



Pectinase Production by *Acinetobacter. oleivorans* DR1 Using Date Fruit Wastes (*Phoenix Dactylifera* L.) as Substrate

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Keywords: Date fruit waste; *A. oleivorans* DR1; Pectinase; Waste utilization; Value-added products.

Abstract

Background: Date (*Phoenix dactylifera* L.) is an important crop in desert regions of the Middle East and contributes significantly to human nutrition. It has been reported that the date palm trees produced million tons wastes across the world. Solid State Fermentation Technology (SSF) using agro-industrial wastes, as substrate for production enzymes by microbial strains, is much more cost-effective technique. In the current research, date fruit wastes were screened as substrate to produce commercially important enzyme i.e., pectinase.

***Acinetobacter. oleivorans* DR1** was screened for pectinase production using date fruit waste as substrate by solid state fermentation.

Methods: Six different varieties of dates fruit samples waste (Majdool, Shishi, Bumaan Keneezi, Khalas, Boojipal) were collected from local market of Ras Al Khaimah, United Arab Emirates.

Results: The *A. oleivorans* DR1 produced higher concentration of pectinase using date fruit wastes as substrate as compared to orange peel, potato peel, citrus peel, and rice husk. *A. oleivorans* DR1 produced maximum pectinase using 1% date fruit wastes with tap water as moistening agent and required pH 8.0, 45 °C incubation temperature, 2% inoculum size and 72 h incubation period.

Conclusion: This is probably the first report of pectinase production by *A. oleivorans* DR1 by utilizing dates fruit waste as substrate. It has been concluded that waste from date fruits is a good source of biomass and can be utilized to produce pectinase for commercial applications and to produce value-added products.



Background

Enzymes are extremely proficient biological catalysts which perform all synthetic and degradative reactions in living organisms. The enzymes are preferred in commercial endeavour than chemicals because of their high catalytic power, specific mode of action, stereo-specificity, eco-friendly nature, and reduced energy requirement etc. Many research activities have been pursued involving enzymes worldwide, for their use in the development of new important industrial processes. There is an ever-increasing demand to reinstate traditional chemical processes with advanced biotechnological processes involving microorganisms or enzymes such as pectinases, xylanases, cellulases, laccases, lipases, and proteases. Today, the pectinases are one of the important enzymes in the commercial sector. It has been reported that microbial pectinases account for 25% of the global food enzymes sales [1]. Pectinases are widely distributed in nature and mainly occur in plants, bacteria, fungi, yeasts, insects, nematodes, and protozoa. Microbial pectinases have found applications in various industries and constitute a major group of industrial enzymes. They are used in extraction and clarification of fruit juices, vegetable oil extraction and processing of alcoholic beverages, manufacturing of jellies and jams, production of single cell proteins, animal feed, dairy products and baby foods.

Date (*Phoenix dactylifera* L.) is an important crop in desert regions of the Middle East and contributes significantly to human nutrition in some regions. Date fruit is highly nutritious and rich in calories [2]. Date palm (*Phoenix dactylifera* L.) is widely planted in hot and dry climate regions of Africa, the Middle East and Asia. Date palm fruit is an important food resource in these regions. Besides food-grade date production, large amounts of dates end up as waste. Agricultural waste is generated in large amounts annually in the form of by-products in agriculture and crop residues [3]. Date residues such as date seeds, Date Press Cake (DPC), and cull dates (out grade dates) are generated by date processing industries and are used as animal feed or ended up in drains and dumps [4]. These residues are rich in carbohydrates, dietary fibers, antioxidants, and phenolic compounds, making them a suitable feedstock for processing to a variety of value-added products [5]. However, date residues are currently considered as waste rather than a resource. Date fruit production and related processing industry generate a large quantity of waste; for illustration, the date juicing industry produces roughly 17–28% Date press cake (DPC), which is mainly discarded in open lands and drains [6]. Waste date has harder texture and more fibers than the commercial edible grade fruit. The large volume of residues produced by the date processing industry is an indirect waste of water and arable land if not efficiently valorized. Furthermore, uncontrolled decomposition of date waste can potentially cause severe health and environmental problems [7]. However, it is an excellent source of sugar to produce refined sugar, concentrated juice, and confectionary pastes as well as fermentation products [8]. Use of the dates fruit waste to produce pectinases can potentially eliminate a pollution problem, improve revenues in date processing and reduce the cost of importing expensive pectinases for use commercially in different industries to produce value added products. Several bacterial and fungal strains have shown great potential to produce different types of pectinolytic enzymes. Most of the Bacterial isolates such as *Bacillus* sp and *Pseudomonas* sp., *Pseudomonas fluorescence* and *Bacillus subtilis*, *Bacillus* sp. MFW7, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus* sp. MBRL576, *Bacillus firmus* I-4071, *Enterobacter aéro-*

genes NBO2, *Pseudoalteromonas haloplanktis* strain ANT/505, *Paenibacillus xylanolyticus*, *Bacillus firmus*, *Bacillus firmus* (P1), *Bacillus coagulans* (P13), *Bacillus endophyticus* (P57) and *Bacillus vietnamensis* (P58), *Bacillus subtilis* BKDS1, *Enterobacter* sp. PSTB-1 and *Staphylococcus aureus* were reported as good pectinase producers [9–21]. Several fungal strains have shown great potential to produce different types of pectinolytic enzymes [22–27].

Keeping in view the demand for pectinase in various industries and to utilize waste of dates fruit, the present research was planned for efficient production of pectinases from microorganism to utilize in different industries for value added products.

Materials and Method

Collection of date fruit wastes

Six different varieties of dates fruit waste samples **Figure 1** (Boojipal, Bumaan, Keneezi, Khalas, Majdool, Shishi) were collected from local market of Ras Al Khaimah. The collected date fruits waste was kept in a large tray and cleaned, and the dust and dirt particles were removed and labelled. Date fruits waste was air-dried, covered with a muslin cloth, and kept at room temperature for 48 h.

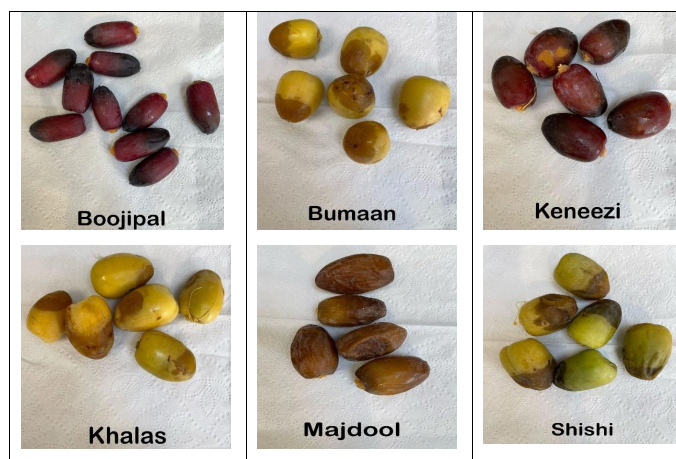


Figure 1: Different varieties of Dates fruit waste.

Bacterial strain

The bacterial strain *A. oleivorans* DR1 was obtained from the culture bank of Kurukshetra University India, which was previously isolated (indigenous source from waste of fruit processing units and vegetables) in the microbiology laboratory of Kurukshetra University, India. The isolate was identified morphologically, biochemically, and genetically on the basis of nucleotide homology and phylogenetic analysis with GenBank Accession Number NC-014259.1 [12].

Solid state fermentation

Solid state fermentation was done by fermentation of *A. oleivorans* DR1 using a 250 mL Erlenmeyer flask containing 5 g date fruit wastes as substrate and inoculated with 10 mL aliquots of *A. oleivorans* DR1 in medium which containing, Sucrose 2.5 g, KNO₃ 0.15 g, KH₂PO₄ 0.25 g, MgSO₄ 0.065 g, CaCl₂ 0.025 g, NaNO₃ 0.125 g, KCl 0.125 g, Yeast extract 0.25 g, at pH 7.0. The flasks were gently tapped intermittently to mix the contents. The fermentation was carried out at 37°C for 72 h, and after that, the solid fermented material was mixed with 30 mL distilled water and stirred for 30 min. Then, the mixture was centrifuged 4000 rpm for 20 min and the supernatant was used

for crude enzyme. Pectinase activity in the crude extract was assayed after 72 h of incubation by DNSA method.

Extraction and assay of pectinase

Pectinase activity was determined by measuring the amount of reducing sugars liberated using Dinitro Salicylic Acid Method (DNSA) [51] Briefly, 250 mL of substrate (polygalacturonic acid 1% w/v) prepared in 0.2 M citrate phosphate buffer pH 7) 100 μ L of (10x) diluted crude pectinase enzyme was added and incubated at 60°C for 10 min. Then 1.5 mL of DNSA reagent was added to the reaction mixture and incubated in boiling water bath for 15 min. to terminate the reaction and cooled to room temperature. A control was run simultaneously which contained all the reagents with heat inactivated 10 μ L enzyme. The absorbance was measured at 540 nm against control. A standard curve of galacturonic acid was also prepared for estimation of enzyme activity. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μ mol of galacturonic acid /mL/min [28].

Optimization of culture conditions for maximum pectinase production under solid state fermentation

To enhance pectinase production fermentation conditions were optimized with respect to pH, temperature, substrate, moistening agent, moisture level, inoculum age, inoculum size, source of carbon and nitrogen. One variable at a time approach was used for optimization and production.

Selection of a suitable Moistening Agent (MA)

Moistening agent was used to provide moisture to substrate for its fermentation. Three types of Moistening Agents (MA) were used for solid state fermentation. Composition (g/L) of which are as follows:

Table 1: Composition of moistening agents.

Composition of Moistening agent	MA 1	MA2	MA3
Sucrose	10	–	–
KNO ₃	0.6	–	0.6
KH ₂ PO ₄	–	1	–
MgSO ₄	0.25	0.25	–
CaCl ₂	0.1	0.1	0.1
NaNO ₃	–	2	–
K ₂ HPO ₄	–	–	0.5
KCl	–	–	0.5

Beside these salt solutions (moistening agents), distilled water and tap water were also employed as the moistening agents. Each Erlenmeyer flask (250 mL) containing 5 g sample of date fruits waste was moistened with 10 mL of moistening agents, autoclaved and then cooled. The flasks were inoculated with 24 h inoculums 10 mL and incubated at 37 °C in solid state fermentation for 72 hrs. After incubation, enzyme was extracted by adding glycine NaOH buffer (pH 10). Incubate for 20 min again. Then squeezing through a muslin cloth followed by centrifugation at 10,000 rpm for 30 min at 4°C the clear supernatant (crude extract) was used for enzyme assay.

Effect of moisture level

The influence of moisture level on the enzyme titre was evaluated under SSF by varying the ratio (w/v) of date fruits waste to tap water as moistening agent (1:1, 1:2, 1:3, 1:4,

1:5). Erlenmeyer flasks (250 mL), each containing 5 g of date fruits waste was moistened with tap water, autoclaved, and inoculated with 24 h old inoculum and incubated at 37 °C in solid state fermentation for 72 h and after fermentation clear supernatant was used for enzyme assay.

Effect of various agro-industrial by product (substrate)

The effect of various substrates on pectinase production in SSF was studied. Erlenmeyer flasks containing 5g of various substrates viz. date fruits waste, sugar cane bagasse, rice husk, citrus peel, potato peel, orange peel, were moistened with 10 mL of tap water, autoclaved and cooled. Control flask which contains only commercial pectin as substrate. The flasks were inoculated with 24 h old inoculums 10 mL and incubated at 37 °C in solid state fermentation for 72 hrs and after fermentation clear supernatant was used for enzyme assay.

Effect of pH

Erlenmeyer flasks (250 mL), each containing 5 g of date fruit waste as substrate and 10 mL of tap water as moistening agent and maintain different pH 3 to 12 by NaOH and HCl. Autoclaved, inoculated, with 24 h old inoculum and then incubated at 72 h and after fermentation clear supernatant was used for enzyme assay.

Effect of temperature

Erlenmeyer flasks (250 mL), each containing 5 g of substrate and 10 mL of tap water, were autoclaved, inoculated with 24 hr old inoculum, and then incubated at different temperatures (20°C to 55°C) for 72 hr and after fermentation clear supernatant was used for enzyme assay.

Effect of incubation period

The time course of pectinase production under SSF was studied. Erlenmeyer flasks (250 mL), each containing 5 gm substrate was moistened with 10 mL of tap water, autoclaved and cooled. The flasks were inoculated and incubated at optimized temperature and the pectinase production was monitored for 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h of incubation. After fermentation at different incubation period, clear supernatant was used for enzyme assay.

Effect of inoculum age

To study the effect of inoculum age, the Erlenmeyer flasks (250 mL), each containing 5 gm substrate was moistened with 10 mL of tap water, autoclaved and cooled. Different ages of inoculum viz. 12 h, 18 h, 24 h, 30 h, 36 h, 48 h, were used to inoculate. Incubate at optimized temperature. After 72 h incubation the enzyme was extracted with glycine NaOH buffer (pH 10) followed by centrifugation at 10,000 rpm for 30 min at 4°C. The clear supernatant obtained was used for pectinase assay.

Effect of inoculum size

The effect of inoculum concentration in the range of 1% to 6% was studied on pectinase production. Erlenmeyer flasks (250 mL), each containing 5 gm of substrate was moistened with 10 mL of tap water, were autoclaved and inoculated with 1% to 6% of 24 h old inoculum. The flasks were incubated at optimized temperature for 72 h and after fermentation clear supernatant was used for enzyme assay.

Carbon sources as additives

Various carbon sources glucose, lactose, maltose, sucrose, xylose, cellulose, fructose, galactose, starch was used in the production medium at a conc. of 1% w/v as carbon source. Production medium containing dates as the only carbon source was used as control. Flasks were inoculated with 48 h old inoculum and incubated for 72 h at optimized temperature and after fermentation clear supernatant was used for enzyme assay.

Nitrogen sources as additives

Different nitrogen sources such as peptone, ammonium sulphate, gelatin, ammonium chloride, urea, ammonium nitrate, ammonium dihydrogen phosphate, potassium nitrate, sodium nitrate were used in production medium at a concentration of 1% w/v as nitrogen source. The medium without any nitrogen source was used as control. Flasks were inoculated with 48 h old inoculum and incubated for 72 h and after fermentation clear supernatant was used for enzyme assay.

Statistical analysis

The tests were performed in triplicates. Data are expressed as mean. Pair wise comparisons were performed. Experimental error was determined for triplicate and expressed as Standard Deviation (SD).

Results

The objective of this research was to utilize dates fruit waste as substrate for pectinase production by *A. oleivorans DR1* under solid state fermentation. The solid-state fermentation involves the growth and fermentation by microorganisms, on moist, water insoluble and solid substrate in absence or near absence of free water. Conditions for pectinase production from agro-industrial by products were optimized using one variable at a time approach.

Moistening agent was used to provide moisture to solid substrate for its fermentation. Maximum pectinase production was observed when tap water was used as moistening agent followed by 8537 U/gds (**Figure 2**). Moreover, when the ratio of substrate to moistening agent concentration was 1:2 (**Figure 3**). Different agro-industrial byproducts viz. date fruit waste, rice husk, sugarcane baggase, citrus peel, potato peel, orange peel were used as substrate for pectinase production as date fruits waste performed best as a substrate in solid state fermentation which 195.18 U/gds (**Figure 4**) and at concentration of 1% which produced 197.849 U/gds (Fig. 5). Maximum pectinase was produced 9617 U/gds (**Figure 6**) at pH 8 by *A. oleivorans DR1*. *A. oleivorans DR1* produced best at 45°C producing 9512 U/gds pectinase (**Figure 7**). Pectinase production was observed at different time intervals of 12 h, 18 h, 24 h and then daily upto 7 days. Maximal pectinase 9420 U/gds production was found at 72 hours of incubation with *A. oleivorans DR1* (**Figure 8**). The maximum pectinase 9618 U/gds production from *A. oleivorans DR1* was found when 48 h inoculum was used. *A. oleivorans DR1* showed maximum pectinase 9528 U/gds (**Figure 9**) production at 2% concentration (**Figure 10**) of 48 h old inoculum. Loss of pectinase was more pronounced in *A. oleivorans DR1* at increased size of inoculum. Various carbon sources glucose, lactose, maltose, sucrose, xylose, cellulose, fructose, galactose, starch was used in the solid-state fermentation medium at a concentration of 1% w/v as additives. None of the additives supported the enzyme production as enzyme production was reduced maximum by 89.5% in *A. oleivorans DR1*, by using ad-

ditives (**Figure 11**). Different nitrogen sources such as peptone, ammonium sulphate, gelatin, ammonium chloride, urea, ammonium nitrate, ammonium dihydrogen phosphate, potassium nitrate, sodium nitrate was used in solid state fermentation medium at a conc. of 1% w/v as nitrogen source. Pectinase production was reduced by addition of additives containing nitrogen in *A. oleivorans DR1* in the range of with 6 out of 9 additives in comparison to the control with 9585 U/gds (**Figure 12**).

Discussions

In the recent years, the potential use of microorganisms as bio-technological sources of industrially important enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity. Furthermore, most commercial pectinases are from microbial sources. In the present study, bacteria are isolated from natural sources and used for pectinase production. The objective of this research was to utilize dates fruit waste as substrate for pectinase production by *A. oleivorans DR1* under solid state fermentation. This study provided a valuable data concerning the assessment and awareness of higher concentration of pectinase production from *A. oleivorans DR1* using date fruit wastes as substrate as compared to potato peel, citrus peel, and rice husk.

Based on morphological, cultural, biochemical characteristics, nucleotide homology and phylogenetic analysis according to Bergey's Manual of Determinative Bacteriology, 8th Edition and by 16S rRNA gene sequencing analysis, isolate was identified as *A. oleivorans DR1* (GenBank Accession Number NC-014259.1), which was obtained from the culture bank of Kurukshetra University India [12].

The solid-state fermentation involves the growth and fermentation by microorganisms, on moist, water insoluble and solid substrate in absence or near absence of free water. In SSF, the solid substrate particles serve as source of carbon, nitrogen, salts, and growth factors and also provide mechanical support and anchorage to microbial cells. Therefore, SSF was used for utilization of agro-industrial by products. Conditions for pectinase production from agro-industrial by products were optimized using one variable at a time approach.

Moistening agent was used to provide moisture to solid substrate for its fermentation. Different moistening agents (**Table 1**) e.g. salt solutions (MA1, MA2, MA3) distilled water and tap water were used for this purpose. To evaluate the effect of moistening agent on pectinase production by the *A. oleivorans DR1*. 10 mL of moistening agent was added to each Erlenmeyer flask (250 mL) containing 5 g sample of date fruit waste sterilized by autoclaving. Flasks were inoculated with *A. oleivorans DR1* and solid state fermentation was carried out for 72h at 37°C under solid state fermentation. Enzyme extracts from SSF were used for pectinase production.

Maximum pectinase production was observed when tap water was used as moistening agent which is 8537 U/gds. Different moistening agents containing nutrients were used for pectinase production from the date fruit waste. Maximum production of 65% to 88% was observed with MA1 which was additionally containing 1% sucrose as compared to other moistening agents MA2 and MA3 (**Figure 2**), which might be due to the fact that date fruit waste already contains a considerable amount of sugars, supplementation of another sugar might have inhibitory effect on enzyme production.

In a similar study, Ahlawat *et al* 2008, reported maximum pectinase from *Bacillus subtilis* SS, when tap water was used as moistening agent and Kashyap *et al* 2002 observed maximum pectinase from *Bacillus sp.* DT7 when distilled water was used as moistening agent. Also, Mrudula and Anitharaj 2011, reported that in the presence of distilled water, maximum pectinase was produced from *Aspergillus niger*.

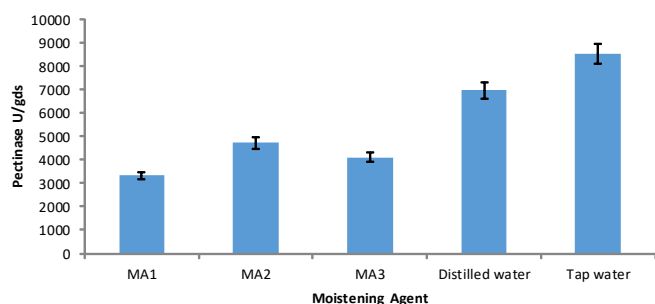


Figure 2: Composition of moistening agents.

The moisture level is a crucial factor that determines the success of the SSF process. The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of solid substrate.

Maximal pectinase was produced when the ratio of substrate to moistening agent concentration was 1:2 (Figure 3). Enzyme production was reduced by lowering or raising the solid liquid ratio as the lowest levels of enzyme production were observed at 1:1, 1:4, 1:5 ratio respectively by *A. oleivorans DR1*. Reduction in enzyme production at high solid-liquid ratio might be due to decrease in porosity, lower oxygen transfer and alteration in date fruit particle structure. On the other hand, low moisture content may lead to poor accessibility of nutrients causing reduction in solubility and in swelling of solid substrate which leads to poor microbial growth and thus reduction in enzyme production.

Similar to present research, substrate to moistening agent concentration ratio of 1:2 was found to be the best by Mrudula and Anitharaj (2011) for pectinase production from *Aspergillus niger*. [29] has also reported a close ratio i.e 1:2.5 for maximum pectinase production from *Bacillus subtilis* SS.

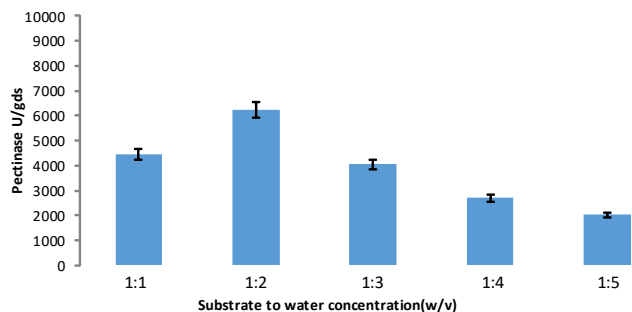


Figure 3: Effect of Moisture level on pectinase production under SSF.

Agro-industrial by products are inexpensive and abundantly available are considered to be the best substrate for SSF. Different agro-industrial by products viz. date fruit waste, rice husk, sugarcane baggase, citrus peel, potato peel, orange peel were used as substrate for pectinase production as date fruits waste performed best as a substrate in solid state fermentation. SSF was carried out by

inoculating this substrate containing optimized moistening agent with different isolates for 72 h. Enzyme extracts were used for analysis. When single substrates were used, date fruit waste was found to be the best for enzyme production which 195.18 U/gds pectinase respectively from *A. oleivorans DR1* (Figure 4) and at concentration of 1% which produced 197.849 U/gds (Figure 5). Control flask which contains only commercial pectin as substrate produced 140.66U/gds.

It was found that a majority of the *Bacillus sp.* have maximum pectinase production ability [30]. Wheat bran has been reported as the prime substrate for pectinase production by [31,29,32] respectively from *Bacillus sp.* DT7, *Bacillus subtilis* SS, *Bacillus sp.* However, [33] reported that *Solanum Tuberosum* (ST) peel produced maximum pectinase from *Bacillus firmus* I-4071, 107, 10104 compared to other substrate. Similar results were reported by [34], stated that higher concentration of pectinase was produced when date fruit waste was used as substrate.

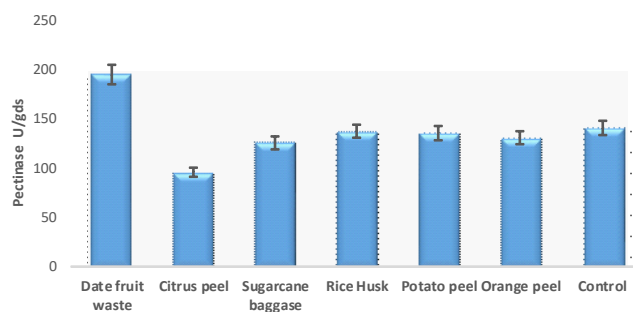


Figure 4: Effect of Agro-industrial by products as substrate.

As in present study date fruit waste was found to be the best for pectinase production by *A. oleivorans DR1*. [35] in their study also found that wheat bran in comparison to rice bran produced more enzyme from *Bacillus sp.* Further [36] reported that wheat bran supported maximum pectinase (589 U g⁻¹) production from *Aspergillus niger*.

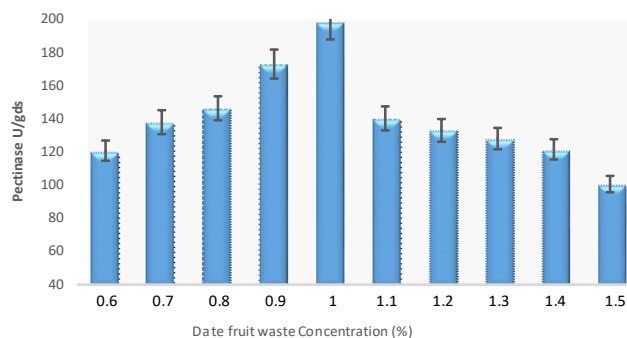


Figure 5: Effect of concentration of Date fruit waste (substrate).

Results of [37] were different who observed that orange peel produced maximum pectinase from *Botryosphaeria rhodina* MAMB-05. [38] revealed that addition of citrus peel into the medium produced maximum enzyme by *Aspergillus niger*.

Higher production of pectinase by these raw materials may be due to the reason that solid substrate not only supplies the nutrient to the microbial cultures growing in it, but also serves as anchorage for the cells allowing them to utilize the substrate effectively [39].

PH of the medium has strong influence on enzyme production. To evaluate the effect of pH, bacteria were grown on opti-

mized solid substrate medium containing 1% of date fruit waste in 1:2 ratio at pH adjusted at 3 to 12 for pectinase production under solid state fermentation for 72 h. Pectinase was estimated in the enzyme extract. Maximum pectinase was produced 9617 U/gds at pH 8 by *A. oleivorans DR1* (Figure 6).

Wide range of pH optimum for pectinase production has been reported in different organism by various workers. Mrudula and Anitharaj [40], found maximum pectinase production at pH 5 from *Aspergillus niger* on orange peel as substrate. [33] observed maximum pectinase production at pH 6 from *Bacillus firmus* -I-10104. [29], has reported maximum pectinase production at pH 9.9 from *Bacillus subtilis* SS.

Pectinase production was found to be maximum from a fungus *Aspergillus niger* using *Ficus religiosa* leaves under solid state fermentation at pH 5 by [41]. Similar results were stated by [42] that maximum pectinase activity at pH 9.0.

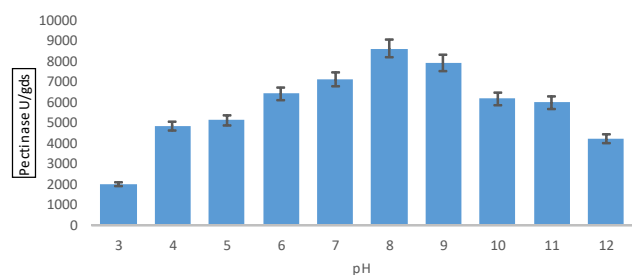


Figure 6: Effect of pH on pectinase production under SSF.

To evaluate the effect of temperature on pectinase production, *A. oleivorans DR1* was grown at different temperatures ranging from 20°C to 55°C for pectinase production under solid state fermentation for 72 h (Figure 7).

A. oleivorans DR1 produced best at 45°C producing 9512 U/gds pectinase. But when the temperature of fermentation was raised to 50 °C or 55°C the level of pectinase reduced drastically to 55% and 43% respectively. Similar results were reported by Bhardwaj and Garg, 2014a stated that *Bacillus* sp. MBRL 576 produced best at 45°C. Similar results were stated by Khan et al., 2017 that maximum pectinase activity at 45°C temperature. [43] reported similar results that maximum pectinase activity at 45°C temperature.

Delftia acidovorans exhibited maximum pectinase at 25°C and by decreasing the temperature further to 20°C, value of pectinase was also declined sharply to 28% according to [44]. [29] Ahlawat *et al* 2007 has observed that maximum pectinase production from a *Bacillus subtilis* SS at a high temperature 70°C. When SSF was carried out at 37°C all the isolates showed decreased production of pectinase. Many other workers have also reported different temperatures for maximum pectinase production, suggesting that the optimal temperature for pectinase production also depends on the strain variation of the microorganism. Pectinase production has been observed at 30°C from *Bacillus* sp. [32], at 37°C from *Bacillus firmus* [33] and at 45°C from *Bacillus* sp. [45]. Temperature of 30°C was also reported to be the best for maximum pectinase production from fungus *Aspergillus niger* using *Ficus religiosa* leaves [41].

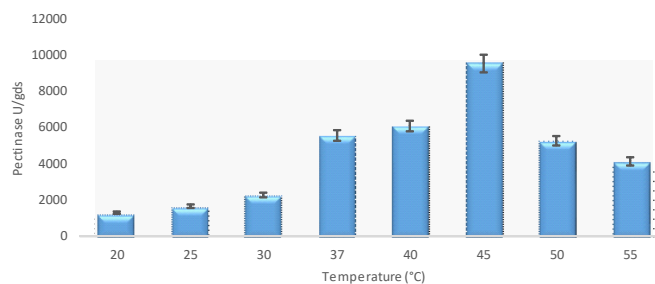


Figure 7: Effect of temperature on pectinase production under SSF.

Pectinase production was observed at different time intervals of 12 h, 18 h, 24 h and then daily up to 7 days. The effect is shown in Figure 8. Maximal pectinase 9420 U/gds production was found at 72 hours of incubation with *A. oleivorans DR1*. Pectinase was detected at low levels at 12 h stage also. Level of pectinase was reduced after 72 h and kept on declining till 168 h. The duration of incubation depends on the growth rate of microorganism and its enzyme production pattern. The present finding of decreased pectinase production after 72 h is attributed to the fact that the gradual depletion of nutrients in the medium might have stressed the bacterial physiology resulting in the inactivation or accumulation of toxic secondary metabolites. In many other studies 72 h was observed as the optimum period for maximum pectinase production e.g. [31] reported it from *Bacillus* sp. DT7 at 37°C; [45] observed it from *Bacillus* sp. at 30°C and [29] reported from *Bacillus subtilis* SS at 70°C. However [33,46] observed that maximum pectinase was produced respectively from *Bacillus firmus* and *Bacillus coagulans* in a longer fermentation period of 96 h.

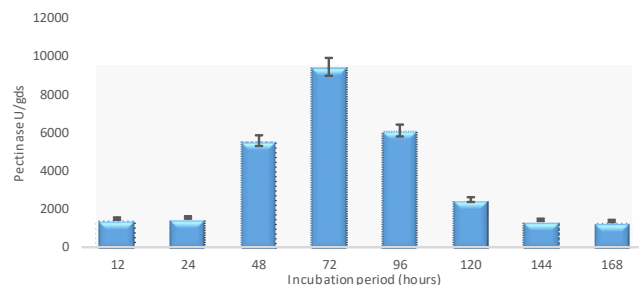


Figure 8: Effect of Incubation period on pectinase production under SSF.

Pectinase production was monitored by varying the inoculum age from 12 h, 18 h, 24 h, 30 h, 36 h, 48 h. The maximum pectinase 9618 U/gds production from *A. oleivorans DR1* was found when 48 h inoculum was used for pectinase production (Figure 9). However some other workers have reported pectinase production at different inoculum age e.g. [29], found 24 h inoculum of *Bacillus subtilis* SS, [32] reported 12 h inoculum of *Bacillus* sp. [47] reported 12 h inoculum of *Bacillus* sp. MG-cp-2 and [48] reported 24 h inoculum for maximum pectinase production of *Aspergillus niger*. Similar results were reported by [28] which stated that maximum pectinase production from *Bacillus* sp. MBRL576, was found when 24 h inoculum was used for pectinase production.

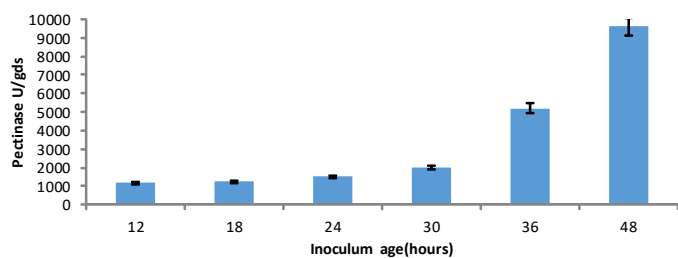


Figure 9: Effect of Inoculum age on pectinase production under SSF.

Pectinase production was analysed by varying the inoculum size from 1% to 6%. The effect is shown in **Figure 10**.

A. oleivorans DR1 showed maximum pectinase 9528 U/gds production at 2% concentration of 48 h old inoculum. Loss of pectinase was more pronounced in *A. oleivorans DR1* at increased size of inoculum. Very high inoculum size may reduce the enzyme production due to utilization of substrate for the growth of large number of cells which leads to decline in oxygen uptake rate and enzyme release [49]. Contrary to the present study [29] and [47] has reported maximum pectinase production with higher inoculum size i.e., 10% (v/w) respectively from *Bacillus subtilis* SS and *Bacillus* sp. MG-cp-2 under solid state fermentation. However, [33] and [32] reported the size of inoculum in terms of volume and observed that 1 mL inoculum produced maximum pectinase from *Bacillus firmus* and *Bacillus* sp. However another research results were reported by [28] stated that *Bacillus* sp. MBRL576 showed maximum pectinase production at 6% concentration of 24 h old inoculum.

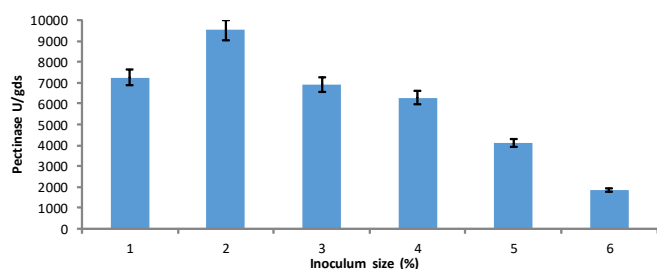


Figure 10: Effect of Inoculum size on pectinase production under SSF.

Various carbon sources glucose, lactose, maltose, sucrose, xylose, cellulose, fructose, galactose, starch was used in the solid state fermentation medium at a concentration of 1% w/v as additives. Production medium without any additive was used as control. Flasks containing this production medium were inoculated with 48 h old inoculum and incubated for 72 h at optimized temperature 45°C. Enzyme extract was used for assay. The effect of various carbon sources is given in **Figure 11**.

None of the additives supported the enzyme production as enzyme production was reduced maximum by 89.5% in *A. oleivorans DR1*, by using additives.

Our results are in agreement with those of [44] who observed that all the different carbon sources exhibited pectinase lower than control from *Bacillus firmus*- I-10104 on *Solanum Tuberosum* (ST) peels, the best carbon source for pectinase production. In contrast to our results [31], observed that pectin, lactose enhanced pectinase production from *Bacillus* sp. DT7 and [40] reported that sucrose promoted maximum enzyme yield from *Aspergillus niger* compared to the other carbon sources used. [40] further reported that addition of different carbon sources

to orange peel resulted in induction and repression of pectinase production in SSF.

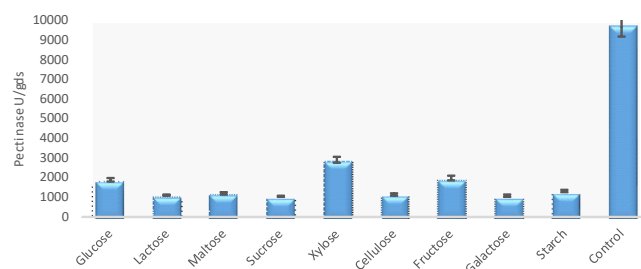


Figure 11: Effect of Carbon sources on pectinase production under SSF.

Different nitrogen sources such as peptone, ammonium sulphate, gelatin, ammonium chloride, urea, ammonium nitrate, ammonium dihydrogen phosphate, potassium nitrate, sodium nitrate were used in solid state fermentation medium at a conc. of 1% w/v as nitrogen source. The medium without any additional nitrogen source was used as control. The results of effect of various nitrogen sources are given in **Figure 12**.

Pectinase production was reduced by addition of additives containing nitrogen in *A. oleivorans DR1* in the range of with 6 out of 9 additives in comparison to the control with 9585 U/gds. The inhibitory effect of nitrogen sources may be due to disturbance in the balance between carbon and nitrogen sources in medium. As a result, the pH control of the system may be lost.

In contrast to our results [31], showed that when various nitrogen sources were supplemented, peptone, yeast extract, ammonium chloride were found to enhance pectinase production up to 24%. Addition of glycine, urea, ammonium nitrate inhibited pectinase production. In a previous study [33], reported that maximum pectinase production from *Bacillus firmus*-I-10104 in the presence of peptone followed by ammonium chloride, urea, and beef extract. [31], observed that maximum pectinase was produced from *Aspergillus niger* when ammonium sulphate was incorporated into the medium.

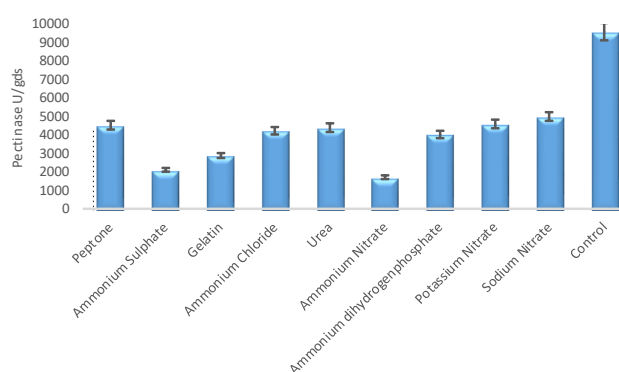


Figure 12: Effect of Nitrogen sources on pectinase production under SSF.

Conclusion

This is probably the first report of pectinase production by *A. oleivorans DR1* using date fruits waste as substrate. In the current study, *A. oleivorans DR1* was screened for the synthesis of pectinase using date fruit wastes as substrate by solid state fermentation. *A. oleivorans DR1* produced higher pectinase using date fruit wastes as substrate as compared to other agro-industrial waste. The physical and chemical parameters of *A.*

oleivorans DR1 of solid-state fermentation were optimized for maximum pectinase production using 1% date fruit wastes as substrate with 1:2 ratio of tap water as moistening agent. The production of pectinase reached maximum by keeping the fermentation of *A. oleivorans DR1* at pH 8.0 and 45 °C for 72 h of incubation. Inoculum size of 2% of 48 h old culture was proved to be the best for pectinase production under SSF. The supplementation of additives viz. carbon sources, nitrogen sources into the medium, exhibited various degrees of pectinase production lower than the control. *A. oleivorans DR1* showed practicable properties for pectinase production using date fruit wastes. Further studies are needed to analyze the commercial feasibility of utilization of date fruit wastes for synthesis of enzymes as well as to produce other value-added products. It has been concluded that date fruit waste can be used inexpensively to produce pectinases by solid state fermentation using *A. oleivorans DR1* for using in various industries for commercial production of value-added products.

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The relevant data and materials are available in the present study.

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Authors' contributions: VB performed all the experiments. VB analysed the data and wrote the manuscript.

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