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Evaluation of agricultural wastes as a sustainable carbon source for the production of β-glucosidase from *Bacillus stercoris*, its purification and characterization

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β-glucosidases are found in all domains of life and has the ability to hydrolyse the glycosidic bond between carbohydrates and non-carbohydrates residues making it a potent enzyme from an industrial and agricultural perspective. The aim of the current study was isolation, screening, and identification of bacterial strains having maximum β-glucosidase activity from soil sample of Jacobabad, Pakistan. The isolates were screened primarily, and efficient isolates were confirmed for β-glucosidase production through secondary screening. Among the different isolated strains, *1j2* was selected as the best β-glucosidase producer. The isolate showed 99% similarity with *Bacillus stercoris* after 16S rRNA sequencing. Different culture conditions were optimized for effective β-glucosidase production. Enhanced level of β-glucosidase was achieved at 40°C, pH 8 at 150 rpm in a β-glucosidase production medium supplemented with a combination of agricultural residues of wheat bran 0.25% (w/v) and orange peel 0.25% (w/v), using 5% inoculum. *Bacillus stercoris* produced a higher yield (2.89U/mg) of β-glucosidase under optimized conditions. Partial purification of β-glucosidase was achieved after 80% ammonium sulfate precipitation. The enzyme was purified and had shown maximum specific activity of 53.2 U/mg with molecular weight of approximately 36 kDa. In all the tested metals Cu^{2+} was observed to have inhibitory effect while Ca^{2+} had not affected the enzyme activity significantly. While organic solvents and surfactants had significantly reduced the β-glucosidase activity. Based on the afore mentioned abilities of purified β-glucosidase from *Bacillus stercoris* strain it could be a promising player for the industrial applications of lignocellulose hydrolysis found in agricultural residues for the cheap production of biofuels.
Keywords: β-glucosidase, agricultural residues, deglycosylation, *Bacillus stercoris.*

INTRODUCTION

In nature, enzymes are widely distributed and since ancient times they have been used in the production of edible products such as wine, beer, sourdough, cheese, and vinegar (Soccol *et al.,* 2006). Enzymes are known as biological catalysts used to accelerate biochemical reactions in living beings. Compared to chemical catalysts, enzymes are degradable and less toxic. Additionally, the selective nature of enzymes causes them to produce a smaller amount of waste products because they are more specific to their substrates. While, chemical catalysts produce toxic and non-biodegradable waste products because of not being substrate specific (Li *et al.,* 2012). Among the important industrial enzymes, cellulases serve as the second largest group and since 1995 its demand has been increased in several industries including textile, paper, detergent, biofuel and animal food and feed industries (Doi, 2008). To completely hydrolyze a cellulose polymer into its monomers, the three components of cellulases act synergistically. Initially endo-1,4-β-D-glucanase acts randomly in between cellulose fibers. The exo-cellobiohydrolase further acts on it and release cellobiose which are made up of two glucose units. Finally, the β-glucosidase acts on the cellobiose and release two glucose monomers (Singhania *et al.,* 2017).

β-glucosidases (BGL) represented by E.C. 3.2.1.21, are enzymes that break down β-glycosidic bonds between two or more sugar molecules, or between a carbohydrate and a non-carbohydrate moiety. Most of the characterized β-glucosidases are retaining catalysts (for example, enzymes from glycosyl hydrolases family GH30, GH3, and GH1), and they perform catalysis in two-step process. Among all the mentioned families of β-glucosidases the key and conserved active site residue is glutamate. The entire catalytic reaction of β-glucosidase is accomplished in two glutamate residues,

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one of which is a universal acid/base mediator and the other one functions as a nucleophile. The initial step, known as glycosylation, involves the nucleophile glutamate attacking the anomeric carbon, forming a glucose-enzyme intermediate product. In the second deglycosylation step, a water molecule serves as a nucleophile and destroys the glycosidic link to release glucose, which is triggered by a catalyst that is an acid/alkaline glutamate residue (Singh *et al.,* 2016).

Enzymes are produced by all living organisms, ranging from simple microbes to higher and complex organisms i.e., plants and animals. Microbial enzymes have numerous advantages over plants and animal enzymes. The microbial enzymes are more active, stable, easy to modify, optimize and have high yield (Gurung *et al.,* 2013; Adrio *et al.,* 2014).

Owing to the current situation of rising daily energy demand and increasing burden on fossil fuels has increased the demand for mass production of biofuels to replace fossil fuels. Pakistan is an agricultural country and produces large amount of waste that is not properly utilized, that could be an important source of energy (Saeed *et al.,* 2015). Lignocellulosic biomass such as sugarcane bagasse, rice straw and wheat grass are hydrolyzed by the cellulase enzyme and used for biofuel production. The process of cellulosic biofuel production involves the degradation of lignocellulosic biomass into sugar, followed by biofuel production through the fermentation process. However, due to insufficient production of BGL, it creates hurdles in the achievement of maximum biofuel production. Due to the high demand of β-glucosidase in the biofuel industry, its higher and sustainable production is needed to meet the current needs of the industry (Srivastava *et al.,* 2019). β-glucosidase can modify natural products and enhance their medicinal properties, including anticancer, antiviral and antioxidant activity. Many plants natural products contain sugar molecule(s) as part of their structure (Franco *et al.,* 2020), and their physiological activity, selectivity and pharmacological properties are often dependent on the sugar component. Thus, to improve their bioavailability and in turn enhance the antioxidant and anti-cancerous potential of these products, their deglycosylation plays an important role. The use of β-glucosidase for deglycosylation is of particular interest due to their high specificity, non-toxicity, robust reactions with limited by-products and no environmental harm (Chen and Chen, 2011).

The current study reports screening for efficient β-glucosidase producing bacterial strain from soil sample of Jacobabad, Pakistan. The isolated strain was identified as *Bacillus stercoris* based on 16S rRNA sequencing. Different agricultural by-products (wheat bran, orange peel, and corn bran) were employed as cheap, raw substrate for the production of β-glucosidase. Optimized selective combination (wheat bran and orange peel) of raw substrates for the enhanced production of β-glucosidase was used for the first time in this study. Experiments were designed to evaluate the effect of raw carbon source on the production of β-glucosidase from indigenously isolated *Bacillus stercoris* strain. The enzyme was purified and characterized. The enzyme could be a potential candidate for biofuel production and deglycosylation of natural products for enhancement of its medicinal value.

MATERIALS AND METHODS

***Sample processing*:** Soil sample in sterile condition was collected from Jacobabad in Sindh, Pakistan. (Temperature: 40°C and pH 8). Serial dilution of the sample was performed by following the standard serial dilution plate technique method with some modification. Several bacterial colonies were obtained which were further screened for β-glucosidase production (Gislin *et al*., 2018; Yin *et al.,* 2010).

***Screening for β-glucosidase producing bacterial strain*:** The isolated pure colonies were processed for β-glucosidase production on VRE (Vancomycin resistant enterococci) medium containing Yeast extract, Sodium chloride, Tryptic soya broth, Esculin, Ferric citrate, Citrate, Sodium azide, Agar at pH 7.0 ± 0.2 (Grabsch *et al*., 2008). The β-glucosidase positive strains were confirmed from the zone of hydrolysis. Based on the screening result *Bacillus stercoris* was selected for further study. Activity of the strain was also confirmed by pouring the supernatant containing crude enzyme extract into the wells on VRE medium plates. The strain was preserved from seed culture and stored in 20% glycerol broth at -20°C.

***Identification of selected β-glucosidase producing bacterial isolates*:** β-glucosidase producing bacterial isolate was identified initially by morphological differences through microscopy (Gram staining), and then by 16S rRNA gene sequencing (Islam *et al*., 2018). DNA was extracted using PureLink™ Genomic DNA mini kit and 16S rRNA (rRNA) gene sequencing was performed by Macrogen (Netherlands).

***Optimization of media components and culture conditions for β-glucosidase production*:** Various physical parameters such as incubation time, temperature, pH, and some media components were optimized with the help of conventional approach, based on one factor at-a-time. Each factor was optimized by transferring 5% of fresh bacterial culture into the production media which was optimized in the current study. In case of temperature the production media was incubated at different temperatures such as 35°C, 40°C, 45°C, 50°C and 55°C at 150 rpm in shaking incubators. Supernatant was collected from the culture through centrifugation and β-glucosidase assay was performed according to the method by (Daroit *et al*., 2007). For optimization of pH, the production media (with different pH ranges i.e., pH 5 to 10) was incubated at optimized temperature after inoculating with fresh bacterial inoculum. Supernatant was assayed for β-glucosidase activity at optimized conditions. The production media was provided with different incubation times to optimize the period in which maximum β-glucosidase

production occurred. Supernatant was assayed for β-glucosidase activity after 24,48 and 72hours of incubation. Different carbon sources such as CMC, corn bran, wheat bran, orange peel and combination of wheat bran and orange peel were used in production media for maximum enzyme production (Ahmed *et al*., 2017). Different nitrogen sources such as tryptic soya broth, beef extract, urea and peptone were added to production media (2g/ml). Supernatant was collected from the culture through centrifugation and β-glucosidase assay was performed which was optimized in the current study.

***Enzyme activity and estimation of protein*:** β-glucosidase activity was detected using *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) as the substrate. The assay was performed as optimized during the current study. The quantity of enzymes required for the release of one µmole of *p*-nitrophenol from *p*-nitrophenyl β-glucopyranoside per minute at optimized conditions is known as one unit of β-glucosidase. The β-glucosidase specific activity (U/mg) was determined as enzyme activity (U/ml)/ protein concentration (mg/ml). For total protein calculation, Lowrey's method was used (Dotsenko *et al*., 2015). Bovine serum albumin (BSA) was used as standard.

***β-glucosidase purification*:** For the partial purification of β-glucosidase different percentages of ammonium sulfates ranging from 20% to 80% were periodically added to the crude enzymes to obtain optimized saturation level of ammonium sulfate. This process was accomplished at low temperature with constantly stirring by magnetic stirrer. For each saturation level, enzyme assay and protein estimation were carried out at optimized conditions. The unwanted ammonium sulfate was removed through dialysis and the samples obtained were then stored for further purification. The dialyzed samples (proteins, after the removal of ammonium sulfate) was passed gently through column packed with Sephadex G-100 linked with Digital Peristaltic Pump Model 3200 with a low speed**.** The column was washed with 50 mM phosphate buffer of pH 8.0. For each fraction, enzyme assay and protein estimation were carried out. Sample of 3 ml was collected from the column after some time, different collected fractions were analyzed for protein concentration and its activity. Those fractions having high activity were pooled together and processed for further analysis (Michlmayr *et al.*, 2010). SDS-PAGE was employed to determine the purity and molecular weight of the purified enzyme (Gurung *et al.*, 2013).

***Biophysical and biochemical characterization of β-glucosidase*:** Purified β-glucosidase was characterized at various temperatures i.e., (35, 40, 45, 50, 55 and 60°C). The effect of different substances including metal ions at a concentration of (5 mM and 10 mM), detergents and organic solvents at 15 % were investigated for purified β-glucosidase activity (Liew *et al*., 2018).

RESULT

***Isolation and screening of isolates*:** After serial dilution of soil sample, many bacterial strains were isolated with different colony morphology i.e., (*1j1, 1j2, 1j3, 1j4, 1j5, 1j6*) and was screened for β-glucosidase production. Among all the tested strains the best β-glucosidase producer strain was *1j2 (Bacillus stercoris)*.

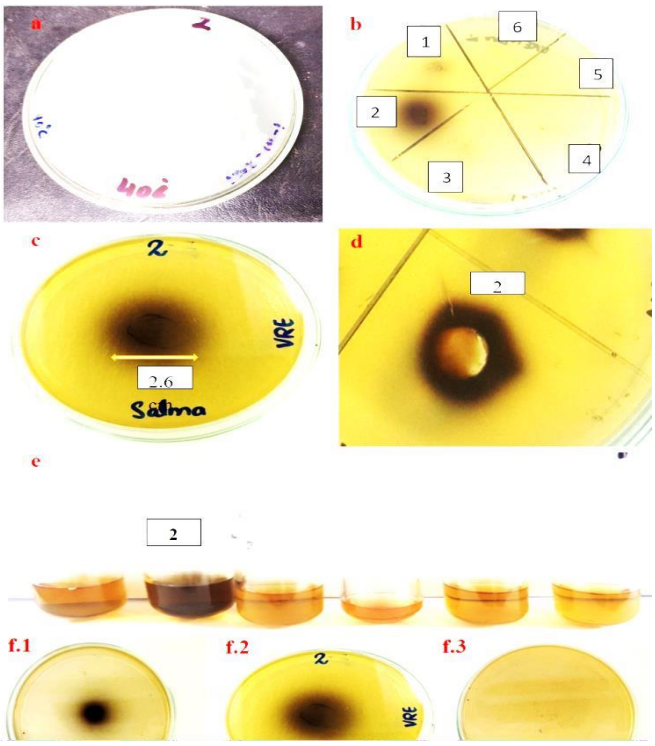

Figure 1. (a) Purified *Bacillus stercoris* colony morphology on nutrient agar plate. (b) Point inoculation of the isolated bacterial isolates on VRE (vancomycin resistant enterococci) agar plates for primary screening of efficient β-glucosidase producer. (c) Zone of hydrolysis (2.6cm) formed by *Bacillus stercoris* β-glucosidase on specific VRE media.(d) Secondary screening of supernatant from *Bacillus stercoris* 24 hours old production medium for β-glucosidase activity in VRE agar wells assay.(e) Confirmation of β-glucosidase producing strains in VRE broth. Among all the strains only the test tube 2 strain which is *Bacillus stercoris* has given the activity in VRE broth that has darken. (f.1, f.2, f.3) shows point inoculation of the strain on VRE media and incubation at different temperatures such as 37°C, 40°C, 45°C. The strain showed activity at 37°C and 40°C but zero activity appeared at 45°C. Between 37°C and 40°C the strain has given maximum activity at 40°C.

Pure colony morphology of *Bacillus stercoris* on nutrient agar plate is shown in Fig.1(a). In primary screening black zones were observed on VRE (vancomycin resistant enterococci) plates by the strains positive for β-glucosidase production as appear in Fig.1(b). The zone of hydrolysis was measured (2.6 cm) as shown in Fig.1(c) and it was observed that *Bacillus stercoris* was the best producers for β-glucosidase among all the test isolates. Secondary screening for the β-glucosidase was confirmed when the 24 hours old production medium of *Bacillus stercoris* was centrifuged and supernatant was incubated in VRE agar well plate and it gave the black zone around the well as shown in Fig.1(d). The β-glucosidase producing ability of the strain was further confirmed by the appearance of black color that resulted from the complex formation between the released esculetin and iron present in VRE broth as shown in Fig. 1(e) in test tube assay. Growth temperature for maximum production of enzyme was confirmed when the strain was inoculated on VRE plates at different temperatures (37°C, 40°C and 45°C). Smaller zone was appeared at 37°C and maximum zone of β-glucosidase was observed at 40°C while there was no zone observed at 45°C Fig.1(f.1) (f.2) (f.3).

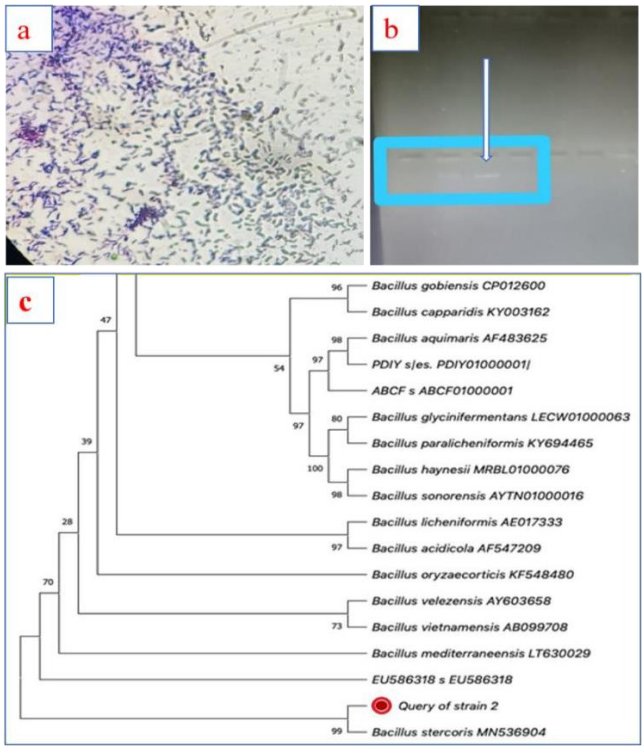

Figure 2. ***(a)*** **Results of Gram staining shows that the Bacillus *stercoris is* Gram + rods (b) Genomic DNA of the isolate *Bacillus stercoris* under UV-illuminator. (c) The Phylogenetic tree constructed by maximum Likelihood method, representing the test strain *Bacillus stercoris labelled as Query of strain 2*.**

Identification of β-glucosidase producing strains*:** According to the morphological tests, the selected isolate *Bacillus stercoris* was found to be Gram positive rods as shown in Fig. 2 (a). The isolated chromosomal DNA was confirmed on the gel as shown in Fig.2 (b). The isolate was identified as *Bacillus stercoris* based on PCR amplification of 16S rRNA gene and sequence analysis. Phylogenetic tree was constructed with the help of MEGA-6 software. Strain has shown homology of 99% with *Bacillus stercoris* Fig.2. (c). ***Optimization of Culture Conditions for β-Glucosidase Production: Different carbon sources such as (1) CMC, (2) corn bran, (3) wheat bran, (4) orange peel and (5) combination of wheat bran and orange peel were used in production media for maximum enzyme production. Among the carbon sources, source no (5) wheat bran and orange peel in combination resulted in maximum production of β-glucosidase i.e., 1.80 U/mg Fig.3(a). Different nitrogen sources (1) Tryptic soya broth, (2) Beef extract, (3) Peptone and (4) Urea when added to the *Bacillus stercoris* containing medium showed maximum specific activity i.e., 2.89 U/mg when tryptic soya broth was used as nitrogen source Fig.3(b).

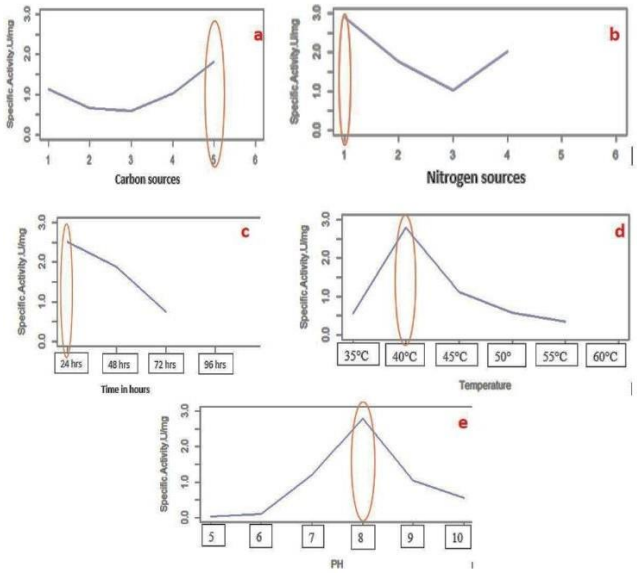

Figure 3. (a) It shows effect of various carbon sources on β-glucosidase production i.e as (1) CMC, (2) corn bran, (3) wheat bran, (4) orange peel (5) combination of wheat bran and orange peel. (b) Different nitrogen sources tested in culture medium for β-glucosidase production were (1) Tryptic soya broth, (2) Beef extract, (3) Peptone and (4) Urea). (c) Shows effect of incubation period in hours (24 to 96 hours) on β-glucosidase production. (d) Shows effect of temperature (35°C to 55°C) on β-glucosidase production. (e), Shows effect of pH (5 to 10) on β-glucosidase production in production medium.

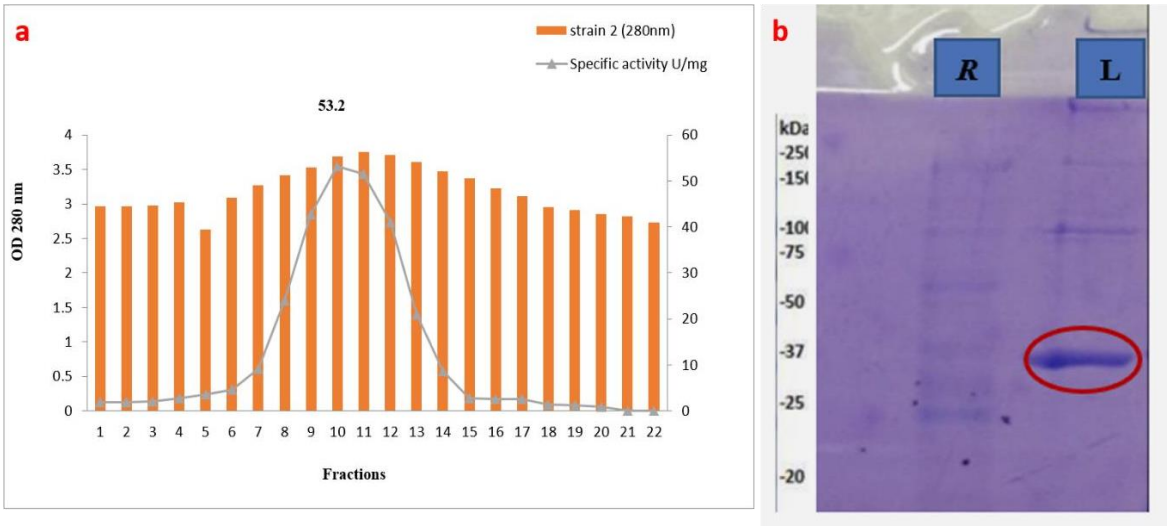

Figure 4. (a) Shows fractions collected from the column, maximum activity was obtained in fraction no.10 (b) Shows SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). Right (R): protein Ladder, left (L): Purified β-glucosidase enzyme from the *Bacillus stercoris* isolated from Jacobabad Pakistan.

Bacillus stercoris was optimized for β-glucosidase production at different incubation times (24, 48 and 72 hours) and maximum β-glucosidase production was observed after 24 hours of incubation. After 48 and 72 hours a decreasing trend in enzyme production was observed Fig. 3(c). *Bacillus stercoris* was optimized for β-glucosidase production at different temperatures i.e., (35°C, 40°C, 45°C, 50°C, 55°C). The strain produced maximum enzyme at 40°C i.e., 2.79 U/mg Fig.3(d). pH is one of the important factors for proper functioning of enzymes so, different substrate solutions were prepared in different buffers of pH (5 to 10) and β-glucosidase assay was carried out. It was observed that maximum β-glucosidase was produced at neutral and alkaline pH. Maximum specific activity was observed at pH 8 (2.44 U/mg) Fig.3(e).

***Purification and Molecular mass of β-glucosidase*:** Optimum partial purification of β-glucosidase was achieved at 80% ammonium sulfate precipitation. Then the partially purified enzyme from *Bacillus stercoris* was loaded on column packed with Sephadex G-100 for further purification. After passing through the column pure enzymes were obtained in fraction no 9,10,11 and 12. Maximum specific activity of purified enzyme was (53.2 U/mg) which was recorded in fraction no 10 as shown in Fig 4. (a). The purity of the enzyme was further analyzed by SDS-PAGE, and it displayed a single band with molecular size of 36 kDa Fig.4 (b).

***Biophysical and Biochemical Characterization of purified β-glucosidase*:** Purified β-glucosidase assay was performed at different temperatures i.e., (35°C to 60°C). Maximum activities were observed at 40°C i.e., (53.2 U/mg) followed by 35°C (Fig 5).

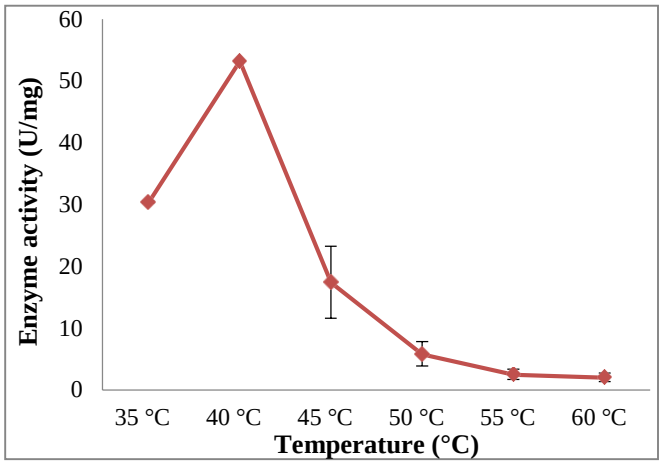

Figure 5. Effect of temperature (35°C to 60°C) on the activity of pure enzyme from *Bacillus stercoris*.

The effect of various metals ions was checked on the activity of purified β-glucosidase. The metals with different concentrations i.e., (5 mM and 10 mM) were mixed with the purified enzyme and enzyme assay was conducted. The activity was not significantly influenced by metals. Although, at higher concentration i.e 10 mM, $CuSO_4$, $HgSO_4$ and $CoCl_2$ had inhibitory effect on β-glucosidase activity as shown in Table 1. The effect of surfactants was observed by treating pure enzyme with different surfactants (Triton 100X, Tween 20, CTAB and SDS) and then enzyme assay was performed. The enzymes showed a significant decrease in activity when treated with 15% surfactants, while 15% Tween-20 has drastically inhibited the *Bacillus stercoris* β-glucosidase activity (Table 1). Enzyme was also evaluated for effect of

different organic solvents, such as Hexane, ethyl acetate, aceto-nitrile, ethanol, and methanol. The enzyme was incubated with 15% of organic solvents and then enzyme assay was performed at 45°C. All the tested organic solvents have decreased the activity of the enzyme but the ethyl acetate and aceto-nitrile have decreased the activity up to 86% and 88% when used in 15% concentration.

Table 1. Effect of various metal ions and other reagents on β-glucosidase activity. Activity was determined by incubating pure enzymes (β-glucosidase) with (5mM and 10mM) metal ions and 15% solution of detergents and organic solvents. The results are expressed as the relative activity compared with the initial activity (control) of 100%.

Metal Ions	**Relative activity (%)**	
Metal ions concentration	5mM	10mM
Control	100.00	100.00
$CoCl_2$	43.60	28.39
$CuCl_2$	43.64	43.01
$HgSO_4$	58.47	35.70
$CuSO_4$	23.87	23.52
$CaCl_2$	101.48	100.00
Other Reagents	**Relative activity (%)**	
Control	100.00	
T-X	32.69	
SDS	10.26	
CTAB	15.87	
T-20	4.09	
Hexane	68.36	
Methanol	63.42	
Ethanol	61.36	
Ethyl acetate	13.98	
Acetonitrile	12.01	

DISCUSSION

Many bacterial strains were isolated and were screened for β-glucosidase production from the soil samples collected from Jacobabad, Pakistan. Among all the tested strains the best producer for β-glucosidase was *Bacillus stercoris*. Several studies, including those by An *et al*. (2005) and Kim *et al*. (2006), have also described the isolation of β-glucosidase producing bacteria from soil.

The medium used for primary screening of β-glucosidase was vancomycin resistant enterococci (VRE), which consisted of esculin as a source of carbon. *Bacillus stercoris* has produced black zones on VRE medium due to the interaction of Fe^{3+} with the liberated esculetin from the β-glucosidase hydrolysis of esculin into esculetin and glucose. The VRE medium has also been used in the studies of Grabsch *et al.* (2008) and Zhang *et al.* (2017) for β-glucosidase screening.

According to morphological tests the *Bacillus stercoris* appeared as Gram-positive bacterial rods. However, to prove its identity 16S rRNA gene sequence analysis was performed and it has revealed that the strain *1j2* belonged to the genus *Bacillus* and showed 99% homology with *Bacillus stercoris.* β-glucosidase producing bacterial strains have been isolated from soil and identified in the previous studies on the basis of 16S rRNA gene sequence analysis (Park *et al.,* 2008).

Optimization of cultural conditions was carried out for *Bacillus stercoris* to produce maximum β-glucosidase. Conventional method for optimization was followed, several factors were optimized such as pH, temperature, incubation period, carbon sources and nitrogen sources.

A number of agro-industrial by-products have been examined as a carbon source for β-glucosidase production (Daroit *et al.*, 2007). In the present study many agro-industrial by-products were evaluated as a carbon source. Maximum β-glucosidase production with a specific activity of 1.80 U/mg was achieved when combination of wheat bran and orange peel was used as a carbon source in the culture medium. The carbon sources used in combination gave more promising results beacasue of numerous nutrients availability for microbes (Teixeira *et al.*, 2016). Combinations of carbon sources have been significant for maximum β-glucosidase production as reported in the study of (Gao *et al.*, 2012). They have used combination of carbon sources and got maximum β-glucosidase production from *Fusarium proliferatum* in the medium. Cotton seed meal, soybean meal and wheat bran when used as substrate for *Aspergillus niger,* have also been reported to produce maximum β-glucosidase (Ohara *et al.,* 2018).

In the current study various nitrogen sources have been tested in production media but maximum β-glucosidase was produced by *Bacillus stercoris* with a maximum activity of 2.89 U/mg when tryptic soya broth was used as a primary nitrogen source. Tryptone is the main ingredient of tryptic soya broth which provides amino acids for the growth of bacteria. Study of Ramachandran *et al.* (2013) and Park *et al.* (2013) shows similarity with the results of current study as they have got maximum β-glucosidase production with a specific activity of (10.4 U/ml) when supplemented the culture medium with tryptone.

Temperature has direct effect on the stability of proteins and metabolism of microorganisms (Somero, 2004). In the present study maximum activity was obtained at a temperature of 40 ^{o}C i.e 2.79 U/mg. The increase in temperature have impact on the substrate structure, it loses its rigidity that are then easily accessed by the enzymes. However, at very high temperatures denaturation of enzymes can occur. Similar results have also been observed for bacterial strain isolated from Eygptian soil environment, that had an optimum temperature of 40^{o}C for maximum β-glucosidase production (El-Sayed *et al.,* 2019). pH of culture conditions ultimately have effect on the active site of the enzyme. It usually changes the shape of an enzyme's active site. In the present study maximum specific activity was obtained at pH:8 i.e (2.44 U/mg) by *Bacillus stercoris*. Our results shows resemblance with a study in

which a *Bacillus sp*, isolated from Eygptian soil produced maximum β-glucosidase at pH 8 while its activity was deceased further at pH 10 (El-Sayed *et al.*, 2019). pH 8 was considered as optimum pH for β-glucosidase producing cellulolytic bacterial strain isolated from dairy effluent and from effluent of biogas production plant (Neesa *et al.*, 2020). Enzyme production was maximum from *Bacillus stercoris* isolate after 24 hours of incubation, with a maximum activity of 2.51 U/mg.These results are in accordance with the study performed by Neesa *et al.* (2020) where the higher production of β-glucosidase was achieved after 24 hours of incubation. In the present study partial purification of crude enzymes was achieved at 80% ammonium sulfate precipitation. The results shows resemblance with the β-glucosidase that were precipitated by using 85% ammonium sulfate in the study carried out by (Dzurendova *et al*., 2021). Proteins were then subjected to column chromatography packed with sephadex G-100 for further purification. Chang *et al.* (2012) used a sephadex column packed with G-100 resin to purify β-glucosidase as well. The purification fold for β-glucosidase was found to be 18.10 and size of purified enzymes was estimated to be 36 kDa on SDS-PAGE. Gao *et al.* (2012) has purifed β-glucosidase with nearly same purification fold and Kuo *et al.* (2018) has estimated molecular mass of β-glucosidase to be 30 kDa from *bruxellensis*.

Purified β-glucosidase from *Bacillus stercoris* was characterized using different parameters such as temperature, metal ions, organic solvents and surfactants. Purified enzyme showed maximum activity at 40ºC (53.2 U/mg) followed by 35ºC where 50% of maximum activity was retained. These results are in accordance with the study conducted by Li *et al.* (2012) where β-glucosidase from soil metagenomics library has also shown maximum activity at 40ºC. Metal ions interfere with the activity of β-glucosidase in different manners. The activity of purified β-glucosidase in our study was not significantly affected by metals but with the use of (5 mM) $CuSO_4$ slight reduction was noted while at higher concentration i.e. 10 mM, $CuSO_4$, $HgSO_4$ and $COCl_2$ have inhibitory effect on β-glucosidase activity. Cu^{2+} has also inhibited β-glucosidase activity in the study of (Bonfá *et al.*, 2018). Heavy metals e.g. Cu^{2+} have strong attraction for thiol groups and are responsible for oxidation of cysteine residue functional groups, which may lead to inhibitory effect on certain enzymes (Hayashi *et al.*, 1999; Saroj *et al*., 2022). Different surfactants interact with hydrolytic enzymes and make certain conformational changes which leads to the loss of activity in such enzymes (Holmberg, 2018). In present study purified β-glucosidase activity was significantly reduced when incubated with 15% surfactants while among surfactants 15% tween-20 has drastically inhibited the β-glucosidase activity. The enzyme used in the study has demonstrated an inhibitory effect towards all tested organic solvents which is similar to another study where enzymatic activity was also hindered by every tested organic solvent against the hydrolysis of genistein and daidzein (Sun *et al*., 2010).

***Conclusion*:** The present study concluded that *Bacillus stercoris* is one of the efficient β-glucosidase producing strain among all the strains isolated from Jacobabad. Optimum β-glucosidase was produced by *Bacillus stercoris* in the culture medium when agricultural wastes (orange peel and wheat bran) in combination was used as carbon source and tryptic soya broth as nitrogen source. Best culture conditions of the medium for the optimum β-glucosidase production were 24 hours of incubation, pH 8, and 40°C. β-glucosidase was partially purified by 80% ammonium sulphate precipitation and full purification was attained through column chromatography packed with Sephadex-G 100. Enzyme was purified up to 18.10-fold and its molecular size was identified as 36 kDa on SDS-PAGE analysis. The purified enzyme was also characterized, and it was observed that purified β-glucosidase was stable at 40°C. Cu^{2+} was observed to have inhibitory effect while Ca^{2+} had not affected the enzyme activity significantly. Surfactants and organic solvents seem to significantly reduce the activity of β-glucosidase produced by *Bacillus stercoris*. The results demonstrate the economic viability and scaling up the production of β-glucosidase using agricultural wastes as a carbon source for the variety of industrial uses, including lignocellulosic hydrolysis to produce biofuels.

***Author contributions*:** Ubaid Ur Rahman and *Salma Qasim contributed equally to this work. Conceptualization: Samiullah Khan and Malik Badshah. Validation: Numan Saleh Zada; formal analysis: Salma Qasim, Numan Saleh Zada and Ubaid Ur Rahman; validation: Sohail Ahmad; investigation: Salma Qasim and Ubaid Ur Rahman; resources: Samiullah Khan and Malik Badshah; data curation: Salma Qasim and Samiullah Khan; writing original draft preparation: Ubaid Ur Rahman, Aamer Ali Shah and Samiullah Khan; writing review and editing: Samiullah Khan and Fariha Hasan; supervision: Samiullah Khan and Malik Badshah; Project administration: Samiullah Khan and Malik Badshah. All authors have read and agreed to the published version of the manuscript.

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