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INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/3050
DOI URL: http://dx.doi.org/10.21474/IJAR01/3050

ISSN NO. 2320-5407

RESEARCH ARTICLE

MICROBIAL DNA EXTRACTION FROM INFECTED SEED POTATO FOR MOLECULAR DIAGNOSTIC APPLICATIONS

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Manuscript Info

Manuscript History

Received: 30 November 2016
Final Accepted: 28 December 2016
Published: January 2017

Key words:-
DNA extraction, Infected Potato, Protocols

Abstract

Transmission of tuber borne diseases is a major cause of yield reduction in potato cultivation. Molecular level diagnosis of the microbial pathogens is a challenge to obtain a sufficient quantity of DNA from infected potato tubers. Therefore, this studyfocused to finda simple protocol to extract microbial DNA from the infected seed potato. Three different DNA extraction protocols were performed and the extracted DNA was tested forthe quantity and quality of DNA using agarose gel electrophoresis. Next Generation Sequencingof 16S rDNA and ITS1 region based amplification was used for the detection of bacterial and fungal pathogens in infected samplesusing Ion Torrent sequencing platform.Both the enrichment and the culture independent boiling methods of extraction yieldedsufficient amount of total genomic DNA from the infected tuber samples.In each method, DNA yield varied depending on the type and severity of infection.The extracted DNA matched with the stringent quality control standards for sequencing on Ion Torrent PGM and amplified both 16S rDNA and ITS1 regions with clear distinctive and reproducible banding patterns in agarose gels.Both bacterial and fungal pathogenswere detected with these modified extraction protocols. However, enrichment method was found to be suitable in detecting pathogens even in latent infections. In conclusion, these DNA extraction protocols provided simple, inexpensive and effective methodology in detectingmicrobial diversityininfected potato tubers.

Introduction:-

One of the most remarkable events in the field of microbial ecology in the past decade has been the advent and development of meta-genomics which is defined as “The direct genetic analysis of genomes contained with an environmental sample” (Thomas*et al*, 2012). Application of meta-genomic approaches on microbial diversity identifications has achieved a considerable progress during the past few years. Culture independent direct DNA extraction is the most crucial step in meta-genomic analysis (Jiang *et al*, 2015). However, many technical challenges regarding efficient DNA extraction protocols from various tissue samples are still remain unanswered.

Potato is a major crop of the world and a number of microorganisms thrive on the nutrients that can be found in potato (Follenweider, 2009). The potato plant is susceptible to many bacterial and fungal diseases which consistently

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cause yield losses in potato production.The presence of microbial populations in infected seed tubers including latent infections are difficult to detect and enumerate with accuracy due to lot of methodological limitations in DNA extraction. Therefore, directmicrobial DNA extraction from potato tubers often yields only very small amounts of DNA insufficient for detection purposes. Like many plant tissues, potato tubers contain acidic polysaccharides (Yusuph*etal*, 2003;Noda *etal*, 2004*)* and various polyphenolics(Friedman, 1997) that are known to inhibit totalnucleic acid extraction. Obviously,such inhibitors are likely to distort PCR and Next Generation Sequence analysis. However, detection of microorganisms present within infected plant tissues is indispensable in phytopathology as they are responsible for many important diseases. Therefore, a broad array of research efforts are directed towards revealing successful DNA extraction protocols. New research findings on culture independent direct microbial DNA extraction methodologies are required in various research areas.

The development of molecular approaches to community analysis have circumvented the need for cultivation because phylogenetically informative DNA sequences can be directly screened from the environment (Laurel *et al*, 2003). The method describes in the present study illustrates a suitable DNA extraction protocolwhich could beused in meta-genomic analysis for microbial detection.

Materials and Methods:-

Seed Potato Sample Collection:-

Both infected and uninfected seed potato tubersrandomly sampled from the imported seed potato consignments at the entry ports of Sri Lanka were collected from the pathology division of National Plant Quarantine Service– Katunayake, Sri Lanka. The collected seed tubers were separately stored in a cold room under 4 ^{0}C at the National Plant Quarantine Service until taken for DNA extraction.

DNA Extraction:-

EnrichmentMethod:-

Seed potato tubers were first washed in tap water and then in distilled water. Surface sterilization was done by washing with 10% NaOCl (Chlorox) for 1 min. Small pieces of seed potato tubers especially from the infected samples, stem end and eyes were crushed and incubated in 15 ml of liquid LB. The mixture was shaken for 12 hrs.under 120 rpm. The liquid culture was centrifuged for 10 min. at 12 400 gunder 4 ^{o}C. The pellet was washed twice using a wash buffer (50 Mm TrisHCl& 5 mM EDTA of pH 8.0) and re-suspended in lysis buffer (100 mMTrisHCl& 100 mM EDTA of pH 8.0, 1.5 M NaCl). The suspension was centrifuged for 15 min. at 12 400 gat4^{o}C. Then NaOAC and 500 µl of ice-cold isopropanol were added to the supernatant and centrifuged for precipitating the DNA. The pellet was washed with70% ethanol and re-centrifuged at 12 400 g for 10 min at 4^{o}C. Ethanol was completely removed by air drying. The DNA was re-suspended inde-ionized water.

LysisMethod:-

Seed potato tubers were washed with tap water and distilled water. Surface sterilization was carried out by shaking with 10% NaOCl for 1 min. and washed thoroughly with autoclaved distilled water. Seed potato tuber was crushed and filtered using a sterile gauze and 50 ml of the filtrate was centrifuged for 5 min. at 750 g. The supernatant was centrifuged again under 10500 g for 10 min at 4 ^{o}C. The cell pellet was re-suspended in 300 µl of TE Buffer. Then 2 µl of lysozyme solution and 1 µl of RNase A were added to the cell suspension. It was mixed by vortexing and incubated at 37 °C for 30 min. Further,300 µl of lysis solution (2X)and 1µl of proteinaseK were added. The mixture was incubated at 65 °C for 15 min and cooled to room temperature and placed on icefor 3-5min. Phenol Chloroform mixture of 700 µl was added and mixed vigorously for 10 sec. The debris was removed by centrifugation for 10 min. at 10 500 g at 4 °C. Ice cold isopropanol was added to the supernatant and mixed by inverting the tube several times. DNA was pelleted by centrifugation for 10 min. at 10,500 g at 4 °C and washed with 70% ethanol and air-driedat room temperature.

Boiling Method:-

Surface sterilization was carried out by washing the infected seed tubers with running water for 5 min.and shakingwith 10 % NaOCl (Chlorox) for 1 min. The tuber was washed thrice with autoclaved distilled water andground manually using a sterile motor and pestle. Then, 10 ml of autoclaved deionized water was added to the crushed potato paste and transferred whole content to an autoclaved container. The mixture was kept for 30-45 min. and filtered using a sterile gauze. The extract was collected and boiled for 5 min.in a water bath adjusted to 95-100 ^{0}C. The mixture was then vortexed for 30 sec.and centrifuged under 12400 g for 10 min. The pellet was washed with 3 ml of wash buffer (50 Mm TrisHCl& 5 mM EDTA of pH 8.0) under 12400 g for 10 min. Then, 3 ml of lysis

buffer (100 mMTrisHCl& 100 mM EDTA of pH 8.0, 1.5 M NaCl) was added and kept for 30-45 min. with proper mixing in each 10 min.intervals. The homogenized mixture was centrifuged at 12400 g for 15 min. The resulting supernatant was then centrifuged under 12400 g for 15 min. with 150 µl of NaOAC and 1:1 ice-cold isopropanol added along the wall of the tube. The pellet was washed with 70% ethanol and re-centrifuged at 12400g for 10 min. Ethanol was completely removed by air drying. The DNA was re-suspended in de-ionized water.

DNA Quantity and Quality Assessment:-
The quality and quantity of DNA from each extraction was verified by 0.8% (w/v) agarose gel electrophoresis looking for no evidence of substantial band shearing or contamination either with RNA or polysaccharide. The concentration of amplified 16s rDNA and ITS1 fragments were tested prior sequencing using a Qubitfluorometric quantitation at Credence Genomics Pvt. Ltd. Colombo,Sri Lanka.

Next Generation Sequencing:-
Based on theresults of amplification of 16s rDNA and ITS1 regions by PCR, the extracted DNA wassubmitted to Credence Genomics Pvt. Ltd. Colombo-Sri Lanka for meta-genomic library preparation and sequencing on Ion Torrent PGM platform to confirm the protocol yielded both bacterial and fungal DNA.

Results:-

In this study three different DNA extraction methods (Enrichment method, Lysis method and Boiling method) were assessed in order to determine the best methodto extract microbial DNA from infected and uninfected seed potato tubers.The enrichment method was capable of yielding DNA from both infected and asymptomatic potato tubers with latent infections. The DNA extracted by enrichment method was found to have higher concentration of approximately 200 ng/ µl(Figure 1).

The culture independent boiling method for meta-genomic DNA extraction was quite efficient in yielding DNA from only infected potato tubers (Figure 2). Quality and quantity of the DNA werefound better in both boilingand enrichment methods.Both enrichment and the rapidlysis methods revealed single, high molecular weight DNA bands with little evidence of shearing and less RNA or polysaccharide contamination. The relative band intensities were varied depending on the sample. However, 1 µl of sample resolved on the agarose gel with the 50 ng lambda DNA standard, consistently yielded approximately 5 ng of DNA per gram of potato tissue (Data not shown).Altogether 3 hrs. were needed for DNA extraction from boilingmethod whereas, enrichment DNA extraction method required approximately 16 hrs.The ratio of absorbance at 260 nm and 280 nm (A260/A280) was 1.75. However, the lysis method for DNA extraction was unable to extract DNA properlyfor producing DNA bands on agarose gel.During the crushing of potato tubers, browning of the solution was observed due to phenolic compound available on the peel. The intensity of browning varied depending on the variety of potato. Upon DNA precipitation, despite the observation of a small brown pellet, the agarose gel failed to reveal any DNA.

PCR amplification of 16srRNAgene (Figure 3) and ITS1(Figure 4) regionsconfirmed the amplificationavailabilityof both bacterial and fungal DNA (Table 1)**.**The DNA was sufficient for high throughput sequencing and passed the quality control measures of Credence Genomics Pvt. Ltd. Sri Lanka for Ion Torrent Next Generation Sequencing.

Table 1:- Concentration of PCR amplified fragments of four DNA samples extracted from Enrichment method

Sample No.	Concentration after PCR ng/µl	
	16s Rdna	ITS1
1.	0.224	0.059
2.	0.137	0.780
3.	0.211	0.214
4.	1.430	0.564

In conclusion the enrichment method extracted a higher value of DNA concentration and the extracted DNA was of high molecular weight for all extracted seed tuber samples.From this study it was suggested that the enrichment method might be a suitable choice for extracting DNA from both infected and uninfected potato tubers because it is capable of yielding DNA from both infected and asymptomatic tubers with latent infections.

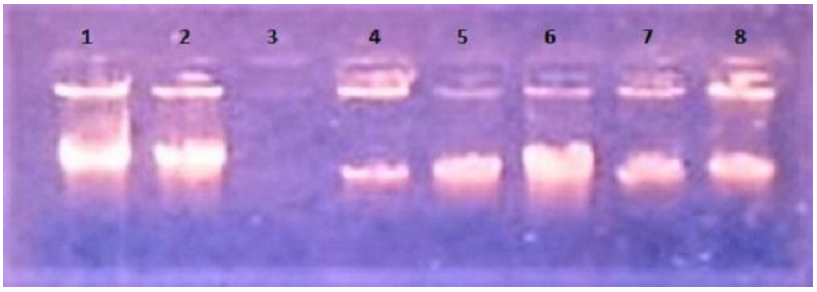

Figure 1:-Enrichment method for genomic DNA extraction from four seed potato samples. Lane 1 & 2: Sample 1; Lane 3 & 4: Sample 2;Lane 5 & 6: Sample 3;Lane 7 & 8: Sample 4

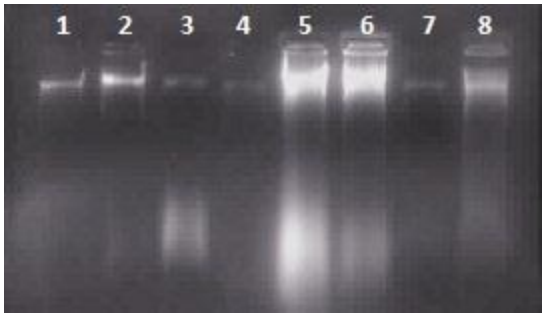

Figure 2:-Boiling method for meta-genomic DNA extraction from four infected seed potato samples - Lane 1 & 2: Sample 1; Lane 3 & 4: Sample 2;Lane 5 & 6: Sample 3;Lane 7 & 8: Sample 4

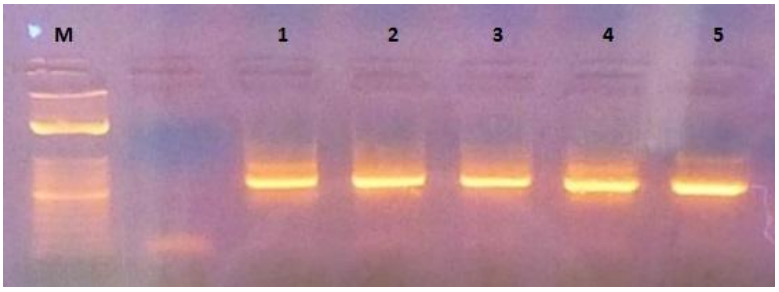

Figure 3:- Amplification of bacterial 16S rDNA from extracted genomic DNA from the samples to confirm the availability of bacterial DNA within the extractions. M -100 bpMarker; Lane 1, 2, 3, 4 : Seed potato DNA Samples. Lane 5: Positive control.

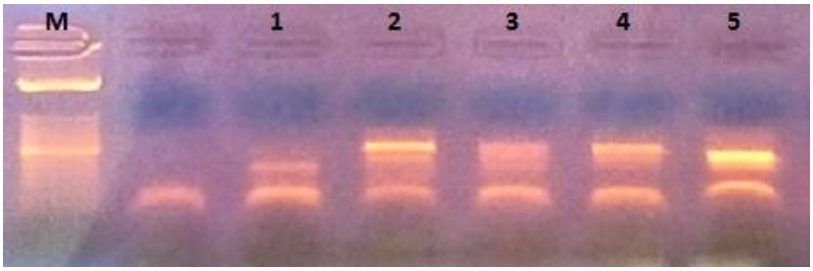

Figure 4 :-Amplification of fungal ITS1 region from extracted genomic DNA to confirm the availability of fungal DNA within the extracted samples. M - 100 bp Marker; Lane 1, 2,3, 4 : Seed potato DNA Samples. Lane 5: Positive control.

Additional comparative studies are necessary to ensure that potato DNA extraction techniques are optimized for other plant materials. Following the results of this study, we recommend boiling method as a method of choice for meta-genomic DNA extraction from infected potato tubers based on the processing time, easiness of use and DNA extraction efficiency.

Discussion:-

Polymerase chain reaction (PCR) and Next Generation Sequencing (NGS) based applications in molecular diagnostics for plant pathogens require good quality DNA for reliable and reproducible results. Imported seed potato tuber lots carry various important diseases therefore tissues from potato tubers are often used for DNA extraction to identify the pathogenic bacteria and fungi. The extraction of DNA from potato tubers often requires tedious protocols.Therefore, it was essential to develop a method reducing the number of handling steps, eliminating the need for long incubations or supplementation with expensive commercial based kits and reagents.

Culture independent boiling method for DNA extraction was efficient only with infected potato tubers where the symptoms are observable. The method is based on direct cell lysis with heat and a lysisbuffer followed by precipitation with isopropanol. This methodwas unable to yield any DNA from uninfected tubers which may be due to low abundance of bacteria available. It was observed that the DNA yield will strongly depend on the amount of tissue taken for extraction and the severity of infection of potato tuber.

In latent infection, the bacteria may be found in all parts of tissues however, their density is usually low. When the pathogen populations are low, they need to be enriched above detection level. In enrichment method for DNA extraction, initial incubation of crushed potato with liquid LB acts as a common enrichment medium for bacteria available within the potato tissues and increased the bacterial cell number. This enrichment medium creates conditions that stimulate growth and multiplication of bacteria possibly facilitating the detection of all available pathogens within the sample which may include pathogens as well as the endophytes. That is similarly important as microbial endophytes that colonize potato tissue are widespread and some of the non-pathogenic endophytes could also turn into phytopathogens that are able to induce infection symptoms (Koiv*et al*, 2015). Therefore, developed enrichment methodallows multiplication of available pathogens which possibly enables isolation of most of the bacteria in the samples.Most sequencing technologies essentially require high nanograms or microgram amounts of DNA (Torsten Thomas, 2012). Therefore, enrichment media is one option employed to increase DNA yields. This method can multiply the number of available microorganisms and thus enrichment methods are widely used in microbial research. But there are potential problems associated with culture based methods.

Culture-based methods are important ininvestigating the microbial communities but they were identified asextremely biased in their evaluation of microbial genetic diversity by selecting a particular population of microorganisms (Rastogi*et al*, 2011). The inability to culture many microorganisms in the provided media produce a major problem where the reports indicated that only 1-4% of the microbes can be cultivated under standard laboratory conditions (Chaudhuri*et al*, 2006). The enrichment media used in culturing microorganisms under laboratory conditions are selective and only a subpopulation of the microbes in an environmental sample are grown in any givenmedium (Schneegurt*etal*, 2003). The analysis using indirect methods such as incubation was also detected as producing artificial changes in the microbial community structure and metabolic activity. Other limitations to culturing include the inability to predict the proper culture medium to select unknown organisms, and the propensity of fast-growing organisms to outgrow and overshadow the more relevant organisms that grow more slowly. These issues can have significant impact on subsequent microbial community analysis.These several important reasons has led to the development of culture independent rapid lysis method for microbial DNA extraction for unbiased detection of microbial pathogens associated with infected seed potato tubers.

However, several limitations are associated with the culture independent microbial DNA extraction from potato tubers. Polysaccharides and polyphenols including flavonoids and other secondary metabolites interfere with the extraction of pure genomic DNA(Sahu*et al*, 2012).Potatoes are a great source of phenolic compounds andthey are present both in the peel and the flesh of potato tubers (Akyol*et al*, 2016). In DNA extraction, brownish pellet indicates the contamination of phenolic compounds (Sahu*et al*, 2012). In all tested DNA extraction protocols, browning of solution was observed which has been attributed to the oxidation of phenolic secondary metabolites in potato tubers. Certain protocols add Polyvinylpyrrolidone (PVP) into extracts to absorb such phenolics preventing their oxidation that renders DNA unusable for downstream applications (Young *et al*, 1993). In many other protocols, an antioxidant such as DIECA (diethyldithiocarbomic acid) is used in very low concentrations (0.05%)

during enrichment to protect bacteria cells from oxidative stress due to the release of the plant compounds (Czajkowski*et al*, 2015). However, any chemical was not applied in these extraction protocols to remove the phenolic contaminants. Several centrifugation and pelleting steps of unlysed tuber materials was effective for removal of polysaccharide and phenolics continuously leeching in to the solution. Also, unlysed tissues settle in the bottom of the falcon tube were removed during the second step. Early removal of debris has also improved the clarity.Mechanical, thermal, and chemical processes that are used to refine DNA may damage DNA through endogenous enzyme hydrolysis, shearing, depurination, cross-linking, acid hydrolysis, and oxidation (Smith*et al*, 2005*)*.These damaged or fragmented template DNA may severely reduce amplification efficiency in PCR or may render target sequences undetectable.

In boiling and enrichment methods for DNA extraction; DNA precipitation included the addition of NaOAc high salt solution before DNA was precipitated with IsoPropanol. Polysaccharides have a similar solubility to DNA and co-precipitate in either iso-propanol or ethanol inhibiting downstream molecular applications (Healey*et al*, 2014). The addition of a high salt buffer increases their solubility in ethanol, allowing their removal once the DNA has been precipitated and pelleted.

To better determine how well the extraction methods performed each of the methods were analyzed separately. The DNA was successful in NGS which may be due to either a lesser degree of inhibitors or a lower degree of damage to the template DNA during extraction. There was no significant difference between the DNA quality recovered by the enrichment method and rapid lysis methods which produced the most consistent, highest quality template.

The extensive research on meta-genomic DNA extraction targeting different substrates highlights the need to ensure that, extraction procedures are well benchmarked, and that multiple methods are compared to ensure representative extraction of DNA (Thomas *et al*, 2012). This research was basically focusing on development of culture independent meta-genomic DNA extraction method from potato tubers. The appliedboiling method extracted the sufficient amount of DNA yields from the tuber material. For most of the analyzed tuber samples, this method recovered the highest levels of amplifiable DNA yields however the yield varies from one sample to another depending on the disease intensity which indicated that overall yield had greater influence over intensity of infection or the level of disease spreading.

Conclusion:-

The optimized enrichment and boiling methods for DNA extraction are simple and rapid therefore, suitable for routine diagnosis assays from potato. The methods yielded sufficient quantities of DNA from the pathogenic microbes associated with the seed tubers. DNA was suitable for the detection of bacterial and fungal pathogens using PCR and NGS based analysis.

Acknowledgement:-

This investigation received financial support from the National Science Foundation of Sri Lanka under Grant No. NSF/SCH/2015/01.

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