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Comparative assessment of acid and enzyme pretreatment of *Spirodella polyrhiza* for bioethanol production

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Abstract

Duckweeds are considered more sustainable compared to conventional crops due to no feed versus fuel competition. However, the feedstocks used for bioethanol production require an efficient pretreatment to increase the sugar yield resulting in reduced cost of the process. To combat low sugar yield challenges, five isolates named AG 1, AG 2, AG 3, AG 4 and AG 5 were isolated from soil. The bacterial isolate AG 3 (*Bacillus* AS1 MK321577) showed the highest amylase activity with the DNS method. The *Bacillus* AS1 MK321577 strain was evaluated for saccharification of *Spirodella polyrhiza* followed by bioethanol production via *Saccharomyces cerevisiae*. The specific activity of amylase was found to be 0.396 U/mg/min after 24 h incubation. Maximum amylase activity was observed in boiled potato peels as carbon sources in the production media at pH 6 and 45 ^{o}C. Placket-Burman's design for optimization of physical and nutritional parameters was used. The parameters selected for optimization were KH_2PO_4, pH, inocula, $CaCl_2$, $MgCl_2$, temperature, starch, $MgSO_4.7H_2O$, reaction time, NaCl and $NaNO_3$. The current study reports that *Bacillus* AS1 MK321577 strain has the highest amylase activity. Acid pretreatment of *S. polyrhiza* was also carried out and compared with the amylase pretreatment. Overall, the highest reducing sugars yield (0.432 mg/ml) and highest bioethanol production (99%) were observed in the amylase pretreated sample. In comparison to acidic pretreatment, enzymatic pretreatment is a more environmentally friendly process and resulted in a 15% enhanced yield of reducing sugars.

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Introduction

Global energy demands are increasing over time and the amount of fossil fuels left in the world is limited. The decreased supply and increased consumption of fossil fuels leads to a rise in their prices. It is well known that the use of petroleum-derived fuels creates a large amount of greenhouse gases (GHG) causing global warming. Environmental problems associated with the use of fossil fuels as well as their expected scarcity in near future require a search for new alternative renewable fuels (Kaur *et al.*, 2018). In order to combat the problem, biofuels are considered the ideal alternatives and especially bioethanol has emerged as the frontrunner renewable fuel. Bioethanol is biodegradable, less toxic and can be blended with gasoline (Danquah and Harun, 2011). The key environmental benefit of bioethanol is that unlike petroleum its consumption does not significantly raise the level of carbon dioxide (CO_2) (Nahar *et al.*, 2019). Bioethanol production is a carbon-neutral process (Wei *et al.*, 2019).

Currently, the bioethanol industry has two major shortcomings i.e., the use of edible feedstock and its chemical pretreatment. The use of edible feedstock for bioethanol production causes an imbalance in the food supply. Developing countries like Pakistan cannot afford bioethanol production from edible sources as the food crisis is increasing with global population growth (Nahar *et al.*, 2019). Alternatively, non-edible feedstocks are considered ideal for bioethanol production. Among non-edibles, duckweed a free-floating aquatic plant is considered an ideal candidate for bioethanol production due to its high starch content and efficient growth rate. Its starch content varies from 3% to 75% depending upon species and geographical conditions.

The protein content varies from 15% to 45% on dry weight bases (Li *et al.*, 2016). During nutrient starvation, the protein content of duckweeds decreases due to a rise in the proportion of starch (Yu *et al.*, 2017). The duckweed species *Spirodella polyrhiza* has low lignin content. Due to the low lignin content in duckweeds, the conversion of starch into bioethanol is relatively easy and cost-effective compared to other energy plants (Liu *et al.*, 2018).

The second major bottleneck associated with bioethanol production is its chemical pretreatment using acid or a base as a catalyst. Enzymes are preferred catalysts for pretreatment especially, amylases which play an important role in producing fermentable sugars which are utilized by yeast strains to produce bioethanol. About 25% to 33% of the world enzyme market is constituted by amylases (Rehman, 2019). Amylases are extracellular enzymes that catalyze the hydrolysis of polysaccharides such as starch (Kannan and Anagaraj, 2019). The major target sites are α – 1, 4 glycosidic linkages in the starch substrate (Pranay *et al.*, 2019). Recently, amylase enzyme hydrolysis has replaced chemical utilization in starch saccharification (Neha *et al.*, 2018). Amylase production can be improved by optimizing various physical and nutritional growth parameters such as incubation period, temperature, pH, inocula size, carbon, and nitrogen sources. Agro-industrial remains are usually considered as the best substrate for effective cost-cutting in the production of amylases. These agriculture wastelands comprise of C- and N-sources required for the growing and breakdown of microorganisms (Djekrif-Dakhmouche *et al.*, 2006; Haq *et al.*, 2005). In the present study, the amylase-producing strain was isolated from Attock fort and was used for the production of amylases that were later on used for pretreatment of *S. polyrhiza* feedstock. The starch content of *S. polyrhiza* was pretreated by amylases of *Bacillus* AS1 MK321577 strain and acidic pretreatment to release reducing sugars. The released glucose was fermented to bioethanol by yeast strain QG1. To the best of our knowledge, no prior study has been conducted on the comparison of bioethanol yield from enzyme and acid pretreated *S. polyrhiza* hydrolysate.

Material and methods

Isolation of amylase producing strains

The soil sample was collected from District Attock, Punjab. It was serially diluted using distilled water. The various dilutions were inoculated on nutrient

agar medium (NaCl, 0.2 g/L, $MgSO_4.7H_2O$, 0.1g/L, K_2HPO_4, 0.17g/L KH_2PO_4, 0.12g/L, starch, 0.2%, bacteriological peptone, 0.5 %, and agar, 1.3 %) and incubated at 37 ºC for 24 h. Five strains were selected from the isolates and named AG 1, AG 2, AG 3, AG 4 and AG 5. They were preserved at -20 ºC for further studies.

Qualitative screening of amylase enzyme activity

Primary screening of amylase producing bacterial strains: A 24 h fresh culture of the selected isolates was point inoculated on starch nutrient agar. The starch hydrolysis was determined by an iodine test. Iodine solution was composed of 2.5 g potassium iodide, 250 mg iodine dissolved in 150 ml of distilled water. The plates were flooded with iodine solution and observed for the zone of starch hydrolysis in mm at regular intervals after 24, 48 and 72 h incubation.

Secondary screening of amylase-producing bacterial strains: A freshly prepared nutrient broth was separately inoculated with each isolate and inoculated at 37 ºC for 48 h. afterward, the broth was centrifuged at 10,000 rpm for 15 min. The cell-free supernatant was poured into respective wells of 5 mm diameter punched by sterile cork borer into the starch agar medium. The plates were then incubated at 37 ºC for 24 h. After incubation, the plates were flooded with iodine solution and the zone of starch hydrolysis was observed. The best amylase-producing strains were selected based on the highest zone of hydrolysis.

Quantitative determination of amylase enzyme activity

The 24 h freshly prepared selected amylase producing strains were inoculated in nutrient broth and incubated at 37 ºC in a shaking incubator at 250 rpm for 24 h. A 50 ml sterilized production media was prepared. The composition of production media was 0.2% starch and 0.5% bacteriological peptone added to 0.1 % minimal salt solution.

The production media was inoculated with a selected strain from the nutrient broth. The samples were collected from the post incubated cultures at 24, 48 and 72 h intervals and centrifuged at 10,000 rpm for 20 min. The supernatant was used for enzyme assay and protein estimation. The pellet was discarded.

Amylase activity was determined according to the Bernfeld method (Ranjan *et al.,* 2020). Amylase produced was quantified by the amount of reducing sugar produced by the activity of the enzyme on the substrate (starch). For determining the enzyme activity, the reaction mixture was prepared by adding 100 µl of crude enzyme solution to 100 µl of soluble starch (as substrate) in 20 mM of sodium phosphate buffer and the reaction mixture was incubated at 45 ºC for 15 min. 100 µl of 3, 5-di nitro salicylic (DNS) reagent was added to stop the reaction, followed by boiling for 15 min. It was cooled by adding 900 µl of distilled water at room temperature and absorbance was recorded at 540 nm using a spectrophotometer (8453 UV– Visible spectrophotometer). Enzyme units (IU/ml/min) were calculated by using a standard curve for glucose.

Identification of the selected bacterial strains

The selected amylase-producing isolates were biochemically identified using Bergey's Manual of Determinative bacteriology (Abootalebi *et al.,* 2020).

The bacterial strain AG-3 was selected for 16S rRNA sequencing. Genomic DNA of the selected amylase-producing bacterial strain was extracted using the cetyl trimethylammonium bromide (CTAB) method for molecular identification, as described previously (Wilson, 2001).

The quality of bands was observed by running the extracted DNA of the selected isolates on 1% agarose gel for 30 min. The isolated DNA samples were sequenced by Macrogen Standard Custom DNA Sequencing Services (Macrogen Inc., Seoul, Korea) for 16S rRNA.

The phylogenetic evolutionary correlation of the obtained sequence was carried out using the Neighbor-joining method in MEGA X as described previously (Saitou and Nei, 1987).

Plackett-Burman Design for Optimization of Amylase Production

Plackett-Burman is very useful where complete knowledge of the system is unavailable or optimization of various factors is required. This design was, therefore, used in this study to optimize the parameters, using Stat-Ease Design-Expert Software version 7.0, for the selected amylase-producing strain. Plackett- Burman is the most popular design used for screening and optimization of enzymes (Giordano *et al.,* 2011). It is considered an economical screening method for estimating main factors using two-level interactions. Plackett-Burman's design gives information about the effect of a single factor on response, i.e., specific enzyme activity. For optimization purposes, factors, i.e., temperature, pH, raw substrate, incubation time, and production media contents, were selected with 3 central points.

Duckweed strains and culture conditions

Duckweeds were collected from a pond in the vicinity of Islamabad. They were brought to the Department of Biochemistry Quaid e Azam University Islamabad and identified as *Spirodella polyrhiza*. *S. polyrhiza* was grown in Hoagland medium (micronutrient solution 0.5 ml, Fe. EDTA Solution 20.0 ml, KNO_3 2.5 ml, $Ca(NO_3)_2 \cdot 4H_2O$ 2.3 ml, $MgSO_4 \cdot 7H_2O$ 1.0 ml, KH_2PO_4 0.5 ml) for two days and its starch content was analyzed by using Lugol solution (add 0.25% w/v iodine and 0.5 % w/v potassium iodide in water). It has a high sensitivity for the determination of starch content in plant material. The reducing sugars were estimated by the DNS method. Glucose was fermented to bioethanol using QG1 yeast strain. The quantification of bioethanol was carried out using an ethanol assay kit.

Determination of starch content in Spirodella polyrhiza

The starch content in *S. polyrhiza* was determined using the Appenorth method (ul ain Rana *et al.*, 2019). The fresh plant material (200 mg) was homogenized completely in 4 mL of 18 % (w/v) of HCl. The homogenized suspension was incubated in a shaking incubator at 5 °C for 60 min and centrifuged at 5000 rpm for 20 min. An aliquot of the suspension was mixed with the same volume of Lugol solution (0.25 % w/v iodine added to 0.5% w/v potassium iodide in distilled water). The addition of Lugol solution results in the appearance of blue color which indicates the presence of starch in the reaction mixture. The absorbance was measured at 530 nm and 605 nm. The following formula was used to calculate the amount of starch per fresh weight (% w/w):

$$S = \frac{[Cs \text{ x Vol (extr)} \times 100]}{FW}$$

Where,

Vol (extr) = volume of plant extract after homogenization in mL

FW = Fresh weight in mg

$$Cs = \frac{A_{605}}{(0.07757 \times P + 4.463)}$$

$$P = \left[(7.295 \text{ x } A_{605}/A_{530} - 4.463)/\left(7.757 - 0.729 \times \frac{A_{605}}{A_{530}}\right)\right] \times 100$$

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Enzymatic pretreatment of S. polyrhiza biomass

The duckweed biomass was dried at 105 °C for 2 days in a desiccator. The dried sample was milled. The saccharification of the starch was carried out by mixing 50 mg dried powder of biomass and 480 µL amylase of *Bacillus* AS1 MK321577 and incubated at 45 °C with shaking at 250 rpm for five hours. The pretreatment was carried out in triplicate. The amount of reducing sugars was measured in mg/mL using the DNS method.

Acid pretreatment of S. polyrhiza biomass

About 1 g of dried and ground biomass was added in 100 mL distilled water and 1% sulphuric acid in a 250 mL flask. The solution was stirred for 30 minutes using a magnetic stirrer, followed by autoclaving at 121 °C for 1 h and then centrifuged at 1000 rpm for 10 min. The solution was centrifuged and hydrolysate was separated and pH was adjusted in the range of 5-5.5 for the fermentation process.

Bioethanol fermentation

The fermentation of the pretreated *S. polyrhiza* biomass was carried out in 5 mL reactors containing 75% hydrolysate, 10% yeast inoculum and 15% fermentation medium (yeast extract, 6.0 g/L; peptone, 5.0 g/L; KH_2PO_4, 4.0 g/L; $(NH_4)_2SO_4$ 2.0 g/L; $MgSO_4.7H_2O$, 1.0 g/L; glucose, 150 g/L; pH adjusted to 5.5 with dilute HCI and heat-sterilized). The fermentation reactors containing either acid pretreated or amylase-pretreated biomass of *S. polyrhiza* were used in duplicate. The acid pretreated reactor contained fermentation medium, yeast inoculum and acidic pretreated hydrolysate. The enzyme pretreated reactor contained fermentation medium, yeast inoculum and amylase-pretreated hydrolysate. A reactor containing fermentation medium, yeast inoculum and glucose was used as a positive control. Similarly, the negative control had only yeast inoculum and fermentation media without any sugar source. A method and apparatus for micro-aeration of large-scale fermentation systems are provided. The micro-aeration system includes a fermentation reactor, a sparging apparatus, and a micro-aeration gas mixture delivered to the fermentation reactor via the sparging apparatus. Sparging was done to ensure anaerobic conditions inside the reactors. The reactors were sealed with rubber stoppers equipped with syringe needles for removing CO_2. Samples were withdrawn for the analysis of the concentrations of bioethanol and residual glucose periodically after 24 h incubation. The bioethanol concentration was quantified by an ethanol assay kit.

Statistical analysis

Data were presented as the mean ± standard deviation of the mean of triplicate samples. Significant differences between means were tested using one-way analysis of variance followed by post-test (Tukey's/Bonferroni's) at a significance level of $p<0.05$ using Prism software (ver. 5.0).

Results and discussion

Qualitative analysis of amylase producing bacterial strains

The five isolates exhibited amylase activity (Table 1) in a 1% starch agar medium. The zone of starch hydrolysis exhibited by five isolates: AG 1, AG 2, AG 3, AG 4 and AG 5 with the highest values of amylolytic index were 32mm, 30mm, 40mm, 29mm and 20 mm, respectively. As bacteria live in a complex environment and they cannot utilize complex molecules such as starch as a source of carbon for metabolism. Therefore, they secrete exo-enzyme such as amylase to degrade complex molecules into subunits which can be utilized by the microorganisms. Amylases catalyze the hydrolysis of polysaccharides such as starch. The major target sites are α – 1, 4 linkages in the starch substrate. The amount of amylolytic index (enzymatic index) is influenced by the speed of microbial growth, the ability of product formation and effectiveness of enzymes in solid media. The study conducted by Satrimafitrah showed that PL 16 with amylolytic index value of 4.04 mm (Satrimafitrah *et al.*, 2020). This shows that strain AG 3 has higher amylase activity as compared to PL 16.

Table 1. Zones of starch hydrolysis for the amylase producing bacterial isolates.

Strains	Zone of Hydrolysis (cm)	Zone of Hydrolysis (mm)
AG 1	3.2	32
AG 2	3.0	30
AG 3	4	40
AG 4	2.9	29
AG 5	2.0	20

Characterization of the selected amylase-producing bacteria

Biochemical and molecular characterization of the selected isolate was carried out as described by (ElSharayidi *et al.,* 2021). It was Gram-positive rod-shaped. The biochemical characterization is shown in Table 2. The DNA bands on agarose gel for strain AG3 (*See* in Suplementary file: Fig. S5).

Table 2. Biochemical identification of *Bacillus* AS1 MK321577 strain.

Biochemical tests	Results
Starch hydrolysis	+
Simon citrate agar test	+
Triple sugar iron test	+
Catalase test	+
Oxidase test	-
Voges-proskauer	-
Methyl red test	+

The strain AG3 phylogenetically was correlated to *Bacillus subtilis*. The optimal tree with the sum of branch length = 0.69281917. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Fig. 1).The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 8 nucleotide sequences.

All positions containing gaps and missing data were eliminated. There were a total of 480 positions in the final dataset. The sequence has been deposited in the GenBank under accession number AS1 MK321577 (Table 3).

Table 3. Description of selected strains based on BLAST analysis.

Isolate no	Identified as	Accession number	New Nomenclature
AG 3	*Bacillus subtilis*	MK321577.	AS1

Quantitative analysis of amylase producing bacterial strains

The strain AG 3 exhibited the highest amylase activity (4U/mg/min) followed by AG 4 (0.306 U/mg/min) and AG 1 (0.096U/mg/min) (Fig. 2). The lowest activity was exhibited by strain AG 5 (0.06 U/mg/min) and AG 2 (0.04U/mg/min). Strains AG 3 was selected for optimization of parameters based on its highest amylase activity compared to others and hence it was chosen for further work throughout the study. Amylase activity exhibited by bacterial strain AG 3 (*Bacillus* AS1 MK321577) isolated from Attock fort has higher specific activity compared to *Bacillus* sp. 3.5AL2 with activity of 1.97 ± 0.41 U/mg protein at the optimal conditions of 60°C and pH 7.0 after 30 min incubation with 1% starch in 0.05 M PBS buff.

Table 4. Analysis of variance (ANOVA) for the factorial model of amylase production from AG3 strain.

Source	Sum of squares	Degree of freedom	Mean Square	F value	p-value pro > F	
Model	149.29	9	16.59	98.57	0.0002	
J-Temperature	74.40	1	74.4	442.17	< 0.0001	Significant
A-Time	22.15	1	22.15	131.65	0.0003	
L-$MgSO_4$	20.51	1	20.51	121.86	0.0004	
F-$Cacl_2$	16.56	1	16.56	98.40	0.0006	
Curvature	42.87	1	42.87	254.78	< 0.0001	Significant
Residual	0.67	4	0.17			
Lack of fit	1.379	2	0.6897	0.205	0.5543	not significant
Pure error	0.67	2	0.34	-	-	
Cor total	192.83	14	-	-	-	

This amylase–producing bacterial strain offers great potential for applications in food and agricultural industries. (Luang-In *et al.,* 2019). A recent study revealed that the bacterial isolate IR-9 has highest activity of 5.9 U/ml, followed by 5.5 U/ml, and 5.4 U/ml by IR-1, and IR-8, respectively. The bacterial strains IR-2, and IR-3 showed 4.9 U/ml and 4.7 U/ml optimum activities in the presence of 3% starch concentration (Ullah *et al.*, 2021).

Table 5. Starch content of *Spirodella polyrhiza*.

Wavelength (nm)	Absorbance	Starch content
605 nm	1.988	0.9 % w/w

The gradual increase in concentration of the starch increases the amylase activity of isolates. The saccharification of 10% kitchen waste using 10 U/g enzymes showed 80% saccharification efficiency at 60 °C after 6 h. (Bhatt *et al.*, 2020). The highest relative amylase activities (2.86) was recorded in isolate SPD24 (Bacillus sp.). The most potent of all the bacteria isolated from soil samples in this study was *Bacillus* species which is in line with the work of Fasiku who reported that 80% of bacteria isolated from soil samples was Bacillus species (fasiku *et al.*, 2020).

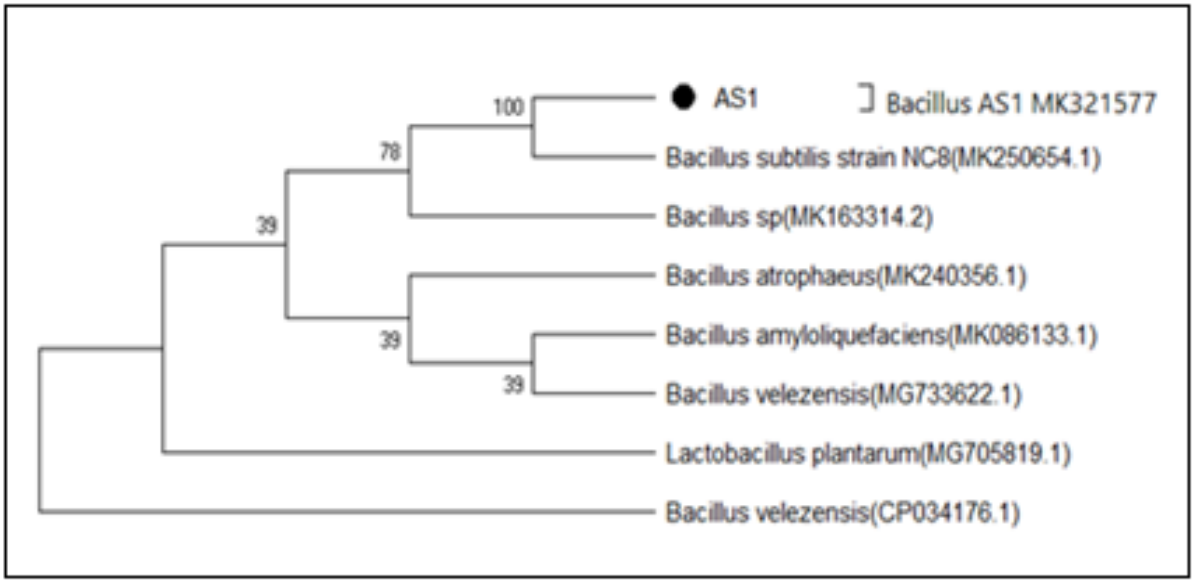

Fig. 1. Phylogenetic tree of the isolated strain *Bacillus* AS1 MK321577.

Optimization of amylase production

Optimization of temperature

Temperature has significant effects on the growth of microorganisms. It affects the rate of enzyme production (Satrimafitrah *et al.*, 2020). The thermo-stability of protein depends upon the nature and conformation of amino acids. Among amino acids, cysteine and valine mostly contribute to the thermostability of proteins (Yablokov *et al.,* 2013). The strain AG 3 exhibited the highest specific activity (0.4294 U/mg/min) compared to other strains at 45 °C after 24 h incubation (Fig. 3). A decline in specific activity was observed with the increase in incubation time and temperature. Amylases are extracellular enzymes, therefore, they become less stable on release in the extracellular environment (Fromm, 2013). According to previous study, *Bacillus* RUI strain has been reported with maximum amylase activity at a temperature of 45 °C (Pinjari and Kotari, 2018).

Optimization of pH

The strain AG 3 showed the highest specific activity (0.3798 U/mg/min) at pH 6 (Fig. 4). The specific activities at pH 5 and pH 7 were 0.3074 U/mg/min

and 0.3420 U/mg/min, respectively at 24 h incubation. Amylase activity was decreased at pH 5 and pH 7 after 24 h incubation. The pH 6 was selected for amylase production as specific activities showed phased decline at this pH. On the other hand, pH 5 and pH 7 showed abrupt decline in specific activities at 48 and 72 h incubation. According to Palmer, changes in pH can cause the cessation of enzyme activity due to denaturation processes in the three-dimensional structure of enzymes (Palmer, 1985).

Most of the bacterial strains that produced amylases at slightly acidic pH or closer to neutral such as *Bacillus* KR-8104 strain have been reported with active alpha-amylase production at a range of pH 5-6 (Hutcheon *et al.*, 2005).

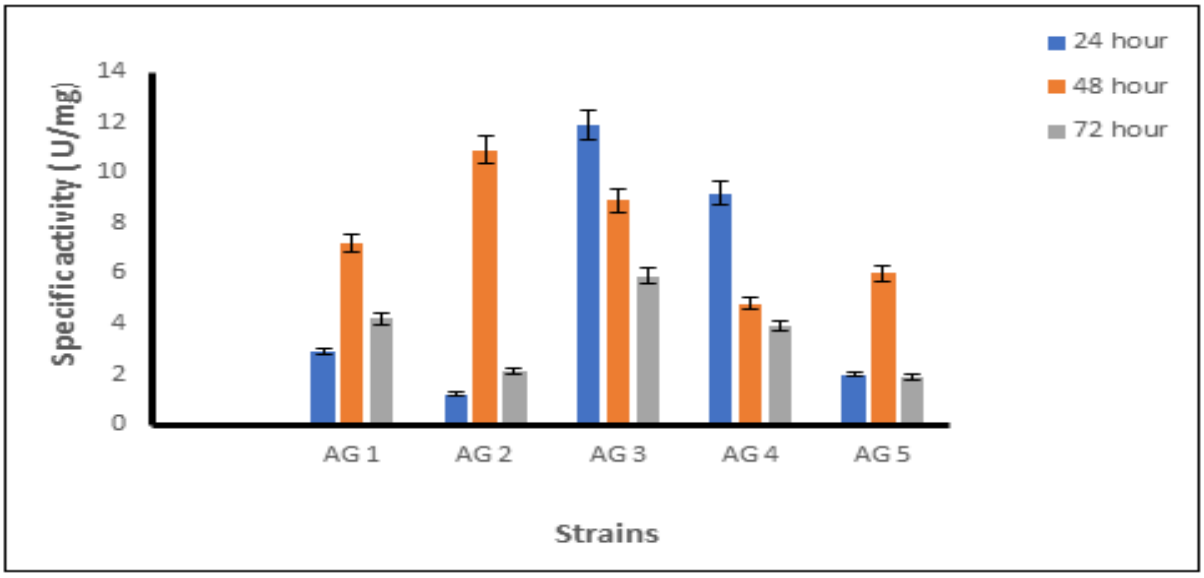

Fig. 2. Specific activities of the isolated bacterial strains after 24, 48 and 72 h incubation.

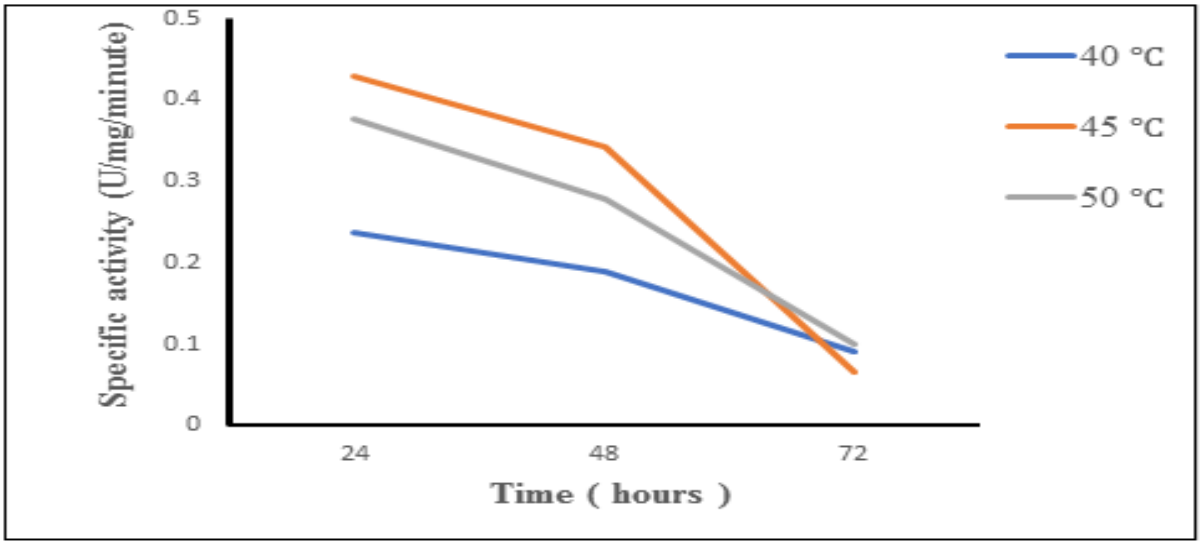

Fig. 3. The specific activity of strain AG3 on selected temperatures at different incubation times (24, 48 and 72 h).

A novel α-amylase by *Bacillus atrophaeus* NRC1 isolated from honey has been reported with maximum amylase activity against starch at pH 6 (Abd-Elaziz *et al.*, 2020).

Optimization of organic substrate

Several organic substrates affected the growth rate of the strain AG 3 (Fig. 5). The strain AG3 was highly efficient and exhibited the highest amylase activity

(0.3825U/mg/min) and (0.2356U/mg/min) on boiled potato peels and boiled banana peels as carbon sources in the production media, respectively (Fig. 6). The use of other organic substrates as carbon sources resulted in reduced specific activities i.e., orange peel (0.2455 U/mg/min), banana peel (0.2183 U/mg/min), rice husk (0.2791U/mg/min) and wheat bran (0.269 U/mg/min). These organic substrates also contained starch contents, but the reduction in enzyme activities might be assumed due to the compactness and structural limitations of these substrates.

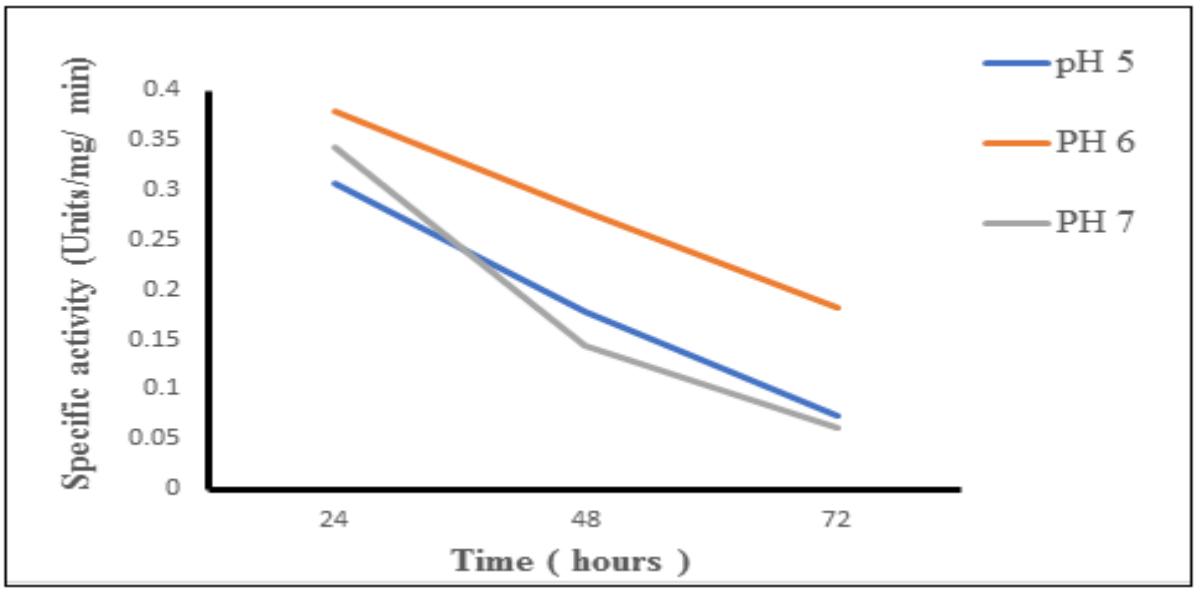

Fig. 4. Specific activity of strain AG3 on different pH at incubation times (24, 48 and 72 h).

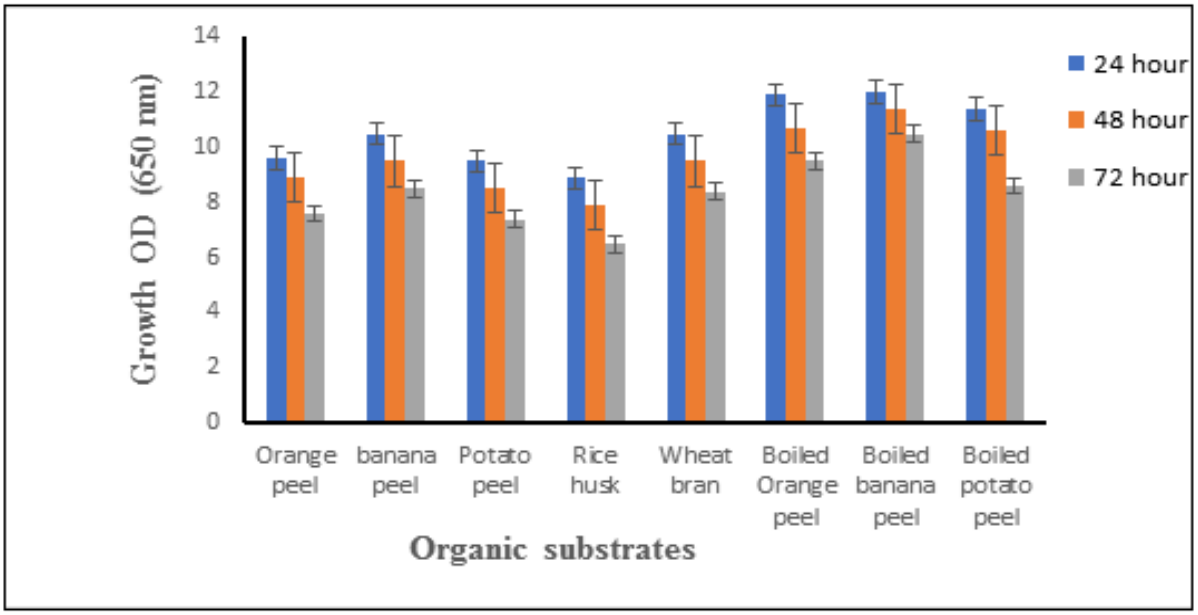

Fig. 5. Growth of strain AG3 on organic substrates at different incubation times (24, 48 and 72 h).

The size of substrate particles, carbon source, protein content, moisture content and compactness offers structural limitations (Leu and Zhu, 2013). A thermophilic strain *Bacillus subtilis* K-18 isolated from the native environment showed maximum amylase activity when grown in media containing potato peel as an organic carbon source. It has been reported as a potent strain for application in industries particularly for bioethanol production (Mushtaq *et al.*, 2017). Previous studies demonstrated

that the use of potato waste as substrate can result in increased bioethanol production (Izmirlioglu and Demirci, 2015).

Optimization of organic and inorganic nitrogen sources

In the present study, organic nitrogen sources studied were yeast extract and bacteriological peptone. Inorganic sources included potassium nitrate, sodium nitrite and sodium nitrate. The use of bacteriological peptone in production media resulted in the highest growth rate of the isolated strain AG 3. (Fig. 7). The highest amylase activity (0.38 U/mg/min) was exhibited by strain AG 3 when grown in media containing sodium nitrate as a nitrogen source for 24 h (Fig. 8).

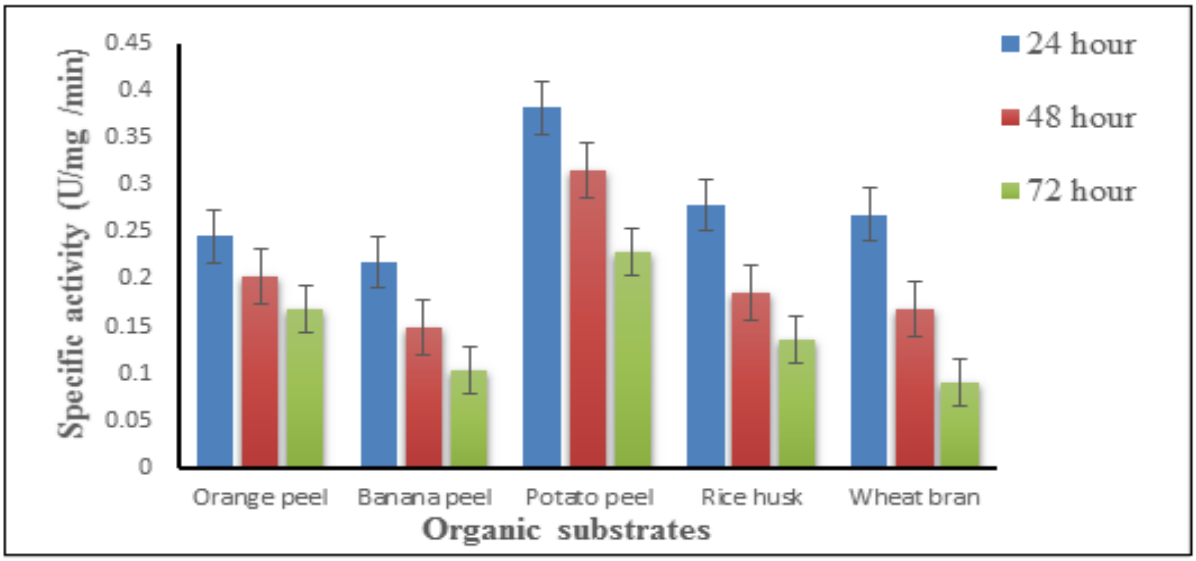

Fig. 6. Specific activity of strain AG 3 on the selected organic substrates at different incubation times (24, 48 and 72 h).

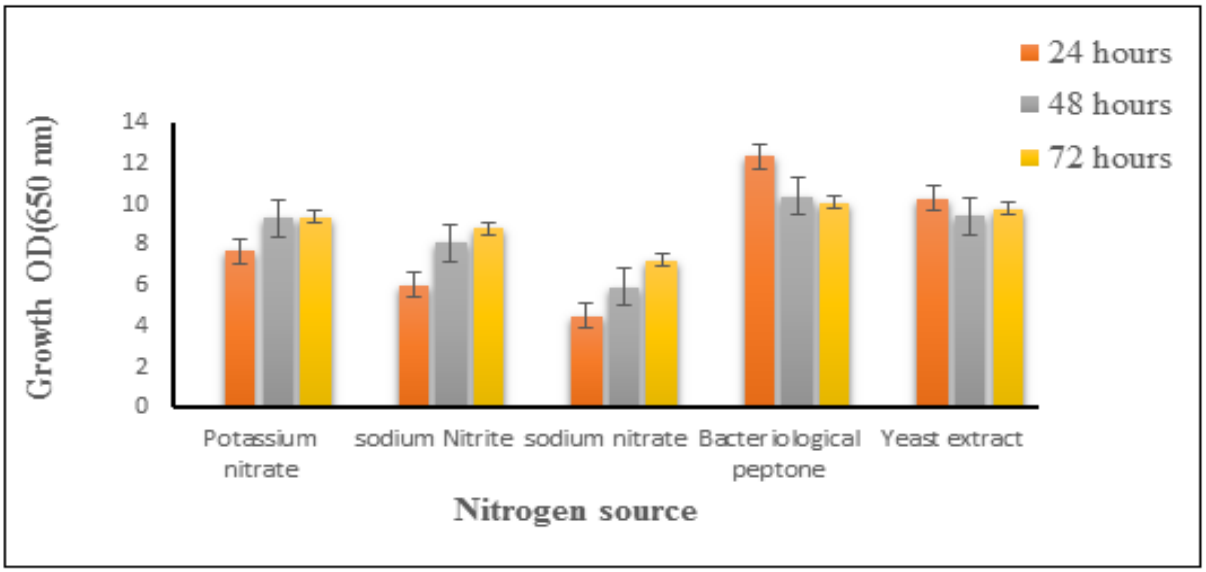

Fig. 7. Growth of strain AG3 on various organic and inorganic nitrogen sources at different incubation times (24, 48 and 72 h).

An alkaliphilic *Bacillus sp.* JB-99 exhibited the highest amylase activity when production media was supplemented with sodium nitrate and potassium nitrate as nitrogen sources indicating the nitrate as the most appropriate nitrogen source for amylase production (Johnvesly and Naik, 2001).

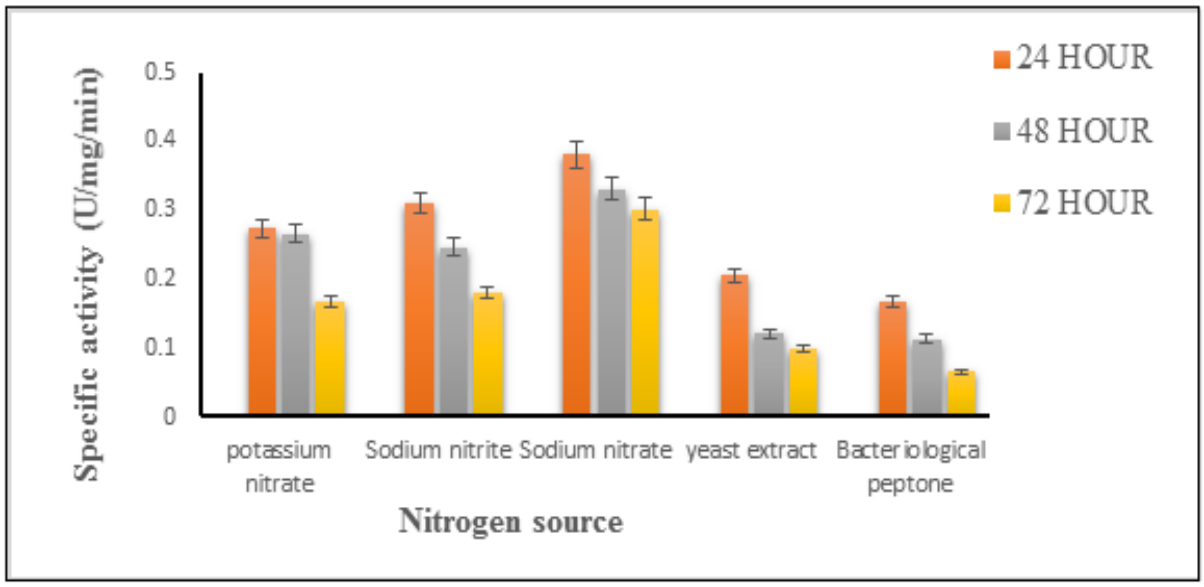

Fig. 8. Effect of incubation time on the specific activity of strain AG3 during different incubation times (24, 48 and 72 h).

Optimization of incubation times

The highest amylase activity (0.4 U/mg/min) was observed on sodium nitrate as a nitrogen source after 24 h incubation for the isolated strain AG 3 (Fig. 9). A decline in amylase activities was observed with the increase in incubation time. This decline in amylase activity can be attributed to the fact that the availability of nutrients becomes limited with time. Additionally, the production of toxic substances in media has also been reported (Raul *et al.*, 2014).

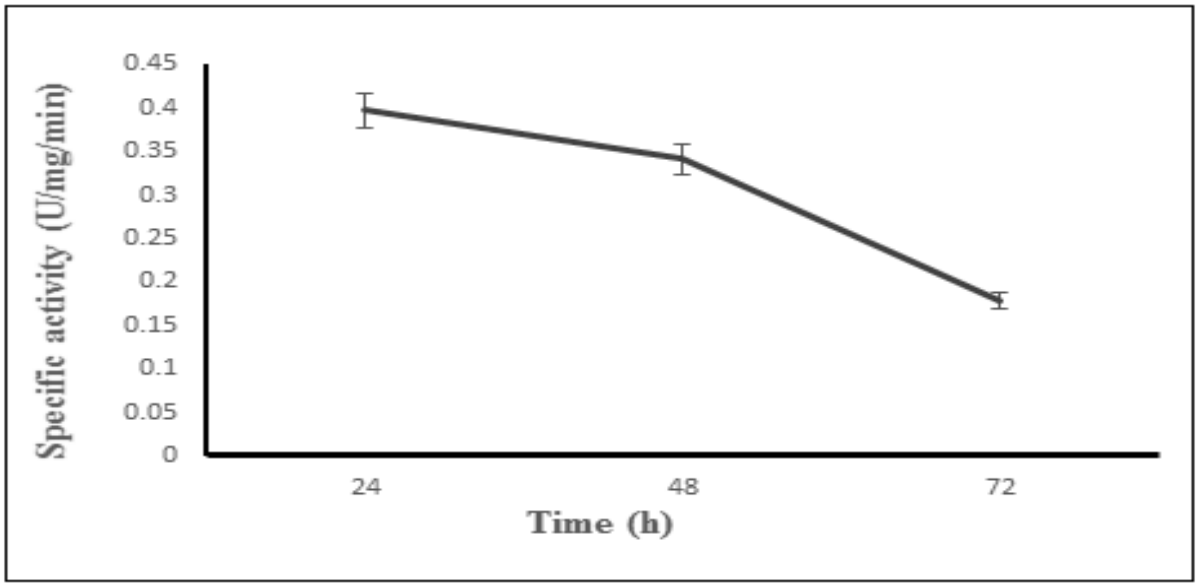

Fig. 9. The specific activity of strain AG3 on different incubation times (24, 48 and 72 h). The other conditions for amylase production were kept constant at optimum.

The incubation time for amylase production varies from strain to strain. It depends upon the nature of microorganisms, the type of organic substrates used as carbon and nitrogen sources in the production media (Sahnoun *et al.*, 2015).

Optimization of media components for amylase production

Maximum amylase production of 12.9 U/mg was observed at run 3 of Placket Burman software. Four factors including temperature, incubation time,

$MgSO_4.7H_20$ and bivalent metal ($CaCl_2$) significantly affected the amylase production (*See* in Suplementary file: Fig. S 2-4). It has been reported that alpha-amylase from *Bacillus subtilis* exhibited enhanced activity when grown in media containing 0.02 % $MgSO_4.7H_2O$. It has been showed that amylase from *Aspergillus oryze* has enhanced activity in the presence of 0.1% $MgSO_4.7H_2O$ ((Kokab *et al.*, 2003) the temperature was the most significant factor for amylase production (Fig.10).

Analysis of variance (ANOVA)

The Table 4 summarizes the results of ANOVA. If the value of probability is less than 0.05%, then the factors are considered significant.

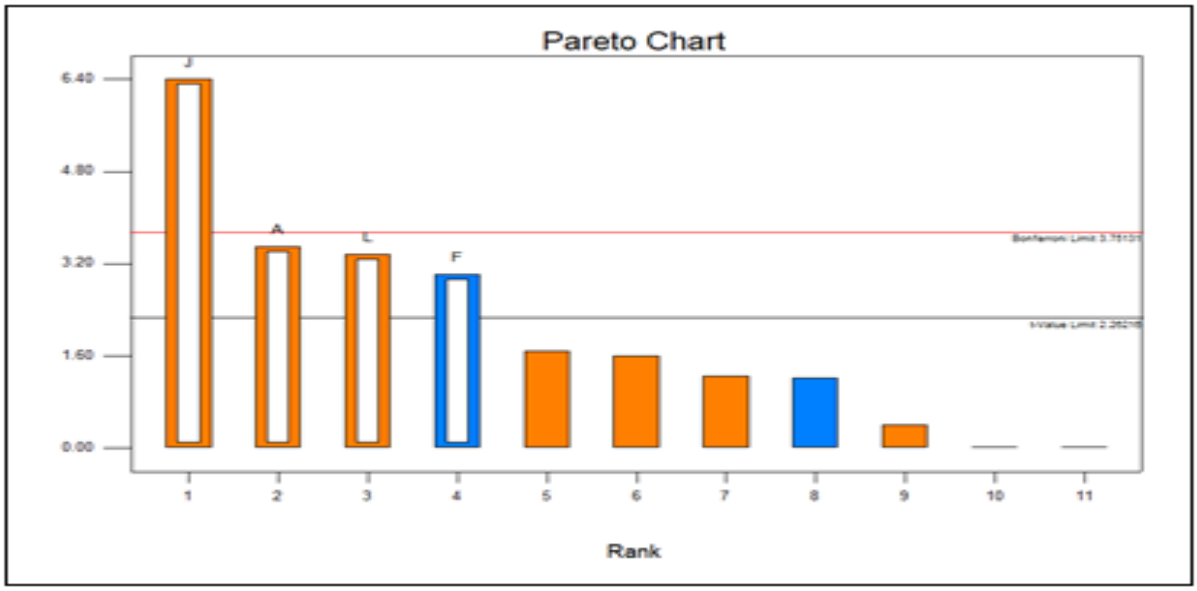

Fig. 10. Evaluation of positive and negative factors based on Pareto chart. Factors are represented as A-Time, B-NaCl, C-KH_2PO_4, D-pH, E-Inoculum, F-$CaCl_2$, G-$MgCl_2$, H-$NaNO_3$, J-Temperature, K-Starch, L-$MgSO_4.7H_2O$. Orange color hollow bars indicate a positive effect while blue color hollow bars indicate negative effect.

The Model F-value of 98.57 shows that the term is significant. There is a 0.02% chance that this Model value-F can occur due to noise. A value less than 0.05 of prob > F implies that terms are significant. In the present study, significant model terms are J, A, L and F. A model term is not considered significant if its value is greater than 0.1000.

A significant curvature exists in the design space as indicated by the curvature F-value of 254.78. Significant curvature is measured by the difference between two average points. It is given as:

Significant curvature = Average of center points – Average of factorial points

There is only a < 0.01% chance that this curvature F-value can occur due to noise. The lack of fit F-value is not significant with pure error. This is indicated by the value 0.205. There is a 55% chance that this large lack of fit F-value can occur due to noise.

It is recommended that a good design must not have a significant lack of fit. Final equations for the coded and actual factors are given (*See* in Suplementary file: Table S1).

Determination of starch content in Spirodella polyrhiza

The *S. polyrhiza* was grown in Hoagland medium (*See* in Suplementary file: Fig. S6). The starch content was analyzed by lugol solution (*See* in Suplementary file: Fig. S7). The starch content was 0.9% w/w (Table 5). A study reveals that the starch content of *S. polyrhiza* K1 reached 24.8% after a harvest regime of 4 days within poly-cultures at the pilot-scale (Chen *et al.*, 2018).

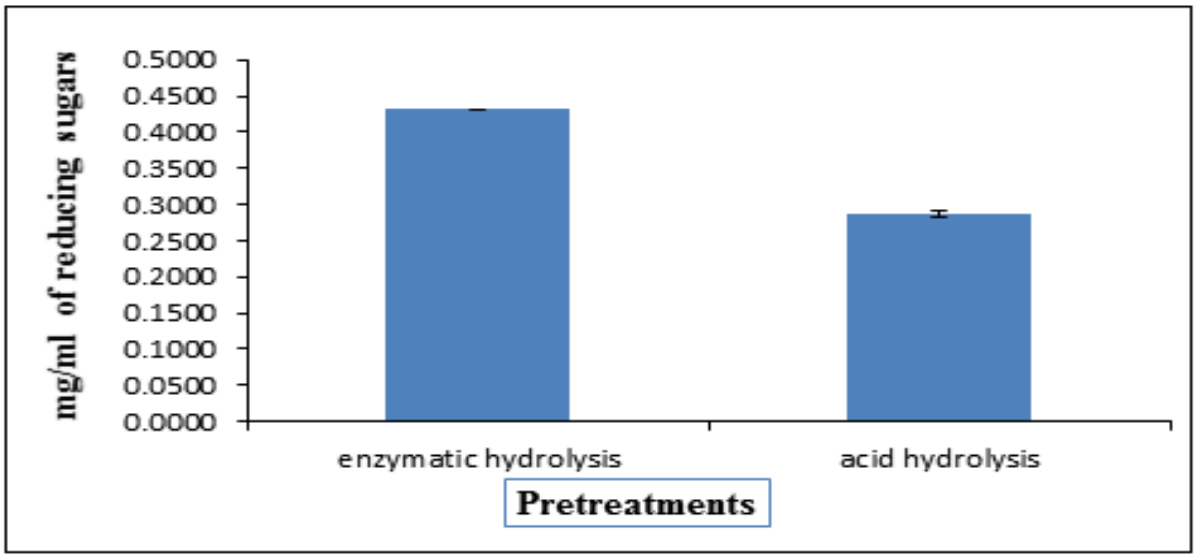

Fig. 11. Enzymatic and acid hydrolysis of the *S. polyrhiza* biomass. Please describe statistical analysis here if you have done for it.

Enzymatic pretreatment of the S. polyrhiza biomass

After enzymatic and acidic hydrolysis of the *S. polyrhiza* biomass, 0.432 mg/ml and 0.28 mg/ml reducing sugars were released in the hydrolysate (Fig. 11) . Enzymatic hydrolysis is a more environmentally friendly pretreatment process compared to acidic hydrolysis and resulted in a higher yield of reducing sugars.

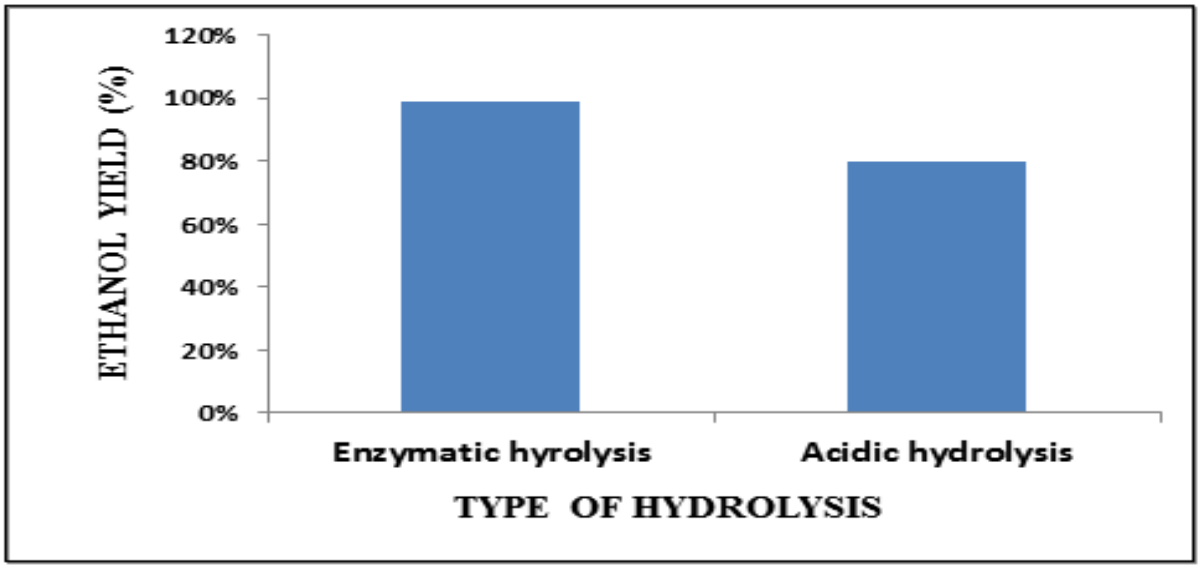

Fig. 12. Percent bioethanol yield of enzyme and acid pretreated hydrolysate of *S. polyrhiza* strains**.**

Ethanol fermentation of the pretreated S. polyrhiza biomass

The reducing sugars in the hydrolysate were subjected to fermentation by yeast strain QG 1 under anaerobic conditions. The bioethanol yields of enzymatic and acidic pretreated hydrolysate using yeast fermentation were 99% and 80%, respectively (Fig. 12). The low bioethanol yield of acid pretreated hydrolysate can be attributed to inhibitory compounds released such as furfurals, which decrease the efficiency of yeast strain. These results indicate that amylase plays an important role in producing fermentable sugars which are utilized by yeast strain to produce bioethanol and duckweed biomass can produce significant quantities of starch that can be readily converted into bioethanol. The bioethanol

yield of starch-rich duckweeds can further be enhanced as reported in a previous study, where the 95 % bioethanol yield of the theoretical yield was obtained after enzymatic pretreatment and yeast fermentation in a 14 L fermenter. (Jiele xu *et al*., 2011).

Conclusions

The bacterial strain *Bacillus* AS 1 MK321577 has a significant, still unexplored enzymatic potential that could be used to achieve a cleaner, environmentally friendly and economically acceptable biofuel production. The duckweed species are capable of heavy metal removal from waste water. Thus, it can be a potential candidate for scheming a duckweed-based heavy metal phytoremediation system. The transfer of duckweeds into wastewater outcomes in high annual yeild of starch. The enzymatic hydrolysis and subsequent yeast fermentation of high-starch duckweed biomass result in biofuels such as bioethanol production which is promising alternative energy source to reduce dependence on depleting crude oil.This *Bacillus* strain is considered a significant source of amylases. We hope this study will aid future large-scale industrial applications for the production of bioethanol.

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Conflict of interest

The authors declare that this research work is not associated with any conflict of interest.

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