industries [11, 30]. However, although xylanase and amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand [2].

On the other hand, *B. amyloliquefaciens* also has a unique ability to produce a broad spectrum of biological control agents for controlling plant diseases. It has been used to inhibit the growth and production of mycelia and sclerotia of *Sclerotinia sclerotiorum* [23], and it had strong antagonistic activity against *Magnaporthe oryzae* [12]. However, effects of the biological agents of *B. amyloliquefaciens* are usually start with an *in vitro* screening against selected pathogens using NB culture assay, and antagonistic activity is quantified in terms of inhibition of pathogen's mycelium growth [31].

Therefore, the purposes of the present work were to isolate and identify a new *Bacillus* isolate capable of producing xylanase and amylase under SSF conditions, and to determine its *in vitro* antagonistic

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potential against four common and destructive soilborne fungal pathogens belonging to *different* genera i.e. *Cochliobolus sativus, Pyrenophora graminea, Fusarium culmorum* and *Fusarium solani*.

2. Materials and methods

Bacterial isolate. The isolate of *B. amyloliquefaciens* SY123A was isolated from soil samples (AL-Jebsah E: 040º44º33.2 / N: 36º03º49.6) [3] collected randomly from different regions of Syria, and screened among 525 isolates on NB culture, the colonies were identified according to Wulff et al. (2002) [31]. A pure culture of SY123A isolate was first grown on NB and incubated for 24 h at 30 °C.

16S rRNA gene sequencing. The *B. amyloliquefaciens* isolate SY123A was identified using 16S rRNA gene sequence analysis as per the standard protocol [25]. The two primers BacF (5'-GTGCCTAATACATGCAAGTC-3') and BcaR (5'-CTTTACGCCCAATAATTCC-3') flanking a highly variable sequence region of 545 bp towards the 5'end of the 16S rDNA region were used. PCR amplification was performed in a final reaction volume of 50 µL. The reaction mixture contained 2 µl (50-100 ng) of extracted genomic DNA, 1x reaction buffer (TrisKCl-MgCl2), 2 mM MgCl2, 0.2 mM dNTP, 1 µM of each primer, and Taq polymerase (5U/µl, QIAGEN). PCR amplification condition was achieved using the following parameters: Initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, primers annealing at 54°C for 1 min, and extension at 72°C for 90 s. The final cycle included extension for 10 min at 72°C to ensure full extension of the products. A total of 30 serial cycles of amplification reaction was performed. PCR products were separated on a 1.5% agarose gel and visualized using UV light (302 nm) after staining with ethidium bromide. Prior to sequencing, PCR products were purified with QIAgen gel extraction kit (28704) according to the manufacturer´s recommendations. Sequencing was carried out on a Genetic Analyzer (ABI 310, Perklin-Elmer, Applied Biosystems, USA). The 123A isolate sequence was compared to sequences deposited in the Genbank database (NCBI). For the local alignment, the BLASX tool (NCBI) was used.

Enzyme production. Enzyme production in SSF was carried out in 250 ml Erlenmeyer flasks, each containing 5 g of solid substrate and nutrients (based on 100 ml of liquid medium) plus distilled water to adjust the moisture content. The fermentation medium consisted of (g/L): wheat bran 10; Na_2HPO_4 .$2H_2O$ 10; KCl 0.5; $MgSO_4.7H_2O$ 0.15, and $NaNO_3$ 6, as a nitrogen source. The culture medium was inoculated with 1mL spore suspension (10^6 spores/ mL) and incubated at 30° C. The enzyme was extracted by adding distilled water containing 0.1% Triton x 100 to make the volume in flask equivalent to 100 mL. At the end of the cultivation stage, the culture was centrifuged (5000 x g for 15 min) and the clarified supernatant was concentrated using ultra filtration (Whatman no. 1. paper). The influence of initial medium pH on xylanase and amylase production was assessed by cultivating the isolate in the basal media of pH ranging from 3.0 to 9.0. The effect of temperature was studied by performing the fermentation at different temperatures from 25 to 65°C.

Xylanolytic activity. Out of 525 isolates the *B. amyloliquefaciens SY123A isolate was considered as the best producer of xylanase.* Xylanase activity was determined using 1% birchwood xylan as substrate [7]. The mixture was incubated at 55 ºC for 5 min, and the reducing sugars dinitrosalicylic acid (DNS) was measured according to Miller (1959) [24]. The released xylose was determined in a *spectrophotometer at 540 nm.* One unit of xylanase activity is expressed as the amount of enzyme which will release 1 µmol of reducing sugars (as xylose *equivalents*) per minute.

Amylolytic activity. The *B. amyloliquefaciens SY123A isolate was also screened out of 525 isolates as the best producer of amylase.* α-amylase activity was determined as described by Okolo et al. (2001) [27]. Reaction mixture contained: 1 % soluble starch, 1.25; 0.1 M acetate buffer (pH 5.0), 0.25 ml; and appropriately diluted crude enzyme extract, 0.25 ml. Liberated reducing sugars (glucose equivalent) were measured 10 min of incubation at 50 ºC using DNS method of Miller (1959) [24]. One unit (IU) of α –amylase is defined as the amount of enzyme that releases 1 µmol of glucose equivalent per min under the assay conditions and enzyme activity is expressed in terms of IU per gram dry fermented substrate.

In vitro evaluation of antagonism. *B. amyloliquefaciens* SY123A isolate was selected out of 525 *Bacillus* isolates on the bases of fungal growth inhibition in ***in vitro*** sensitivity experiments against four fungal pathogen. These fungi including the virulent isolate (*Cs* 16) of barley *C. sativus* [4],

virulent SY3 of barley *P. graminea* [5] and *F. culmorum* (*F3*) and *F. solani* (*F3*5) isolates of wheat [1]. 123A isolate was streaked as thick bands on four opposite edges on the NA plates. Then 5 mm diameter disc of each fungus was cut from of an actively growing culture by a sterile cork borer and placed onto the center of above NA plates. The Petri dishes were sealed by parafilm and incubated at 25 ± 1 °C in dark for 4 days. Where mycelia disc on NA medium without bacteria was served as control. The antagonistic effect showed by bacteria was measured as zone of inhibition (the distance between the edge of antagonistic bacterial growth and the edge of tested fungal isolates) according to Foysal et al. (2011) [14]. Experiments were performed in triplicate. The percentage of inhibition of radial growth (PIRG) was calculated according to Rabindran and Vidyasekaran (1996) [29].

Statistical analysis

All experiments were conducted twice in triplicate. Data were analyzed for enzyme production and antagonistic activities using analysis of variance (ANOVA), and means with $p < 0.05$ were considered statistically significant.

3. Results and discussions

B. amyloliquefaciens SY123A was isolated from soil samples collected from various regions of Syria. After being plated on NB medium and cultured for 24 h at 37 °C, the colonies of this isolate were creamy white and slimy with a ridged surface and irregular margins (Fig. 1A) which is in close agreement with Li et al. (2016) [19]. Those findings led to consider the isolate belonging to *B. amyloliquefaciens* which was then confirmed by the 16S rRNA sequencing. The 16S rRNA sequence was compared with the other sequenced bacteria in National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) in GeneBank showed similarity of derived sequences with some sequences belonging to the 16S small sub-unit rDNA of other bacteria. The sequence of 123A was most closely related to *B. amyloliquefaciens* with similarity of 100% (Table 1). The nucleotide sequence was deposited in GenBank under accession number MT159380.

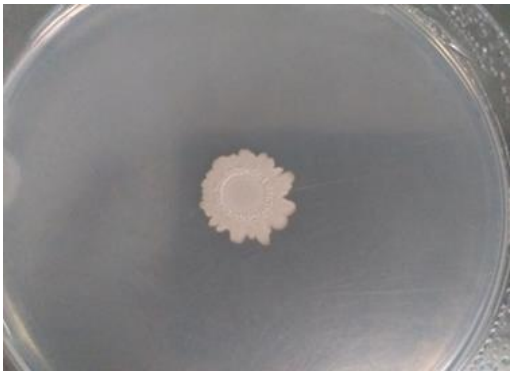
The results showed that the *amyloliquefaciens* isolate SY123A had a best capability to produce xylanase (391.38 U/g) and amylase (195.46 U/g) out of 525 bacillus isolates after 48 h of incubation. The data presented in Figure 2 confirmed that pH 7.0 and pH 6.0 was the most conducive for xylanase and amylase production, respectively. This was consistent with the previous report stating that the enzyme activity of xylanase was generally stable in neutral conditions, and readily inactivated under both acidic and alkaline conditions [22]. Our results are in line with O. El- Tayeb (2007) [13] who reported that pH 6.0 was optimal to produce amylase from *Bacillus amyloliquefaciens* (strain 267CH). However, pH of the medium can influence the dissociation degree of the enzyme, the charge condition, the structure, and function of the protein [21].

The optimum range temperature for xylanase and amylase production was 40 °C for xylanase and 37 °C for amylase (Table 3), which is in distant from that of the xylanases produced by *B. amyloliquefaciens* CH51 strain (25 °C) [16], and with xylanases produced by *B. amyloliquefaciens* strain SK-3 (70 °C) [17]. On the other hand, our results are in agreement with those of Nusrat and Rahman (2007) [26] who stated that, α-amylase production from *Bacillus amyloliquefaciens* was maximum at temperature 37°C, and with those of Haq et al. (2010) [15] who demonstrated that the better activity of α-amylase was at 37°C in the mutant strain of *Bacillus amyloliquefaciens EMS6*.

On the other hand, ***in vitro*** sensitivity of four fungal pathogens to antagonistic *B. amyloliquefaciens* SY123A demonstrated that this isolate was inhibited the growth of phytopathogens by 47–98% in comparison to the controls (Figs. 1B and 4). The inhibition rate of SY123A was highest (98%) against *P. graminea* and lowest (47%) against *F. culmorum*. It showed 87% and 81% antagonistic activity against *C. sativus* and *F. solani* respectively. The broad-spectrum antagonistic activity against these plant pathogens indicated that isolate SY123A can be a potential biocontrol agent for the diseases caused by these fungal pathogens. This study supports by the results obtained by Li et al. (2014) [18] who reported that *B. amyloliquefaciens* had an ability to suppress soil-borne pathogens. Similarly supportive results were obtained by Xu et al. (2013) [32] on the inhibitory *effects* of *B. amyloliquefaciens* against *Fusarium oxysporum* f.sp. *cucumerinum*.

Table 1. 16S rRNA gene sequence similarity between *B. Amyloliquefaciens 123A* and microorganisms strains at NCBI.

Microorganism	16S rRNA gene sequence similarity (%)
pG1 protein [*Bacillus amyloliquefaciens*]	100.00%
hypothetical protein CJP14_20620 [*Bacillus velezensis*]	98.68%
pG1 protein [*Bacillus amyloliquefaciens*]	98.70%
hypothetical protein B5V88_02535 [*Bacillus sporothermodurans*]	94.81%
hypothetical protein PuT2_15495 [*Pusillimonas sp.* T2]	94.74%
hypothetical protein BTR23_20630 [*Bacillus pseudofirmus*]	97.22%
hypothetical protein B6A27_12100 [*Anoxybacillus sp. UARK-01*]	92.21%
hypothetical protein CNQ80_10340 [*Bacillus cereus*]	97.10%
hypothetical protein BN871_AB_00880 [*Paenibacillus sp. P22*]	92.31%
hypothetical protein B2J90_00160 [*Bacillus cereus*]	93.55%
hypothetical protein B7467_04935 [*Staphylococcus lugdunensis*]	92.65%
hypothetical protein HMPREF0769_11381 [*Staphylococcus aureus subsp. aureus MN8*]	91.18%
hypothetical protein BVG16_32145 [*Paenibacillus selenitireducens*]	93.55%



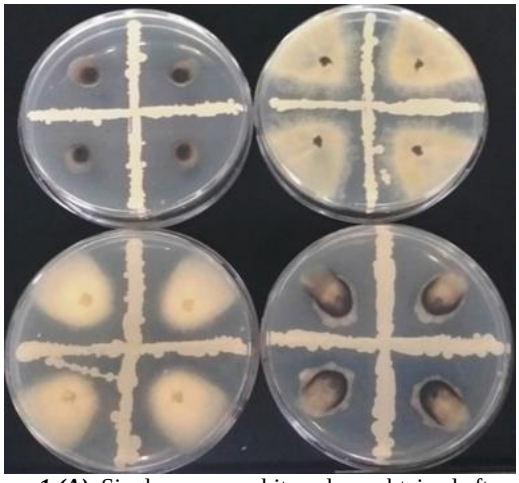


Figure 1 (A). Single creamy white colony obtained after sub-culturing of *B. amyloliquefaciens* SY123A on NA medium. (B) Efficacy of *in vitro* antagonistic SY123A against *P. graminea* (1), *F. culmorum* (2), *F. solani* (3) and *C. sativus* (4) on NA

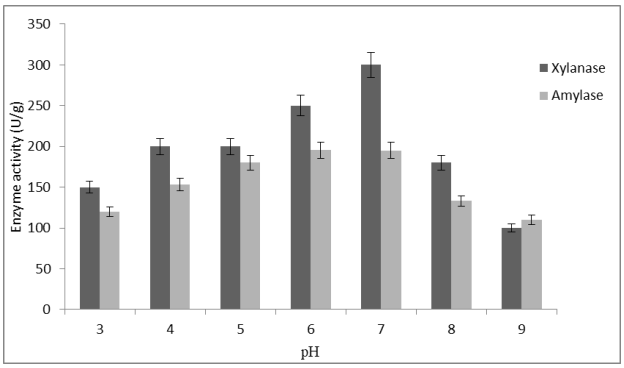


Figure 2. Effect of initial pH values on xylanase and mylase production by *B. amyloliquefaciens* SY123A under SSF

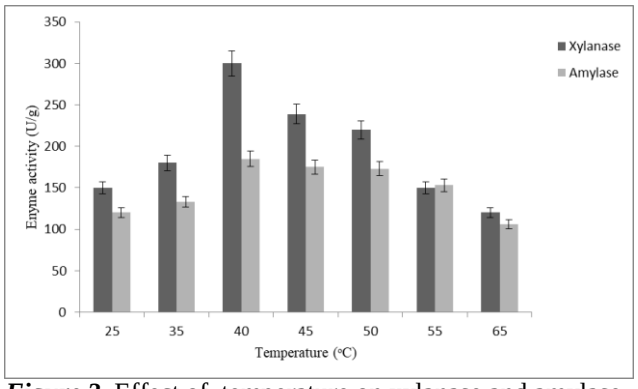


Figure 3. Effect of temperature on xylanase and amylase production by *B. amyloliquefaciens* SY123A under SSF.

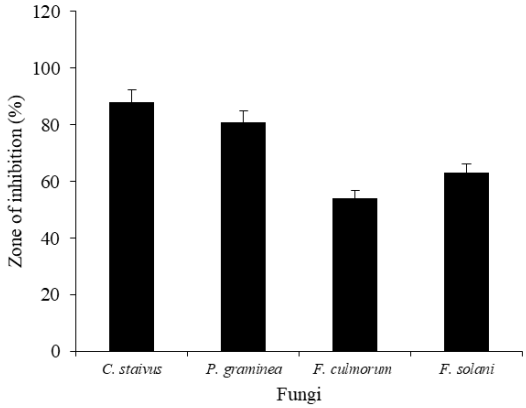


Figure 4. Antagonistic efficacy of *B. amyloliquefaciens* isolate SY123A against four soilborne fungal pathogens. Zone of inhibition (%) was calculated according to formula described by Rabindran and Vidyasekaran (1996) [29].

On the other hand, *B. amyloliquefaciens* isolates have been reported to produce major lipopeptide-like compounds that belong to the iturin homologs [28]. The iturin homologs penetrate into the cytoplasmic membrane by the hydrophobic tail, followed by auto-aggregation resulting in pore formation, which causes cellular leakage, these mechanisms might explain why isolate SY123A could effectively inhibit the mycelial growth of the four studied pathogens. However, this inhibitory effect shown by *B. amyloliquefaciens* could be further explored as a potentially efficient biological control agent against these pathogens in agricultural applications. Furthermore, although the mechanism of *B. amyloliquefaciens* SY123A to control these fungi is still unclear, we will further study this biological control mechanism.

In this study, *B. amyloliquefaciens* SY123A, was isolated from soil and identified based on the morphology of colonies and 16S rRNA sequencing. SY123A not only had the capacity for high level of xylanase and amylase production, but also had broad-spectrum antagonistic activities against several plant pathogens, such *Cochliobolus sativus*, *Pyrenophora graminea*, *F. culmorum* and *F. solani*. These results could suggest SY123A as a very promising isolate for agro-industry and the environmental aspects.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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References

1. Alazem M., *Characterization of Syrian Fusarium species by cultural characteristics and aggressiveness*. Thesis, University of Damascus, Faculty of Agriculture, **2007**, 72.
2. Al-Maqtari QA, AL-Ansi W, Mahdi AA, Microbial enzymes produced by fermentation and their applications in the food industry - A review. *Int. J. Agri. Inn. Res*., **2019**, *8*, 62-82.
3. Ammouneh H, Harba M, Idris E, Makee H., Isolation and characterization of native *Bacillus Thuringiensis* isolates from Syrian soil and testing under insecticidal activities against some insect pests. *Turk. J. Agri. Forest*. **2011**, *35*, 421-431.
4. Arabi MIE, Jawhar M. Identification of *Cochliobolus sativus* (spot blotch) isolates expressing differential virulence on barley genotypes in Syria. *J. Phytopathol.* **2004**, *152*, 461-464.
5. Arabi MIE, Jawhar M, Al-Safadi B, Mirali N. Yield responses of Barley to leaf stripe (*Pyrenophora graminea*) under experimental conditions in Southern Syria. *J. Phytopathol*. **2004**, *152***,** 519-23.
6. Aunstrup K. Production, isolation, and economics of extracellular enzymes. Wingard LB, Katchalski-Katzir E, Golsdstein L (Eds.), *Appl. Biochem. Bioeng. Enzy. Technol*. **1979**, *2*, 28-68
7. Bailey MJ, Bailey P, Poutanen R., Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol*., **1992**, *2*3, 257–270.
8. Bhardwaj N, Kumar B, Verma P., A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bior. Biop*., **2019**, *6*, 40.
9. Caulier S, Nannan C, Gillis A, Licciardi F, Bragard C, Mahillon J., Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. *Front Microbiol*. **2019**, *10*, 302.
10. Devaraj K, Aathika S, Periyasamy K, Manickam Periyaraman P, Palaniyandi S, Subramanian S., Production of thermostable multiple enzymes from *Bacillus amyloliquefaciens* KUB29. *Nat. Prod. Res*. **2019**, *33*, 1674-1677.
11. de Souza PM, de Oliveira Magalhães P., Application of microbial α-amylase in industry - A review. *Braz. J. Microbiol*. **2010**, *41*, 850–861.
12. Dong Y, Li H, Rong S, Xu H, Guan Y, Zhao L, Chen W, He X, Gao X, Chen R, Lihua Li L, Xu Z., Isolation and evaluation of *Bacillus amyloliquefaciens* Rdx5 as a potential biocontrol agent against *Magnaporthe oryzae*, *Biotech. Biotechnol. Equip*. **2019**, *33*, 408-418.

13. El-Tayeb O, Mohammed F, Hashem A, Abdullah M., Optimization of the industrial production of bacterial alpha amyalse in Egypt. IV. Fermentor production and characterization of the enzyme of two strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens. Afr. J. Biotechnol*., **2007**, *7*(24), 4521–4536.
14. Foysal MJ, Rahman MM, Alam M., Antibiotic sensitivity and *in vitro* antimicrobial activity of plant extracts to pseudomonas fluorescens isolates collected from diseased fish. *Int. J. Nat. Sci*., **2001**, *1*, 82–88.
15. Haq I, Ali S, Javed MM, Hameed U, Saleem A, Adnan F, Qadeer MA, Production of alpha amylase from a randomly induced mutant strain of *Bacillus amyloliquefaciens* and its application as a desizer in textile industry. *Pak. J. Bot*., **2010**, *42*(1), 473–484.
16. Kim GM, Kwon GH, Lee AR, Lee KW, Park JY, Cha J, Song YS, Kim JH., Characterization of a 27 kDa fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH51 isolated from cheonggukjang. *J. Microbiol. Biotechnol*. **2009**, *19*, 997–1004.
17. Kumar S, Haq I, Prakash J. et. al., Purification, characterization and thermostability improvement of xylanase from *Bacillus amyloliquefaciens* and its application in pre-bleaching of kraft pulp. *3 Biotech* **2017**, *7*, 20.
18. Li B, Li Q, Xu Z, Zhang N, Shen Q, Zhang R., Responses of beneficial Bacillus amyloliquefaciens SQR9 to different soilborne fungal pathogens through the alteration of antifungal compounds production. *Front Microbiol*. **2014**, *5*, 636.
19. Li X, Zhang, Y, Wei, Z, Guan, Z, Cai, Y, Liao, X., Antifungal activity of isolated *Bacillus amyloliquefaciens* SYBC H47 for the biocontrol of peach gummosis. PloS one, **2016**, *11*(9).
20. Lisboa MP, Bonatto D, Bizani D, Henriques JA, Brandelli A. Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. Int Microbiol. **2010**, *9*, 111–118.
21. Lošdorfer Božič A, Podgornik R., pH dependence of charge multipole moments in proteins. *Biophys. J*. **2017**, 113, 1454–1465.
22. Mamo G, Thunnissen M, Hatti-Kaul R, Mattiasson B., An alkaline active xylanase: Insights into mechanisms of high pH catalytic adaptation. Biochimie. **2009**, *91*(9), 1187-96
23. Mansourt A, Niday A, Patrice S., Biological control of *Sclerotinia sclerotiorum* (lib.) de bary with *Trichoderma harzianum* and *Bacillus amyloliquefaciens*. *Crop Prot*. **2008**, *27*, 1354–1359.
24. Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugars. Ann. Chem. **1959**, *31*, 426-428.
25. Nair JR, Singh G, Sekar V., Isolation and characterization of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. *J. Appl. Microbiol.* **2002**, *93*, 772–780.
26. Nusrat A, Rahman SR., Comparative studies on the production of extracellular α-amylase by three mesophilic *Bacillus* isolates. *Bangladesh J. Microbiol*. **2007**, *24*(2), 129–132.
27. Okolo BN, Ire S, Ezeogu LI, Anyanwu CU, Odibo FJC**.,** Purification and some properties of a novel raw starch-digesting amylase from *Aspergillus carbonarius*. *J. Sci. Food Agri.*, **2001**, *81*, 329-336.
28. Qiu Y, Zhu Y, Zhan Y, et. al. Systematic unravelling of the inulin hydrolase from *Bacillus amyloliquefaciens* for efficient conversion of inulin to poly-(γ-glutamic acid). *Biotechnol. Biofuels*. **2019**, *12*, 145.
29. Rabindran R, Vidyasekaran P., Development of a formulation of *Pseudomonas fluorescens* PfALR2 for management of rice sheath blight. *Crop Prot*., **1996**, *15*, 715 - 721.
30. Reddy NS, Nimmagadda A, Sambasiva Rao KRS (2003**)** An overview of the microbial α-amylase family. *Afri. J. Biotech*. **2003**, *2*, 645-648.
31. Wulff EG, Mguni CM, Mansfeld-Giese K, Fels J, Lübeck M, Hockenhull J., Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. campestris. *Plant Pathol*. **2002**, *51*, 574–584.
32. Xu Z, Shao J, Li B, Yan X, Shen Q, Zhang R., Contribution of bacillomycin D in *Bacillus amyloliquefaciens* SQR9 to antifungal activity and biofilm formation. *Appl. Environ. Microbiol*. **2013**, *79*(3), 808-15.